JOURNAL OF CLINICAL MICROBIOLOGY, June 2000, p. 2369–2373 Vol. 38, No. 6
0095-1137/00/$04.00+0

Correlation between In Vitro and In Vivo Antifungal Activities in Experimental Fluconazole-Resistant Oropharyngeal and Esophageal Candidiasis

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Received 18 November 1999/Returned for modification 23 December 1999/Accepted 1 February 2000

Oropharyngeal and esophageal candidiasis (OPEC) is a frequent opportunistic mycosis in immunocompromised patients. Azole-resistant OPEC is a refractory form of this infection occurring particularly in human immunodeficiency virus (HIV)-infected patients. The procedures developed by the Antifungal Subcommittee of the National Committee for Clinical Laboratory Standards (NCCLS) are an important advance in standardization of in vitro antifungal susceptibility methodology. In order to further understand the relationship between NCCLS methodology and antifungal therapeutic response, we studied the potential correlation between in vitro susceptibility to fluconazole and in vivo response in a rabbit model of fluconazole-resistant OPEC. MICs of fluconazole were determined by NCCLS methods. Three fluconazole-susceptible (FS) (MIC, ≤0.125 μg/ml) and three fluconazole-resistant (FR) (MIC, ≥64 μg/ml) isolates of *Candida albicans* from prospectively monitored HIV-infected children with OPEC were studied. FR isolates were recovered from children with severe OPEC refractory to fluconazole, and FS isolates were recovered from those with mucosal candidiasis responsive to fluconazole. Fluconazole at 2 mg/kg of body weight/day was administered to infected animals for 7 days. The concentrations of fluconazole in plasma were maintained above the MICs for FS isolates throughout the dosing interval. Fluconazole concentrations in the esophagus were greater than or equal to those in plasma. Rabbits infected with FS isolates and treated with fluconazole had significant reductions in oral mucosal quantitative cultures ($P < 0.001$) and tissue burden of *C. albicans* in tongue, soft palate, and esophagus ($P < 0.001$). In comparison, rabbits infected with FR isolates were unresponsive to fluconazole and had no reduction in oral mucosal quantitative cultures or tissue burden of *C. albicans* versus untreated controls. We conclude that there is a strong correlation between in vitro fluconazole susceptibility by NCCLS methods and in vivo response to fluconazole therapy of OPEC due to *C. albicans*.

Oropharyngeal and esophageal candidiasis (OPEC) is a common opportunistic infection in immunocompromised patients. *Candida albicans*, the most common cause of OPEC may become resistant to fluconazole and other antifungal azoles. Fluconazole-resistant OPEC is a refractory infection which may cause debilitating pain, dehydration, and malnutrition in patients with AIDS (9, 19, 21, 24).

In addressing the emerging problems of mucosal and disseminated candidiasis, the Antifungal Subcommittee of the National Committee for Clinical Laboratory Standards (NCCLS) developed standardized methods for in vitro antifungal susceptibility (15). The demonstration of reliable correlations between MICs and clinical antifungal efficacy is paramount to successful clinical application of these standardized methods. Assessment of clinical antifungal efficacy in patients with OPEC may be confounded by variations in host response, compliance with antifungal therapy, usage of concomitant antifungal agents, different antiretroviral regimens, and selective effects of antibacterial antibiotics administered for concomitant bacterial infections.

We therefore investigated the correlation between in vitro antifungal susceptibility performed by NCCLS methods and in vivo antifungal efficacy in a rabbit model of fluconazole-resistant OPEC. This model resembles the human infection, but permits evaluation of in vitro in vivo correlations, with all hosts being uniformly immunosuppressed, receiving the same oral antibiotics, and consistently receiving the same dosage of fluconazole without concomitant antifungal therapy.

