

Rapid, Transient Fluconazole Resistance in *Candida albicans* Is Associated with Increased mRNA Levels of *CDR*

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Fluconazole-resistant *Candida albicans*, a cause of recurrent oropharyngeal candidiasis in patients with human immunodeficiency virus infection, has recently emerged as a cause of candidiasis in patients receiving cancer chemotherapy and marrow transplantation (MT). In this study, we performed detailed molecular analyses of a series of *C. albicans* isolates from an MT patient who developed disseminated candidiasis caused by an azole-resistant strain 2 weeks after initiation of fluconazole prophylaxis (K. A. Marr, T. C. White, J. A. H. vanBurik, and R. A. Bowden, *Clin. Infect. Dis.* 25:908–910, 1997). DNA sequence analysis of the gene (*ERG11*) for the azole target enzyme, lanosterol demethylase, revealed no difference between sensitive and resistant isolates. A sterol biosynthesis assay revealed no difference in sterol intermediates between the sensitive and resistant isolates. Northern blotting, performed to quantify mRNA levels of genes encoding enzymes in the ergosterol biosynthesis pathway (*ERG7*, *ERG9*, and *ERG11*) and genes encoding efflux pumps (*MDR1*, *ABC1*, *YCF*, and *CDR*), revealed that azole resistance in this series is associated with increased mRNA levels for members of the ATP binding cassette (ABC) transporter superfamily, *CDR* genes. Serial growth of resistant isolates in azole-free media resulted in an increased susceptibility to azole drugs and corresponding decreased mRNA levels for the *CDR* genes. These results suggest that *C. albicans* can become transiently resistant to azole drugs rapidly after exposure to fluconazole, in association with increased expression of ABC transporter efflux pumps.

Fluconazole-resistant *Candida albicans* has become a major cause of oropharyngeal candidiasis in patients with human immunodeficiency virus (HIV) infection (21). Patients at highest risk for infection with resistant organisms are those that are severely immunosuppressed and have low CD4 cell counts and who have had previous exposure to high total doses of fluconazole (>10 g) or long durations of fluconazole treatment (2, 11, 14, 31). Recently, infections caused by fluconazole-resistant *C. albicans* have been reported to occur in patients not infected with HIV (13, 16, 17). To date, four patients with hematological disorders (i.e., leukemia and myelofibrosis) are known to have developed fungemia with azole-resistant *C. albicans* (13, 16, 17). All either were receiving immunosuppressive chemotherapy or had undergone marrow transplantation (MT) when they developed fungemia, and all of these patients developed resistance within 2 to 3 weeks of exposure to fluconazole. This rapid development of infection caused by azole-resistant *C. albicans* is a phenomenon that has not been reported to occur in patients with HIV infection (21).

The development of resistance depends on host factors, such as patient adherence to the antifungal regimen and drug-drug interactions, as well as factors intrinsic to the organism, such as susceptibility to the drug (reviewed in reference 31). In patients with HIV infection, the administration of azole drugs results in growth of both intrinsically resistant non-*C. albicans* *Candida* species (i.e., *C. glabrata* and *C. krusei*) and resistant *C. albicans* strains (18, 19).

The molecular mechanisms and genetic alterations that ren-

der strains of *C. albicans* azole resistant have been best identified by using matched sensitive and resistant isolates from patients with HIV infection (23, 32). Alterations of the target enzyme of azole drugs (lanosterol demethylase, or Erg11), including increased expression and point mutations in the *ERG11* gene (30, 31), as well as mutations in genes encoding other enzymes involved in the ergosterol biosynthesis pathway (e.g., *ERG3*) have been described (8, 9, 15). Also, several investigators have correlated increased expression of the *CDR* genes, which are members of the ATP binding cassette transporter (ABCT) efflux pump superfamily, and of the gene (*MDR1*) for the major facilitator efflux pump with the development of resistance (1, 23). To date, 10 *CDR* genes (*CDR1* through *CDR10*) have been cloned, but only *CDR1* and *CDR2* have been associated with azole resistance (20, 22, 23, 31). The study of a series of 17 isolates from a patient with HIV infection has demonstrated that all of the mechanisms described above contribute to the final resistant phenotype in one strain of *C. albicans* (29, 32). These isolates, and all other resistant clinical isolates studied, have a stable resistant phenotype that is maintained for many generations of growth in azole-free media (32).

