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Isolation and Characterization of Fluconazole- and Amphotericin B-Resistant *Candida albicans* from Blood of Two Patients with Leukemia

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Infections with fluconazole-resistant *Candida albicans* isolates have rarely been described in clinical settings other than oropharyngeal candidiasis in patients with late-stage AIDS. We report on two patients with leukemia who developed fungemia caused by fluconazole-resistant *C. albicans* after receiving fluconazole prophylaxis (400 mg/day) and empiric amphotericin B therapy (0.5 mg/kg of body weight per day). The fluconazole MICs for the isolates were ≥64 μg/ml, and the isolates were resistant to other azoles and had membrane sterol changes consistent with a mutation in the $\Delta^{5,6}$-sterol desaturase gene. The lack of ergosterol in the cytoplasmic membrane of the fluconazole-resistant strains also imparted resistance to amphotericin B. Both patients were successfully treated with high-dose amphotericin B (1 to 1.25 mg/kg/day) and flucytosine (150 mg/kg/day).

Fungal infections remain a major problem for patients with leukemia and in bone marrow transplant recipients. The most common etiologic agents in these infections are *Candida* species. The prophylactic use of fluconazole has been shown to reduce both the incidence of systemic candidiasis and the number of deaths from fungal infection in bone marrow transplant recipients (1). A potential problem with the prophylactic use of any antimicrobial agent is the selection or development of resistant organisms. Intrinsically fluconazole-resistant strains of *Candida* (*Torulopsis*) *glabrata* and *Candida krusei* have become important pathogens in our hospital and other centers with the widespread use of fluconazole (10, 16, 17). Infections with fluconazole-resistant *Candida albicans* have rarely been described in clinical settings other than late-stage AIDS patients with oropharyngeal candidiasis (12).

There are a limited number of reports on the mechanisms of azole resistance in clinical *C. albicans* isolates. These mechanisms include reduced drug permeation involving changes in phospholipids and the membrane sterol composition (2), changes in the affinity of the target for the drug, cytochrome P-450-dependent 14α-sterol demethylase ($P\text{-}450_{DM}$) (14), changes in $\Delta^{5,6}$-sterol desaturase, another enzyme in ergosterol biosynthesis (4), and changes in specific efflux pumps which result in failure to accumulate the drug (9, 13).

Here, we report on two leukemic patients who developed candidemia caused by fluconazole- and amphotericin B-resistant *C. albicans* isolates after fluconazole prophylaxis and empiric amphotericin B therapy and the characterization of these resistant isolates.

(This work was presented in part at the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif., 17 to 20 September 1995 [8]).

Case report for patient 1. Patient 1, an 18-year-old male with acute lymphocytic leukemia status after autologous bone marrow transplantation in October 1994, was admitted in December 1994 for evaluation of a relapse of disease. At that time, he was diagnosed with herpes zoster infection and received high-dose acyclovir for the duration of hospitalization. On admission, ceftazidime was initiated for febrile neutropenia and was continued for the duration of the hospitalization. Ten days after admission, a Hickman catheter was placed for central venous access, and he began reinduction chemotherapy with mitoxantrone and etoposide for 3 days (days 1 to 3), followed by cytarabine over 3 days (days 8 to 10). On the day prior to initiation of chemotherapy, norfloxacin (400 mg/day) and fluconazole (400 mg/day) were initiated for gut decontamination and fungal prophylaxis, respectively. Vancomycin was added to the antimicrobial regimen on day 5 due to persistent fevers. Blood samples for culture were obtained from the Hickman catheter once every 24 h when the patient's temperature exceeded 100.5°F.