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MATERIALS AND METHODS

Organisms. Three isolates each of fluconazole-resistant (FR) *C. albicans* (fluconazole MIC, ≥64 μg/ml) (isolates 105, 126, and 272) and fluconazole-susceptible (FS) *C. albicans* (fluconazole MIC, ≤0.125 μg/ml) (isolates 117, 196, and 409) were used for all experiments. Each isolate was obtained from a different human immunodeficiency virus (HIV)-infected child being monitored at the National Cancer Institute in the National Institutes of Health Warren Grant Magnuson Clinical Center. FR isolates were recovered from children with severe OPEC refractory to fluconazole, whereas, the FS isolates were recovered from children with asymptomatic oropharyngeal candidiasis responsive to fluconazole. The isolates were identified as *C. albicans* by germ tube formation, corn meal agar morphology, and 20C Analytic Profile Index strips (Biomerieux, Marcy l'Etoile, France). All isolates were stored on potato dextrose agar (PDA) slants at −70°C. Isolates were subcultured from PDA slants onto Sabouraud dextrose agar plates for use in in vitro and in vivo studies.

MICs. Broth macrodilution testing was performed according to NCCLS document M27-A (15). Briefly, stock solutions of fluconazole (Roerig-Pfizer, New York, N.Y.) were made with RPMI 1640 with MOPS (morpholinepropanesulfonic acid) buffered to pH 7.0 (Biowhittaker, Walkerville, Md.). The yeast inoculum size ranged between 0.5×10^3 and 2.5×10^3 CFU/ml. Dilutions of the inoculum were made with RPMI 1640 buffered to pH 7.0 with MOPS. Serial twofold dilutions were further performed to achieve final concentrations from

0.125 to 64 μg/ml for fluconazole. The tubes were incubated at 35°C in air. The MICs were recorded at 24 and 48 h. Growth of *C. albicans* with fluconazole was compared to that of the inoculum control tube. ATCC strains 90028 (*C. albicans*), 6258 (*Candida krusei*), and 22019 (*Candida parapsilosis*) were included for quality control.

Animals. Twenty-five female New Zealand White rabbits weighing 2.5 to 3.5 kg were used throughout these experiments. Animals were individually housed and provided food and water ad libitum, following National Institutes of Health and American Association for the Accreditation of Laboratory Animal Care guidelines on care and use of laboratory animals (14). Silastic venous catheters were surgically placed under aseptic operative conditions for nontraumatic venous access, as previously described (26). The central venous catheter facilitated administration of parenteral medications and withdrawal of blood for plasma pharmacokinetics. Rabbits were euthanized by intravenous pentobarbital bolus (300 mg/kg of body weight) at the completion of each experiment.

Immunosuppression. Methylprednisolone given intravenously at 5 mg/kg of body weight/day (days 1 to 17) was administered for induction and maintenance of cell-mediated immunosuppression. Corticosteroids in rabbits induce involution of mucosa-associated lymphoid tissue (MALT) and loss of mucosal immunity of the gastrointestinal tract similar to those observed in AIDS (22). Specifically, the domes and follicles of MALT are reduced in size, and the dome epithelial layer is markedly depleted of lymphocytes as well as M cells. There also is a striking depletion of follicular B cells. Moreover, numerous open lesions develop in the lumenal surface of dome epithelium in a pattern that is consistent with extensive apoptosis of M cells. These immunologic and histologic effects of corticosteroids lead to impaired mucosal immunity and permit the establishment of chronic OPEC.

Antibiotics. Gentamicin (80 mg/liter) and vancomycin (50 mg/liter) were administered in each rabbit's drinking water starting on day 1 and continuing through day 18 in order to reduce mucosal bacterial colonization competitive with *C. albicans*.

Inoculation. Organisms from stock isolates were stored in skim milk at −70°C, streaked onto Sabouraud glucose agar (SGA) plates, and incubated at 37°C for 24 h. Three to five discrete colonies were then inoculated into 50 ml of Emmon's modified Sabouraud broth (pH 7.0) and incubated at 37°C for 16 h on a shaking incubator at 80 rpm. The *Candida* suspension was then centrifuged, washed three times in sterile 0.9% NaCl, counted by hemacytometer, and diluted to the desired concentration of blastoconidia (2.5×10^8 CFU/ml) in a 5-ml volume of saline per rabbit. Inoculum size was confirmed by plating serial dilutions onto SGA check plates. The inoculum of *C. albicans* was administered daily for 7 days (days 4 through 10 of immunosuppression and antibiotics) as a 2-ml volume orally via a 16-gauge Teflon catheter attached to a 3-ml syringe.