In a study of two strains of *C. albicans* from patients with leukemia, Nolte et al. found that the resistant isolates had membrane sterol changes that were consistent with alterations in the $\Delta 5,6$ -sterol desaturase gene, *ERG3* (17). In this study, *C. albicans* isolates were found to be resistant to fluconazole and amphotericin B (AmB) after only 2 weeks of antifungal drug exposure. Although these patients were known to be colonized with *C. albicans*, surveillance cultures were not available for analysis. The authors suggested that because of the short duration of azole exposure, the more likely mechanism of resistance was selective growth pressure of an already resistant

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strain rather than induction of resistance (17). Members of our group recently reported a similar case of development of a disseminated infection caused by a resistant *C. albicans* strain in an MT patient (13). Fortunately, serial surveillance colonizing isolates were available for study, and restriction fragment length polymorphism (RFLP) analysis of genomic DNA demonstrated that one susceptible strain of *C. albicans* became resistant after only 2 weeks of antifungal drug therapy. In this study of the series of nine isolates, we showed that *C. albicans* can become transiently resistant to fluconazole as a result of increased expression of the *CDR* efflux pump gene family.

MATERIALS AND METHODS

Yeast growth and storage. Yeast nitrogen base (YNB) (Difco, Detroit, Mich.), Sabouraud dextrose agar (Difco), and RPMI 1640 medium (American Bioorganics, Niagara Falls, N.Y.) were prepared and sterilized according to the manufacturers' recommendations. Individual yeast colonies from surveillance cultures were grown at 30°C in YNB, confirmed to be *C. albicans* by germ tube testing and RFLP analysis (13), and stored frozen at -70°C in YNB containing 10% glycerol.

Antifungal susceptibility testing. Powder formulations of fluconazole (Roerig-Pfizer, New York, N.Y.), itraconazole (Janssen Pharmaceutica, Beerse, Belgium), ketoconazole (Janssen Pharmaceutica) and AmB (Sigma, St. Louis, Mo.) were suspended in distilled water, filter sterilized, and stored frozen at -70°C. Isolates were tested for susceptibility to each antifungal agent by the broth macrodilution method published by the National Committee for Clinical Laboratory Standards (NCCLS) (5). Susceptibilities to fluconazole (initially and after serial growth) were confirmed in a reference laboratory. E tests (AB Biodisk) for fluconazole were performed according to the manufacturer's recommendations.

Testing for stability. Isolates 2, 5, and 8 were serially cultured with 1-to-5,000- μ l dilutions every 2 or 3 days in drug-free YNB media, and susceptibility to fluconazole was monitored by weekly E tests. MICs for the final transfers of the most resistant isolates (5, 7) were confirmed by the NCCLS macrodilution technique in our laboratory and in the reference laboratory.