His hospital course was complicated by persistent fevers of more than 100.0°F from the day of admission throughout his hospitalization, severe mucositis, prolonged neutropenia, and postherpetic neuralgia pain due to herpes zoster infection. On day 12 following documentation of a bone marrow biopsy specimen clear of leukemia, sargramostim was initiated to accelerate neutrophil recovery. On day 15, fluconazole prophylaxis was discontinued and empiric amphotericin B therapy was initiated for persistent fevers. Patient 1 was enrolled in a clinical trial comparing the efficacy of liposomal amphotericin B and conventional amphotericin B and received liposomal amphotericin B at 5 mg/kg of body weight per day on days 15 to 17. Blood samples for culture obtained on days 17 and 18 grew yeasts, later identified as *C. albicans*. On day 18, empiric liposomal amphotericin B was discontinued and conventional amphotericin B at a dosage of 1.25 mg/kg/day was initiated, along with flucytosine therapy at 150 mg/kg/day. Patient 1 continued on this regimen for the duration of his hospitalization. All

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blood samples for culture obtained following the initiation of conventional amphotericin B and flucytosine therapies were negative for fungi or bacteria.

On day 35, patient 1 was transferred to the intensive care unit due to severe gastrointestinal (GI) bleeding and deteriorating clinical status. He remained severely neutropenic with an absolute neutrophil count (ANC) of $<100/\mu l$ until day 49. On day 51, he died as a result of persistent GI bleeding. An autopsy was not performed.

Case report for patient 2. Patient 2, a 41-year-old female with acute myelogenous leukemia (AML) status postinduction, was admitted in December 1994 for evaluation of relapsed disease. She was afebrile with an ANC of $364/\mu l$ on admission. Norfloxacin (400 mg/day) and fluconazole (400 mg/day) were initiated for gut decontamination and fungal prophylaxis, respectively, on the day of admission. On the day after admission, she spiked a temperature to 105.9°F and was given ceftazidime, amikacin, and vancomycin. Subsequently, blood samples for culture obtained 2 days after admission grew *Pseudomonas aeruginosa*. Intravenous acyclovir was initiated on day −1 for viral prophylaxis. As part of a phase II clinical trial evaluating cytokine therapy in AML, PIXY 321, a fusion protein of granulocyte-macrophage colony-stimulating factor and interleukin 3, was initiated 3 days after admission (day −1) and was continued until day 50. Reinduction chemotherapy with cytarabine for 6 days (days 1 to 6) and mitoxantrone for 1 day (day 2) was initiated 4 days after admission. On day 2, ceftazidime was discontinued and imipenem was initiated for persistent fevers and symptoms consistent with sepsis. On day 11, fluconazole prophylaxis was discontinued and empiric amphotericin B (0.5 mg/kg/day) was initiated for persistent fevers. *C. albicans* was isolated from cultures of blood obtained from the patient's Hickman catheter on days 19, 20, and 21. On day 21, the amphotericin B dose was increased to 1.0 mg/kg/day and flucytosine therapy at 150 mg/kd/day was initiated. All subsequent blood samples obtained for culture were negative for fungi.

Patient 2 remained neutropenic until day 31. Flucytosine was discontinued on day 38 due to poor compliance. Amphotericin B was discontinued on day 42. Her hospital course after engraftment was complicated by changing mental status, persistent fevers, recurrent gram-negative bacteremia, poor caloric intake, severe GI toxicity, and thrombocytopenia. Patient 2 was discharged on day 63 with no evidence of a fungal infection.

Surveillance cultures. Weekly throat and perianal swab samples for fungi were collected from all leukemic and bone marrow transplant recipients at Emory University Hospital and plated onto Sabouraud's dextrose agar with penicillin and streptomycin. Isolates of *C. albicans* were identified by germ tube formation. Patient 1 was persistently colonized with *C. albicans* in the throat and perianal area from the day after admission until death. Patient 2 was colonized with *C. albicans* in the throat and perianal area from hospital days 5 through 33 and then again on day 56.

Antifungal susceptibility testing. Broth macrodilution MICs were determined by the National Committee for Clinical Laboratory Standards M27-P method (7). Yeasts at a final concentration of 0.5×10^3 to 2.5×10^3 cells/ml were incubated in air at 35°C for 48 h with twofold dilutions of fluconazole (0.125 to 64 μg/ml) or amphotericin B (0.0313 to 16 μg/ml). The MIC was that concentration that completely inhibited the growth (amphotericin B) or that produced an 80% reduction of turbidity by comparison with that produced by the drug-free control (fluconazole). The broth macrodilution MICs were confirmed by broth microdilution testing at the Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio. The MICs of fluconazole and amphotercin B for both isolates were >64 and 1 μg/ml, respectively. The MICs of fluconazole, ketoconazole, and itraconazole were also determined by the agar dilution method with high-resolution medium (Oxoid) and were >100, 6.3, and >25 μg/ml, respectively.