Antifungal therapy. Fluconazole (kindly provided as a gift from Roerig-Pfizer, New York, N.Y.) was administered at 1 mg/kg of body weight/day intravenously twice daily for 7 days (days 11 to 17).

Measurement of in vivo antifungal activity. Therapeutic response to fluconazole was measured by daily oral mucosal quantitative cultures, daily rectal quantitative cultures, postmortem histologic evidence of candidiasis, and postmortem quantitative cultures of tongue, soft palate, buccal mucosa, and esophagus. The tongue, soft palate, buccal mucosa, and esophagus were resected en bloc postmortem. Tissues were homogenized and quantitatively cultured by serial 10-fold dilutions as previously described (28). Other representative tissue sections were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with Gomori methenamine silver and periodic acid-Schiff.

Determination of concentrations of fluconazole in plasma and esophageal tissue. Concentrations of fluconazole in plasma and tissue were determined by high-performance liquid chromatography, as previously described (27). Plasma drug pharmacokinetic parameters, including the mean peak plasma drug concentration, were determined in rabbits that received a single 1-mg/kg dose of fluconazole intravenously. Briefly, the area under the plasma concentration-time curve from 0 to 12 h (AUC_{0-12}) was calculated by using the linear trapezoidal rule up to the final measured concentration and then extrapolated to infinity. Plasma pharmacokinetic variables were calculated and compartmentally analyzed for best fit. The terminal half-life ($t_{1/2}$) in the postdistributive phase was determined by using weighted, nonlinear least-squares regression analysis (7). The postdistributive phase of the curve analyzed for the terminal rate constant was selected as the most terminal linear portion of the log[concentration] · time curve. Clearance (C) was calculated as dose/AUC_{0-12}. Concentrations of fluconazole were determined in esophageal tissue 24 h after administration of the last dose. Standard curves were established by using esophageal tissue from normal untreated rabbits.

RESULTS

Correlation between in vitro antifungal susceptibility and in vivo microbiologic response to antifungal therapy. Rabbits infected with FS isolates and treated with fluconazole ($n = 12$) had a significant reduction in oral mucosal quantitative cultures ($P < 0.001$) and a significant reduction in tissue burden of all mucosal tissues ($P < 0.001$) relative to untreated controls ($n = 13$) (Fig. 1 and 2).

Rabbits infected with FR isolates were unresponsive to fluconazole ($n = 12$), with no significant reduction in daily oral mucosal quantitative cultures during therapy and with no significant reduction of *C. albicans* tissue burden in the tongue, soft palate, and esophagus in comparison to untreated controls ($n = 14$) (Fig. 1 and 3).

Histology. Untreated control animals infected with FS and FR isolates and treated animals infected with FR isolates of *C. albicans* demonstrated pseudohyphae, hyphae, and blastoconidia invading the nonkeratinizing squamous epithelium of the mucosal surfaces (Fig. 4). There was no histologic evidence of fungal elements in fluconazole-treated rabbits infected with FS isolates of *C. albicans*, which was consistent with the microbiologic clearance of infection due to these isolates.

Relationship between MICs and concentrations of fluconazole in plasma and esophageal tissue. The peak level of fluconazole in plasma (C_{max}) was 1.27 ± 0.09 μg/ml. This value is similar to that achieved in humans receiving the same dosage. The esophageal/plasma fluconazole concentration ratio was 1.4 ± 0.5, indicating high-level penetration of fluconazole into the esophagus. The plasma pharmacokinetics of fluconazole closely followed a two-compartment model: AUC_{0-12} = 8.2 ± 0.5 μg/ml · h; $t_{1/2}$ = 12.5 ± 4.6 h; Cl = 0.21 ± 0.06 liter/h; and C_{min} = 0.43 ± 0.03 μg/ml. Thus, the fluconazole dosage of 1 mg/kg maintained levels of approximately 4 to 10 times above the MIC for the FS *C. albicans* isolates throughout the dosing interval. In comparison, the MICs for the FR isolates of *C. albicans* were ≥50 times the levels of fluconazole in plasma.