Sterol intermediate assay. The sterol membrane components of one sensitive isolate (isolate 1) and one resistant isolate (isolate 5) were analyzed by the method described by Barrett-Bee et al. (3). Cell protein extracts were prepared from 1-liter cultures grown to mid-log phase. Cells were washed with sodium phosphate (pH 7.5) and resuspended in phosphate buffer, and glass beads were added to create a slurry. Slurries were iced and vortexed five times, for 1 min each time. Glass beads were removed by filtration through a disposable filter column (Fisher), and cell debris was removed by centrifugation first at 2,500 \times g (10 min) and then at 10,000 \times g (10 min). Equal amounts of protein extracts, calculated with a Bradford assay (Bio-Rad [Hercules, Calif.] protein assay) were used to assay for cell membrane constituents. The assay was performed as previously described (30). Briefly, cell extracts were mixed with [¹⁴C]mevalonic acid, 2 mM MnCl₂, 2 mM MgCl₂, and cofactor mix in the presence of increasing concentrations of fluconazole. The reaction mixtures were incubated in glass tubes for 1 h, the reactions were terminated with 1 ml of 15% KOH in 90% ethanol, and the reaction products were incubated at 80°C for 45 min. Petroleum ether extractions were prepared and dotted onto a thin-layer chromatography plate (EM Science, Gibbstown, N.J.). The plate was developed in toluene-ether (9:1), dried, and exposed to a phosphorimaging screen (Storm 1860; Molecular Dynamics), and quantification was carried out with the Molecular Dynamics ImageQuant program.

DNA extraction, Southern blotting, DNA sequence analysis, and RFLP analysis. Genomic DNA from fluconazole-susceptible isolate 1 and resistant isolate 5 was prepared and purified after cell shearing with glass beads (7). PCRs with primers spanning the length of the *ERG11* gene were carried out at 50°C, as described previously (29). The reaction products were cleared by dilution in 2 ml of distilled water, centrifuged through a Centricon 100 concentrator (Amicon, Beverly, Mass.), and recovered according to the manufacturer's instructions. Nucleotide sequences of all fragments were determined with an automated DNA sequencer with *Taq* dye-primer and dye-terminator chemistries (Applied Biosystems, Foster City, Calif.) and compared to the published sequences in GenBank (10).

RFLP analysis of the initial, resistant isolate 8 and the susceptible isolate 8 obtained after 12 serial transfers in the absence of fluconazole (isolate 8T12) was performed to determine genetic relatedness. RFLP analysis was also performed on the resistant isolate 5 and the susceptible isolate 5 obtained after 33 transfers in the absence of fluconazole (isolate 5T33). Genomic DNA was prepared as described above and digested with restriction enzymes, and Southern blots were prepared by standard techniques (12). The *C. albicans* strain-specific Ca3 probe was used for hybridization (24).

RNA preparation and Northern analysis. Yeast RNA was prepared by methods previously described (25). Ten micrograms of each RNA was denatured in a loading buffer (50% formamide, 20% MOPS [morpholinepropanesulfonic acid] [pH 7.0], 17.5% formaldehyde), electrophoresed on 1% agarose, and blotted

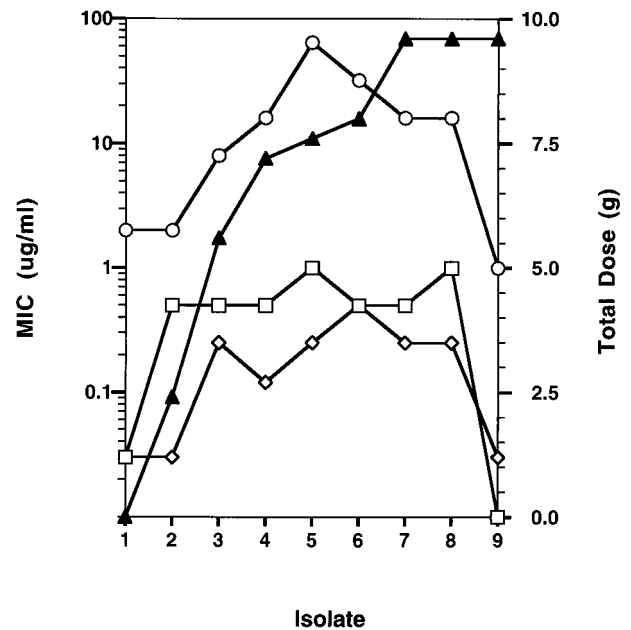


FIG. 1. Susceptibilities of isolates and exposure to fluconazole. MICs of fluconazole (○), ketoconazole (□), and itraconazole (◇) for each isolate are shown on the left y axis, and the total cumulative doses of fluconazole administered to the patient (▲) are shown on the right y axis. Isolates 1 through 9 were obtained on days -9, -2, 6, 15, 17, 18, 22, 23, and 28 relative to transplantation (day 0), respectively. Fluconazole was administered from days -7 to 7 and from days 12 to 21, and AmB was administered from days 7 to 10 and from days 20 to 25.