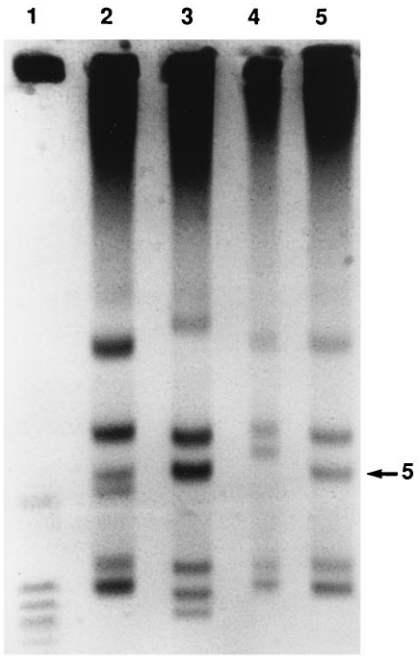

FIG. 1. Transverse alternating field electrophoretic karyotyping of fluconazole-resistant isolates of *C. albicans*. Lanes: 1, *Sacchromyces cerevisiae* size markers; 2, *C. albicans* B792 (reference strain); 3, *C. albicans* B311 (reference strain); 4, *C. albicans* isolate from patient 1; 5, *C. albicans* isolate from patient 2. The arrow marks the chromosome homolog 4-5 pair that showed a size difference.

Strain typing. Electrophoretic karyotyping was done by Tim Lott at the Centers for Disease Control and Prevention by using transverse alternating field electrophoresis (5). The conditions for electrophoresis were as follows: 15°C, 100 mA, a 72-h run time, a 5-min switch time, and 1% agarose. The karyotype analysis showed a difference in the chromosome homolog 4-5 pair between the two strains (Fig. 1).

Fluconazole uptake. Accumulation of fluconazole was measured as described previously (9). The accumulation of fluconazole by the strains from patients 1 and 2 and by strain Y01.345 is shown in Fig. 2. Y01.345 is a standard, fluconazole-sensitive strain used in the uptake assay. The values in Fig. 2 are the means ± standard deviations of three measurements. The strains from both patients 1 and 2 accumulated large amounts of fluconazole.

Membrane sterol analysis. Membrane sterols were extracted, converted to trimethylsilyl derivatives, and analyzed with a gas chromatographic mass spectrometer as described previously (2). Neither strain had detectable ergosterol. Membranes of the isolate from patient 2 contained 3β-ergosta-7,22-dienol and 3β-ergosta-8-enol, and membranes of the isolate from patient 1 contained 3β-ergosta-7,22-dienol and 3β,5α-ergosta-7,22-dienol. These membrane sterol patterns are indicative of a mutation in the $\Delta^{5,6}$-sterol desaturase gene.

Fluconazole IC_{50} determinations. Fluconazole inhibition of sterol biosynthesis was analyzed by measuring the incorporation of [^{14}C]mevalonic acid into ergosta-7,22-dienol and ergosta-8-enol in cell lysates (6). Inhibition of sterol synthesis

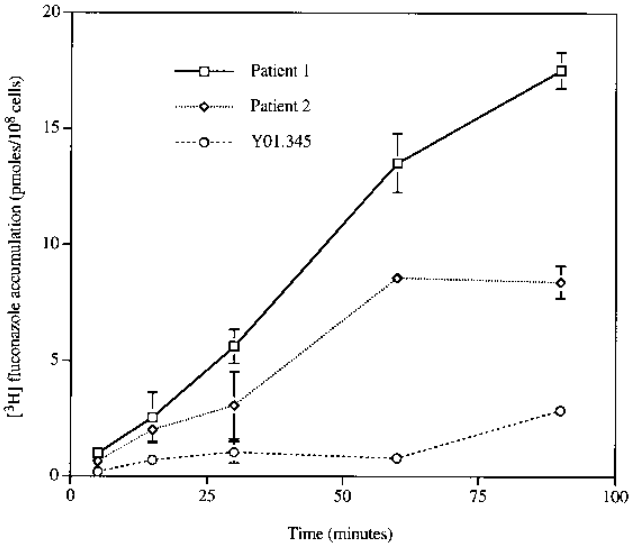

FIG. 2. Accumulation of fluconazole by fluconazole-resistant (strains from patients 1 and 2) and a fluconazole-sensitive (strain Y01.345) strains of *C. albicans*. Points are means ± standard deviations.