DISCUSSION

This study describes a strong correlation between in vitro and in vivo antifungal susceptibility to fluconazole in an immunosuppressed rabbit model of OPEC. Animals infected with FS isolates of *C. albicans* had virtually complete eradication of infection in response to fluconazole. Rabbits infected with fluconazole-resistant isolates of *C. albicans* had no response to fluconazole and were comparable to untreated control animals in the extent and severity of mucosal infection. Resistance to fluconazole occurred in the presence of sustained circulating concentrations of fluconazole in plasma and esophageal tissue.

The recent development of standardized methods by the NCCLS subcommittee on antifungal compounds has greatly contributed to investigation and understanding of the relationships between in vitro, in vivo, and clinical responses to antifungal compounds (1, 2, 6, 13, 18, 20). Patients infected with FR *C. albicans* tend to have profound immunosuppression related to more advanced HIV infection (11, 24). The relative contribution of the immunosuppression versus the intrinsic microbiologic resistance may be difficult to elucidate in a clinical context. The findings from these studies indicate that in vitro resistance to fluconazole, as measured by NCCLS macrodilution methods, correlates significantly with in vivo refractoriness to antifungal therapy for mucosal candidiasis.

As clinical microbiology laboratories utilize NCCLS methodologies to guide the management of patients with mucosal candidiasis, a firm scientific foundation of in vitro and in vivo correlations will provide more meaning to these interpretations. The findings in this study further contribute to the accumulating body of data on the predictive utility of the NCCLS methodology for antifungal susceptibility for antifungal azoles.

To our knowledge, this is the first reported model of fluconazole-resistant OPEC due to *C. albicans*. The findings from