onto nitrocellulose by standard techniques (12). Hybridizations were carried out with probes for the *ERG7*, *ERG9*, and *ERG11* genes and with probes for the efflux pump genes *MDR1*, *CDR*, and *ABC1*. Membranes were washed, exposed to X-ray films, stripped (12), and rehybridized to labeled actin probes in order to correct for differences in amounts of RNA loaded. Actin probes were labeled with [γ -³²P]ATP by using T4 polynucleotide kinase. All other probes were labeled with [³²P]dATP by the random priming method.

RESULTS

Susceptibilities of yeast isolates. Yeast isolates for susceptibility testing were obtained from a patient who developed disseminated infection with *C. albicans* despite receipt of antifungals (fluconazole and AmB) while undergoing MT (13). Four rectal isolates (isolates 1 to 4) and four bloodstream isolates (isolates 5 to 8) were found to have increasing susceptibilities to azole antifungal drugs (Fig. 1). The ninth isolate, which was obtained from the lung postmortem, was susceptible to fluconazole. As shown in Fig. 1, the fluconazole-resistant isolates were also resistant to ketoconazole and itraconazole. MICs of the azole drugs correlated with exposure to fluconazole during the transplant course. The patient received a total dose of 9.6 g of fluconazole (Fig. 1). Unlike the previously collected colonizing and invasive isolates, the ninth isolate, obtained from lung tissue at autopsy (7 days after fluconazole was discontinued), was susceptible to all azoles. All isolates were susceptible to AmB, with an MIC of 0.5 μ g/ml.

Production of sterol intermediates. Membrane sterol components were synthesized from labeled mevalonic acid by using cell extracts in the presence of increasing doses of fluconazole (3). The results of assays of cell extracts of a sensitive isolate (isolate 1) and a resistant isolate (isolate 5) are shown in Fig. 2. The levels of lanosterol were increased and those of ergosterol were decreased in the presence of fluconazole, consistent

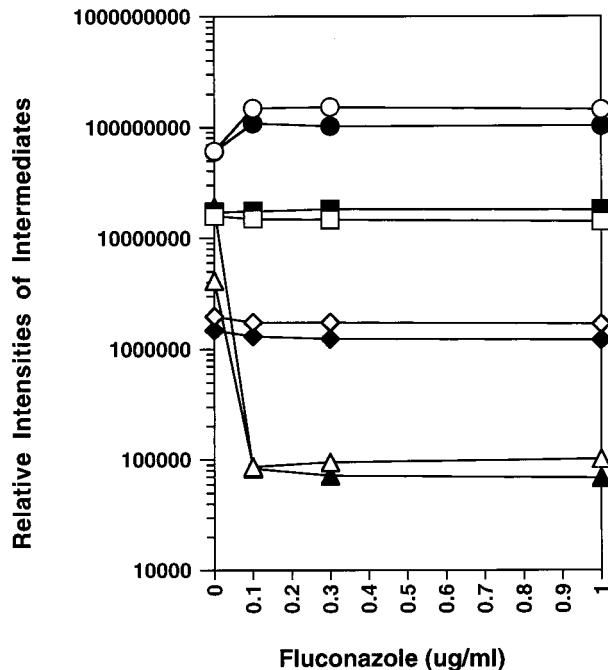


FIG. 2. Results of sterol intermediate assay. Relative intensities of intermediates, measured as described in the text, for susceptible isolate 1 (open symbols) and for resistant isolate 5 (solid symbols) are shown. Cell extracts were incubated in increasing concentrations of fluconazole (x axis). Relative intensities of the intermediates lanosterol (\circ), squalene (\square), 2,3-oxidosqualene (\diamond), and ergosterol (\triangle) were measured by phosphorimaging.

with the fluconazole activity (inhibition of lanosterol demethylase) in the ergosterol biosynthetic pathway. However, the production of intermediates in response to fluconazole did not differ in the sensitive strains and resistant strains, suggesting that the resistant phenotype in isolate 5 is not caused by an alteration of any enzyme involved in the ergosterol synthesis pathway, including the target enzyme of fluconazole, Erg11.