was calculated as a percentage reduction of incorporation of radioactivity into sterols in fluconazole-treated samples versus that in control samples without the drug. The IC_{50}, the concentration of fluconazole required to inhibit sterol synthesis by 50%, was then calculated. The fluconazole IC_{50}s were 89 and 62 nM for the strain from patient 2, and 178 and 191 nM for the strain from patient 1 in two separate experiments. The IC_{50} for the fluconazole-sensitive control strain Y01.345 was 42 nM.

Mouse model of systemic candidiasis. Specific-pathogen-free female CR CD1 mice with an average weight of 20 g were inoculated with a range of different cell numbers of the strains from patients 1 and 2. Y01.02, a *C. albicans* strain sensitive to both fluconazole and amphotericin B, at an inoculum size of 4×10^5 cells/mouse is the standard control for this model. The results are given in Table 1. For mice infected with the standard sensitive strain at an inoculum of 4×10^5 cells, the mean survival time (MST) was <2 days. At an inoculum size of 5×10^6 cells, the MSTs were 3 days for mice infected with the strain from patient 2 and >3.5 days for mice infected with the strain from patient 1. Thus, both resistant patient isolates were less virulent than the standard sensitive strain, and the strain from patient 1 was less virulent than the strain from patient 2 in this mouse model.

Discussion. The number of reported cases of fluconazole-resistant candidemia is low, despite widespread use of the drug. In a recent review, only 31 patients were reported to have developed candidemia while receiving fluconazole, and only 19 patients with preexisting candidemia were reported to have failed fluconazole therapy (12). When the data were analyzed by species, *C. albicans* was isolated from the blood of 8.3% of patients who developed candidemia while on fluconazole and 50% of patients whose candidemia was unsuccessfully treated with fluconazole at dosages ranging from 100 to 400 mg/day. Although it is difficult to determine precisely the cause of fluconazole failure in these patients, MICs were not greater than 3.25 μg/ml for any of the *C. albicans* isolates tested. Our patients differed from those described in other reports in that fluconazole MICs were ≥64 μg/ml for isolates of *C. albicans* from cultures of blood from both patients. Breakthrough candidemia occurred in both patients, despite fluconazole prophylaxis and low-dose amphotericin B therapy.

Resistance to azoles in *C. albicans* seems to be associated mainly with long-term therapy in severely immunocompromised patients such as those with advanced AIDS or mucocutaneous candidiasis (12). In AIDS patients with oropharyngeal candidasis, azole resistance is typically due to acquisition of resistance in a previously sensitive strain and correlates with the cumulative dose of the drug. Our patients developed candidemia caused by fluconazole-resistant *C. albicans* after approximately 2 weeks of fluconazole prophylaxis at 400 mg/day. There was no other recent history of azole exposure. Since the exposure to fluconazole was relatively short in our patients, it is likely that the patients were colonized with the fluconazole-resistant strains prior to the initiation of fluconazole prophylaxis. We know that both patients were persistently colonized with *C. albicans* prior to the development of candidemia. Unfortunately, the surveillance culture isolates were not available for antifungal susceptibility testing. The blood culture isolates had different electrophoretic karyotypes, implying independent acquisition of the resistant strains by the patients rather than nosocomial transmission between them.