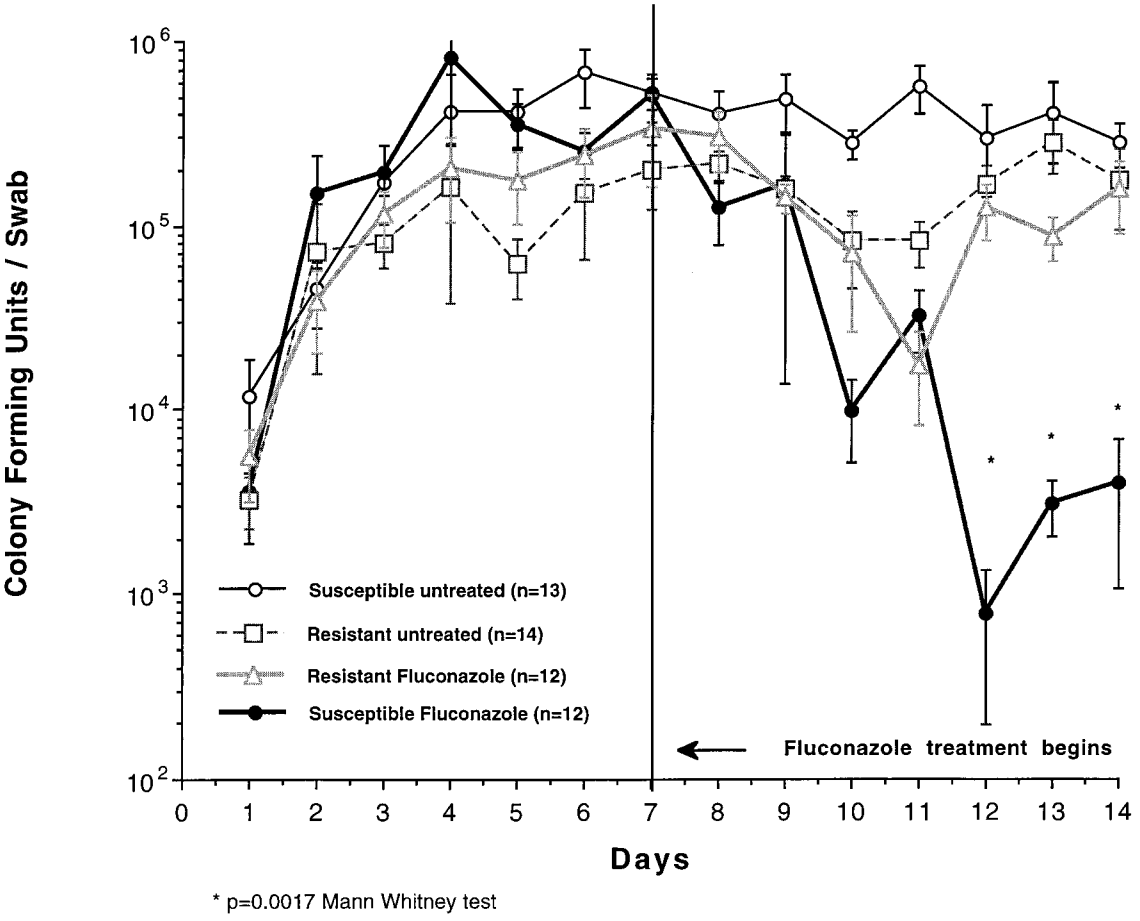

FIG. 1. Serial quantitative oral mucosal surveillance cultures in rabbits infected with either FS or FR *C. albicans*. FR isolates were not cleared from the oral cavity of treated rabbits.

this study complement the important work by Anaissie and colleagues (1) and others (3, 13, 25), who have investigated experimental azole-resistant disseminated candidiasis. For example, a murine model of disseminated candidiasis due to *Candida krusei* was found to be less susceptible to fluconazole than was a model of disseminated candidiasis due to FS *C. albicans* (1). Najvar and colleagues (13) reported that a murine model of disseminated candidiasis due to FR *C. albicans* was less responsive to fluconazole than was a model of disseminated candidiasis due to FS *C. albicans*. Given the problematic nature of investigating in vitro-in vivo correlations in human candidiasis, carefully defined and clinically applicable laboratory animal models of mucosal and disseminated candidiasis are critical tools in understanding the relationships of in vitro antifungal drug resistance and in vivo therapeutic outcome.

Patients with fluconazole-resistant OPEC usually have severe impairment of mucosal immunity. Corticosteroids in this model contribute to ablation of mucosa-associated lymphoid cell components (22). The resulting depletion of B- and T-cell domains and regression of lymphoid tissues in the gastrointestinal tract bear features similar to those encountered in the MALT of HIV-infected patients. FR *C. albicans* has been described predominantly in the setting of OPEC, rather than in disseminated candidiasis. Thus, an immunosuppressed animal model of OPEC may be particularly valuable for correlation

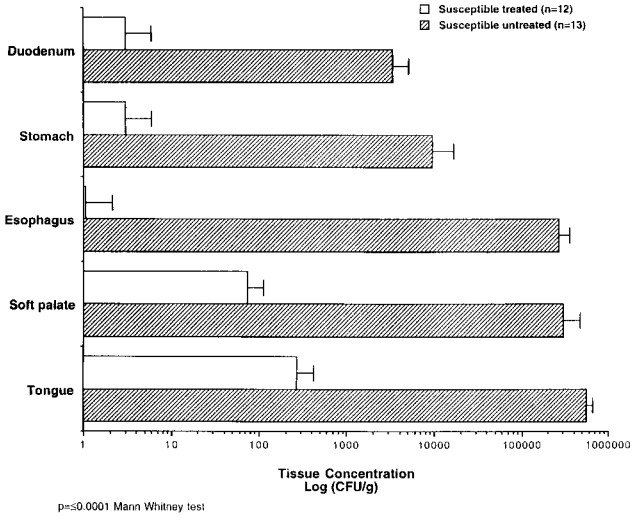

FIG. 2. Concentrations of FS *C. albicans* in oral, esophageal, gastric, and duodenal tissues of rabbits treated with fluconazole in comparison to those of untreated controls.