DNA sequence analysis. In order to verify that fluconazole resistance was not caused by a change in *ERG11*, the genes from both fluconazole-susceptible isolate 1 and -resistant isolate 5 were sequenced. Comparison with the published sequence for *ERG11* (11) revealed that the isolates contained several silent nucleotide changes (T462C, A504G, C558T, C805T, A1167G, C1257T, A1587G, and T1617C), as expected for different strains of *Candida*, as well as a conservative substitution for Asp of Glu at position 116 (D116E). However, this substitution was present in both the sensitive and resistant isolates and thus does not account for resistance in this series.

Expression of ergosterol synthesis enzymes and efflux pumps. In order to determine if changes in the ergosterol synthesis pathway account for the azole drug resistance observed in the series, mRNA levels were quantified by Northern analysis for several ergosterol biosynthesis genes, including *ERG7*, *ERG9*, and *ERG11*. The mRNA levels for these ergosterol biosynthetic genes varied only by factors of 2 to 3 and did not correlate with the resistance pattern within the series of isolates (data not shown).

mRNA levels for several ABC transporter genes (*CDR*, *ABC1*, and *YCF*) and a major facilitator efflux pump gene (*MDR1*) were measured in the series of isolates. Northern blot analysis indicated that mRNA levels of the efflux pump gene *MDR1* did not change significantly throughout the series, but levels of *CDR* were increased in isolates 2 to 8 and were decreased in the

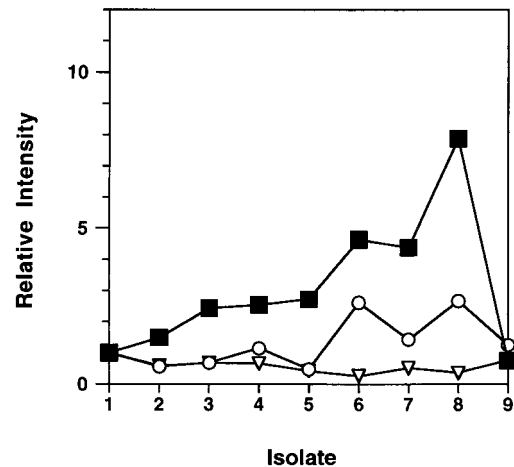


FIG. 3. Northern analysis of mRNA of efflux pump genes. Signal intensities of mRNA levels of *CDR* (\blacksquare), *ABC1* (\circ), and *MDR1* (∇) were quantified by phosphorimaging relative to actin probe and normalized to the intensity of the first isolate (as described in the text).

ninth isolate (Fig. 3), corresponding to the resistance pattern of these isolates. Small (two- to threefold) increases in mRNA for *ABC1* were observed in isolates 6 through 9. *YCF* mRNA was not detectable in any of the nine isolates. Note that the *CDR* probe used in these experiments cross-hybridizes with multiple *CDR* genes (*CDR1* through *CDR4*) (32). In order to determine if *CDR* gene amplification accompanied increased mRNA levels, genomic DNA was subjected to Southern blotting and hybridized to *CDR*. No difference in intensity between the susceptible and resistant isolates was apparent (data not shown).