A number of different mechanisms of azole resistance in *Candida* spp. have been reported. Changes in the target enzyme $P\text{-}450_{DM}$ may decrease the binding affinity of azoles (3, 14). Alternatively, mutations causing overexpression of P-

TABLE 1. Virulence determiantions of fluconazole-resistant (strains from patients 1 and 2) and fluconazole-sensitive (strain Y01.02) strains of *C. albicans* in a mouse model of systemic candidiasis

Strain	Inoculum size (no. of yeasts/mouse)	No. of mice	No. of survivors								Predicted MST (days)
			Day 1 at h:						Day 2	Day 3	Day 4	
			5	6	7	8	8.5	24				
Strain from patient 2	5×10^7	4	4	4 (4)[a]	0							<1.0
	1×10^7	4	4	4	4	4 (1)	3	3 (3)	0			~1.75
	5×10^6	4	4	4	4	4	4	4	4 (4)	0		3.0
	1×10^6	4	4	4	4	4	4	4	4	4	4 (4)	>4.0
Strain from patient 1	5×10^7	4	4	4	4 (4)	0						<1.0
	1×10^7	4	4	4	4	4	4	4 (1)	3	3 (3)	0	4.0
	5×10^6	4	4	4	4	4	4	4 (1)	3	3	3 (3)	>3.5
	1×10^6	4	4	4	4	4	4	4	4	4	4 (4)	>4.0
Y01.02	4×10^5	5	5	5	5	4	4 (3)	1 (1)	0			<2.0

[a] For MST purposes, values in parentheses indicate the number of mice dead on the following day.

450_{DM} may occur, but they are only partially responsible for azole resistance. Resistance may also occur by changes in the sterol synthesis pathway which compensate for the lack of ergosterol. Mutations in the $\Delta^{5,6}$-sterol desaturase lead to the accumulation of aberrant sterols, which can partially overcome the effect of reductions in membrane ergosterol content (3). Finally, a number of strains have been shown to have reduced cytoplasmic concentrations of fluconazole either through a barrier to drug influx or a multidrug resistance-like energy-dependent efflux mechanism (9, 13).

Both of the fluconazole-resistant *C. albicans* isolates described here had similar membrane sterol patterns, indicative of a lesion in the $\Delta^{5,6}$-sterol desaturase gene. Although the membrane sterol analysis was not quantitative, it is likely that 3β-ergosta-7,22-dienol was the major sterol in the membranes of both resistant strains. The lack of ergosterol in the membranes of these strains also explains their resistance to amphotericin B. In addition, the alterations in the membrane sterol composition probably explain both the relatively slow growth in vitro and the reduced virulence of the two resistant strains in the mouse model of systemic candidiasis. The altered membrane sterol content apparently did not reduce the permeability of the cells to fluconazole; in fact, the resistant isolates accumulated large amounts of the drug.

Although the amphotericin B MICs of 1 μg/ml for these isolates are high for *Candida* spp., the MIC methods that we used are not optimal for the detection of amphotericin B resistance. Antibiotic medium 3 broth (BBL/Becton Dickinson, Cockeysville, Md.) and the Etest (AB BioDisk, Solna, Sweden) with RPMI 1640 buffered with morpholinepropanesulfonic acid have recently been shown to give higher amphotericin B MICs for amphotericin B-resistant isolates (11, 15).

The fluconazole IC_{50} for the strain from patient 1 was four to five times greater than that for the sensitive control strain. Since $P\text{-}450_{DM}$ is the target for fluconazole, it follows that either the demethylase enzyme is mutated so that it becomes less sensitive to fluconazole or it is overexpressed such that more fluconazole is required to inhibit total enzyme activity. The modest elevation of the fluconazole IC_{50} relative to that for the control strain suggests that $P\text{-}450_{DM}$ is overexpressed or mutated in the strain from patient 1. However, in this strain it is the altered $\Delta^{5,6}$-sterol desaturase rather than the overexpression or mutation of $P\text{-}450_{DM}$ that accounts for the high fluconazole MIC.

The prophylactic use of fluconazole in leukemia and bone marrow transplant recipients has been shown to reduce the incidence of systemic candidiasis (1, 16). When breakthrough candidemia occurs it is usually due to fluconazole-resistance *C. glabrata* or *C. krusei*. The emergence of fluconazole- and amphotericin B-resistant strains of *C. albicans* in this patient population is a troubling new development. Future studies will define the prevalence of resistant *C. albicans* in Emory University Hospital.

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