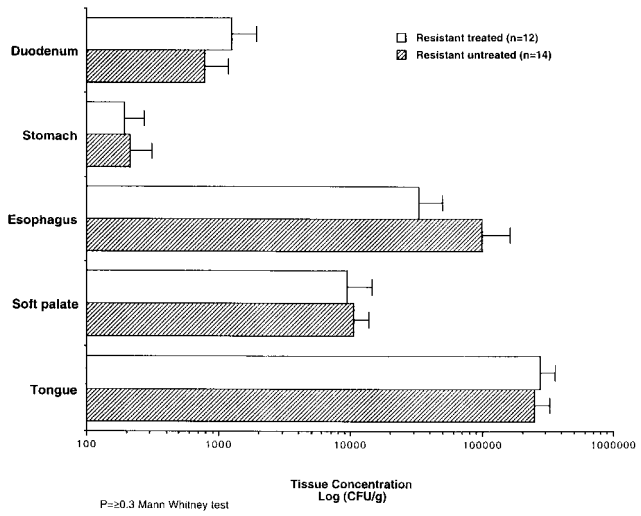

FIG. 3. Concentrations of FR *C. albicans* in oral, esophageal, gastric, and duodenal tissues of rabbits treated with fluconazole in comparison to those of untreated controls.

between in vitro antifungal susceptibility methodology and in vivo antifungal response of FR *C. albicans*.

The persistent and profound mucosal immunosuppression, as well as the continuous exposure of antibiotics, in this animal model leads to sustained concentrations of *C. albicans* in oropharyngeal and esophageal tissue of approximately 10^5 CFU/g. In comparison, the course of infection in the immunocompetent model is one of initial colonization followed by gradual clearance. Colonization occurs in the oropharynx and esophagus at a significantly lower concentration in tissue of approximately 10^1 to 10^3 CFU/g.

In the initial posttreatment phase, there is a trend toward declining CFU per swab followed by a return to elevated levels of ≥105 CFU/swab. This trend may reflect initial postinoculation host clearance of *C. albicans* followed by regrowth under the influence of persistently impaired mucosal immunity. These trends may also reflect a subpopulation of *C. albicans* that may be more susceptible to fluconazole, after which the more resistant population overgrows the mucosal surfaces under azole selective pressure.

The lack of response to fluconazole in HIV-infected patients with refractory mucosal candidiasis also has been attributed to inadequate bioavailability or lack of tissue delivery of fluconazole to the site of infection(s). Our findings indicate that esophageal fluconazole levels equal or exceed plasma fluconazole levels.

The mean fluconazole C_{max} of 1.27 μg/ml in this study is similar to that obtained in humans and is approximately 10 times greater than that of the MIC for the susceptible isolates. Additionally, the plasma fluconazole concentrations were sustained above the MIC throughout the 12-h dosing interval. The C_{max} for the given dosage in this model is similar to that achieved at 1 mg/kg/day in human children and adults (4, 10).