Stability of resistance. To determine the stability of the resistance phenotype, the isolates were serially transferred in fluconazole-free medium. As seen in Fig. 4A, each of the resistant isolates lost resistance with serial passage, but the isolates did so at various rates. MICs for the susceptible transferred isolates 5 (5T33) and 8 (8T12) were verified by macrodilution testing as 4 and 1 $\mu\text{g/ml}$, respectively. The final susceptible isolates were also susceptible to ketoconazole and itraconazole (Fig. 4B), findings consistent with the cross-resistance associated with the *CDR* efflux pumps. The isolates remained susceptible to AmB, with MICs of 0.5 $\mu\text{g/ml}$. RFLP typing verified that the susceptible isolates 5T33 and 8T12 were the same strain as the original resistant isolates (Fig. 5A). In addition, Northern blot analysis verified that *CDR* mRNA levels were lowered in the susceptible isolates 5T33 and 8T12 (Fig. 5B and C). The *CDR* mRNA levels of isolate 5T33 decreased by a factor of 2 after approximately 400 generations (33 transfers) of growth in azole-free media, and the *CDR* mRNA levels of isolate 8T12 decreased by a factor of 10 after approximately 150 generations (12 transfers) of growth (Fig. 5C). These findings correspond with loss of resistance in the isolates after serial transfer (Fig. 5A).

DISCUSSION

In patients with HIV infection, the use of prophylactic fluconazole to decrease the incidence of oropharyngeal candidiasis has been found to be effective but is not recommended because of the frequent development of toxicities and drug resistance (27). Fluconazole administered prophylactically in patients undergoing MT has been shown to decrease the inci-

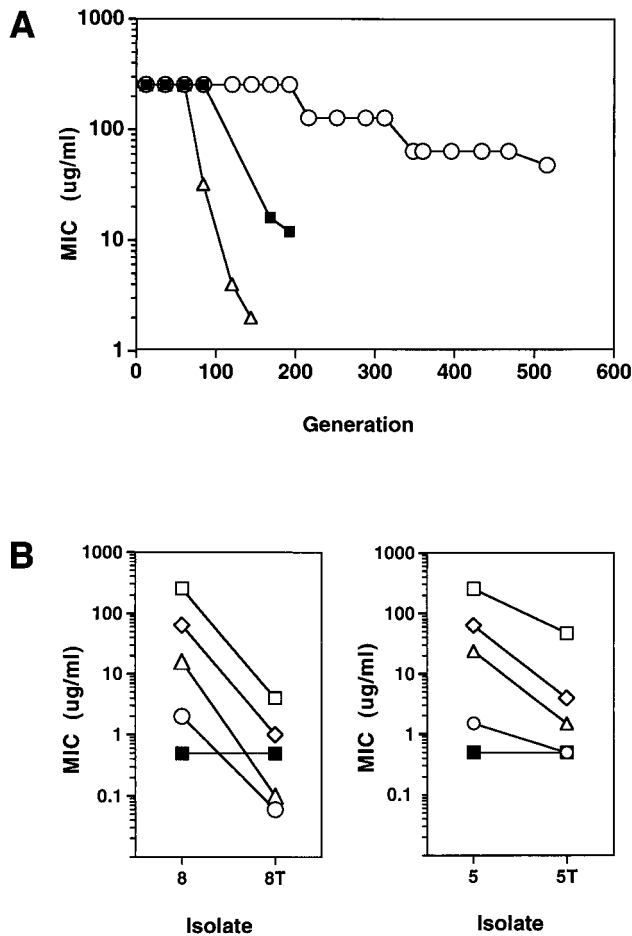


FIG. 4. Stability of fluconazole resistance after growth in drug-free media. (A) Fluconazole MICs for isolates 2 (■), 5 (○), and 8 (△), serially transferred in drug-free media, are shown. Each transfer represents approximately 12 generations of growth. MICs were determined by E test and confirmed by the NCCLS method (see text). (B) Susceptibilities of transferred isolates to other azoles. MICs for the initial fluconazole-resistant (isolates 5 and 8) and -susceptible (isolates 5T and 8T) isolates are shown. 5T, 5T33; 8T, 8T12. Fluconazole (□), ketoconazole (△), and itraconazole (○) MICs were determined by E test, and additional MICs of fluconazole (◇) and AmB (■) were determined by macrodilution methods.