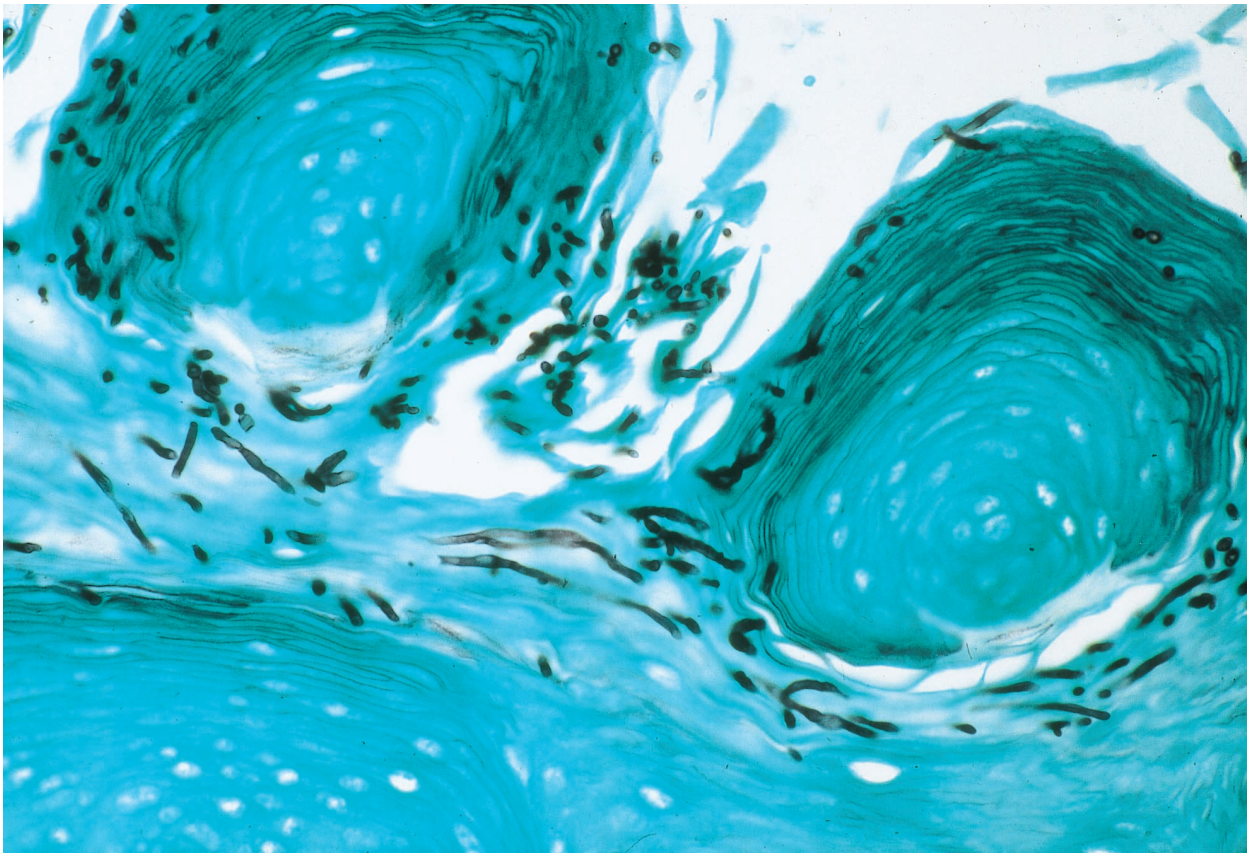

FIG. 4. Dorsum of tongue in fluconazole-treated rabbit infected with FR *C. albicans*. Pseudohyphae, hyphae, and blastoconidia are invading the nonkeratinizing squamous epithelium (Gomori methenamine silver stain, original magnification ×400). Similar histology was present in untreated controls infected with FR or FS isolates of *C. albicans*.

In comparison, the MICs for the resistant isolates exceeded the peak plasma drug concentration by approximately 50 times. Plasma drug concentrations never exceeded the MIC at any time during the dosing interval. The concentrations of fluconazole in esophageal tissue exceeded the MICs for the FS isolates by approximately 10 times, but were approximately 50-fold lower than the MICs for the FR isolates.

The isolates chosen for this study reflect the extremes of susceptibility for documentation of the validity of in vitro and in vivo correlations of fluconazole in OPEC. The encouraging results obtained in this study warrant further investigation of other key in vitro and in vivo correlations of fluconazole and other antifungal compounds. Examples would include studying the in vivo response of organisms for which fluconazole MICs are close to or at the NCCLS breakpoint for susceptibility, investigating the validity of the susceptible-dose dependent interpretive category, and analyzing whether dose escalation can be used to successfully treat infections caused by organisms for which fluconazole MICs are >32 μg/ml.

Therapeutic options often are limited in patients with azole-resistant OPEC, particularly when there is cross-resistance to other antifungal triazoles. While cyclodextrin itraconazole may provide high local concentrations of antifungal compound, some patients are unable to tolerate the cyclodextrin vehicle of this compound. Intravenous amphotericin B may be the only alternative antifungal compound available to treat esophageal candidiasis and advanced oropharyngeal candidiasis. New antifungal compounds, such as broad-spectrum triazoles and echinocandins, are being developed which may be utilized against fluconazole-resistant OPEC (8, 12, 16, 23; J. A. Vasquez, D. Boikov, M. E. Lynch, and J. D. Sobel, Abstr. 36th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F30, p. 105, 1996). As standardized methods for in vitro antifungal susceptibility are developed and refined for these new compounds, implementation of a clinically applicable animal model of azole-resistant OPEC will be important in establishing predictive in vitro and in vivo correlations.

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