dence of mucosal and disseminated candidiasis (6, 26), with one study showing a decrease in mortality related to antifungal prophylaxis (26). The increased use of azole drugs in transplant and cancer patients has resulted in an evolution of the spectrum of infections and an increased incidence of the frequently azole-resistant *Candida* species *C. krusei* and *C. glabrata* (33–35). The results of this study and others (17) emphasize that the use of azole drugs in MT patients can potentially lead to the development of infection caused by azole-resistant *C. albicans* as well.

Previous studies have documented that *C. albicans* can become resistant to fluconazole through mechanisms that involve modifications of the target enzyme, encoded by *ERG11*, as well as increased levels of mRNA for efflux pumps. In this study, molecular analysis showed that one strain of *C. albicans* became resistant to multiple azole drugs in association with increased expression of *CDR*. Analysis of mRNA expression of several *ERG* genes, the sequencing of *ERG11*, and assay of cell sterol constituents revealed no differences between sensitive and resistant isolates. This verifies that the ergosterol synthesis

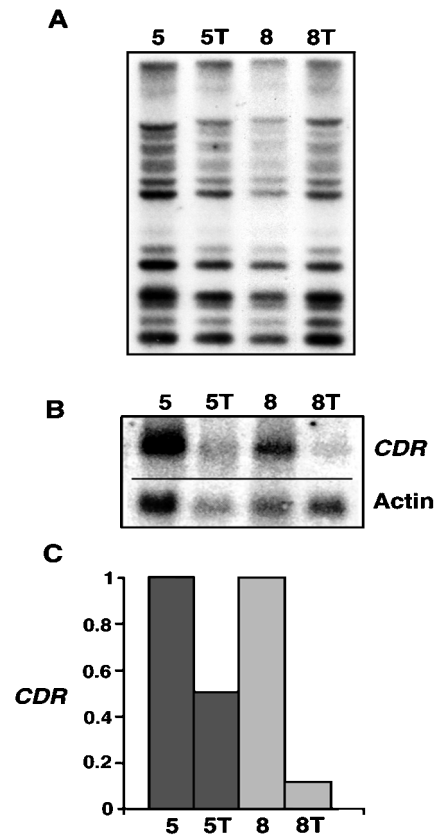


FIG. 5. Analysis of revertant isolates. Resistant isolates 5 and 8 are compared with the sensitive isolates obtained after serial transfer (isolates 5T and 8T). (A) RFLP analysis of genomic DNA from isolates, after digestion with *EcoRI*, and hybridization with the Ca3 probe. 5T, 5T33; 8T, 8T12. (B) Intensities of mRNA signals from Northern blots, probed with a *CDR* probe (upper panel). The blot was stripped and rehybridized with an actin probe (lower panel). (C) Levels of *CDR* mRNA were quantified by phosphorimaging of the Northern blot signals, calculated relative to actin controls, and normalized to the level for the resistant isolate for each sample.

pathway enzymes are not involved in azole resistance in this series of isolates. Increased mRNA expression for *CDR* corresponded to increased resistance to fluconazole, ketoconazole, and itraconazole in these isolates, with no effect on susceptibility to AmB. These results support previous observations that the ABC2 pumps are involved in efflux of all azole drugs, causing clinical cross-resistance to fluconazole, ketoconazole, and itraconazole (23). The correlation between azole cross-resistance and *CDR* expression is further supported by the loss of resistance to all azoles in the resistant isolates after serial transfer in the absence of drugs and the corresponding decrease in mRNA levels of *CDR*. AmB susceptibility was maintained in this series, as there was no change in cell membrane sterol content in either sensitive or resistant isolates.

Although we have documented a strong correlation between increased mRNA levels of *CDR* and resistance, we have provided no definitive proof that efflux pump overexpression is the cause of azole resistance. Also, since we have not examined the transcriptional regulation of the *CDR* genes or the mRNA half-life in our series of isolates, we do not know the precise mechanism that results in increased mRNA levels of *CDR* (increased transcription versus decreased mRNA degradation). Finally, the *CDR* probe employed in these experiments cross-hybridizes with multiple *CDR* genes (*CDR1* through

CDR4). The increased mRNA levels can thus reflect increased expression of any one gene or of a combination of *CDR* genes. In order to clarify these issues, further studies are in progress.

The rapid development of resistance in *C. albicans* noted in this series has not been observed in patients previously exposed for short durations to azoles, and until recently, the secondary development of resistance had been considered to be a problem limited to patients maintained on fluconazole for long durations (21). The results of this study suggest that *C. albicans* can become resistant to azoles rapidly, in association with increased mRNA levels for *CDR*. The haploid yeast *C. glabrata* can become resistant to azoles more frequently and more rapidly than can the diploid yeast *C. albicans* (28, 31). Warnock et al. described a case in which a strain of *C. glabrata* became resistant to fluconazole after only 9 days of drug exposure, with subsequent study of the organism revealing that the resistance was associated with increased expression of the gene (*ERG11*) for the target enzyme (28). A likely explanation for the difference between the two yeasts is that the diploid nature of *C. albicans* allows expression of both susceptible and resistant versions of an enzyme, with the susceptible enzyme maintaining the susceptible phenotype of the cell. The diploid nature of *C. albicans* may thus explain why most isolates become resistant to fluconazole only after long durations of drug exposure.

The azole drug resistance in this series of isolates was transient in vitro, as susceptibility resulted after serial transfer in drug-free media. This transient nature of resistance was also observed in vivo, as the azole drug-susceptible isolate (isolate 9) was obtained after the drug was no longer being administered to the patient (13). This transient phenotype has not been found in resistant *C. albicans* from patients with HIV (21, 31). One possible explanation is that azole resistance in patients with HIV, which develops after prolonged drug exposures, may result from stable genomic alterations (i.e., point mutations or deletions), while the rapid, transient resistance observed in these isolates may be caused by other factors that regulate efflux pump expression (i.e., overexpression of *trans*-acting factors). Although *CDR* gene regulation has not been well characterized, it is likely to be affected by the presence of azole drugs.

In a previous study, investigators successfully induced the development of azole resistance by simulation of chronic fluconazole exposure in vitro (4). In that study, serial passage of a laboratory strain of *C. albicans* in media containing low levels of fluconazole resulted in the expression of a resistant phenotype. This resistance, however, was not associated with an increased expression of *ERG11* or known efflux pumps. Interestingly, this isolate developed resistance rapidly (within 15 to 20 days) and transiently, with a reversion to a susceptible phenotype after removal of the drug (4). In this study, the resistant *C. albicans* organism isolated in vivo after a brief exposure to the drug was either an isolate that became resistant rapidly or possibly a resistant substrain that had a growth advantage when fluconazole was administered. Since only a single isolate was obtained at each time point, it is impossible to distinguish between these two possibilities. Thus, although precise mechanisms have yet to be defined, it appears that *C. albicans* can develop an unstable resistance to azoles rapidly after exposure to fluconazole, both in vitro and in vivo.

The ability of a colonizing *C. albicans* strain to develop azole drug resistance rapidly and transiently is an observation of concern in relation to profoundly immunosuppressed MT patients, given their dependence on prophylactic antifungal drugs. This observation has not been made in patients with HIV infection, but it is important to note that the differences in the two patient populations are multiple and include mech-

anism and degree of immunosuppression as well as previous and concurrent exposure to different medications. This study emphasizes the need for further investigation into the clinical significance of azole resistance in MT patients as well as the molecular mechanisms involved in the development of rapid, transient drug resistance by *C. albicans*.

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ERRATUM

Rapid, Transient Fluconazole Resistance in *Candida albicans* Is Associated with Increased mRNA Levels of *CDR*

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