

Patients with Long-Term Oral Carriage Harbor High-Persister Mutants of *Candida albicans*[∇]

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Fungal biofilms produce a small number of persister cells which can tolerate high concentrations of fungicidal agents. Persisters form upon attachment to a surface, an important step in the pathogenesis of *Candida* strains. The periodic application of antimicrobial agents may select for strains with increased levels of persister cells. In order to test this possibility, 150 isolates of *Candida albicans* and *C. glabrata* were obtained from cancer patients who were at high risk for the development of oral candidiasis and who had been treated with topical chlorhexidine once a day. Persister levels were measured by exposing biofilms growing in the wells of microtiter plates to high concentrations of amphotericin B and plating for survivors. The persister levels of the isolates varied from 0.2 to 9%, and strains isolated from patients with long-term carriage had high levels of persisters. High-persister strains were isolated from every patient with *Candida* carriage of more than 8 consecutive weeks but from no patients with transient carriage. All of the high-persister isolates had an amphotericin B MIC that was the same as that for the wild type, indicating that these strains were drug-tolerant rather than drug-resistant mutants. Biofilms of the majority of high-persister strains also showed an increased tolerance to chlorhexidine and had the same MIC for this antimicrobial as the wild type. This study suggests that persister cells are clinically relevant, and antimicrobial therapy selects for high-persister strains in vivo. The drug tolerance of persisters may be a critical but overlooked component responsible for antimicrobial drug failure and relapsing infections.

Candida species are opportunistic pathogens that are typically present in the oral cavities of healthy individuals (2, 14, 24). In immunocompromised patients, the severity of *Candida* infection can range from a superficial annoyance to a life-threatening systemic infection of the organs and sepsis. While superficial infections of the oral or vaginal mucosa are easily treatable with azoles, 5 to 8% resist therapy, producing relapses (11, 30). Systemic invasive fungal infections, characterized by the hyphal growth of *Candida albicans*, are the cause of high rates of morbidity and mortality, which approach 40% (8). Difficult-to-treat *Candida* infections also occur on prosthetic devices, such as catheters and heart valves, and an infected prosthesis requires device removal to avoid systemic infection (22).

Candida forms biofilms on the surfaces of mucosal tissues and prostheses that are highly tolerant to antifungal agents (18, 26). The recalcitrance of biofilm infections to antimicrobials is not obvious, since planktonic populations of disease-causing strains can be highly susceptible to antifungals, including azoles, echinocandins, and amphotericin B (AMB) (16).

We reported that upon attachment, *C. albicans* forms a small subpopulation (~1%) of persister cells that are completely tolerant to the currently used systemic antifungals (16) and

resemble well-characterized dormant persisters formed by pathogenic bacteria (12, 13, 18, 27, 28, 31, 32). The concentration-dependent killing of a *C. albicans* biofilm with a fungicidal agent such as AMB shows a sharply biphasic pattern, with the bulk of the cells rapidly dying and a small plateau of surviving persisters being present. Vital staining shows live persister cells present in a biofilm killed by exposure to a high level of AMB, and these cells can be sorted out from the bulk. Surviving persisters produce a new biofilm with a similarly small population of persisters, indicating that these cells are not classical resistant mutants but phenotypic variants of the wild type. Persisters exhibit multidrug tolerance, which is a hallmark of a biofilm infection.

Attachment to a surface is an important step in fungal pathogenesis, including pathogenesis resulting in vaginitis, oral thrush, and catheter biofilm infections (6, 10, 15). It seems that persisters, which form upon attachment of the pathogen to a surface, may play an important role in the tolerance of *Candida* infections to antifungals.

In *Escherichia coli*, the periodic application of a high concentration of a bactericidal antibiotic in vitro leads to the selection of high-persister (*hip*) mutants (20, 21). Importantly, these mutants have the same MIC as the wild type, but they produce considerably more persister cells. While the persister phenotype itself is not due to a mutation, high-persister strains carry mutations that cause an increased incidence of persisters. One of these mutants was mapped to an allele of a *hipA* gene coding for a toxin of the *hipBA* toxin/antitoxin module (13, 21). The mechanistic basis of HipA-dependent persister formation was recently identified (27). HipA is a protein kinase (3) that phosphorylates elongation factor EF-Tu, which leads to the inhibition of protein synthesis. This creates a dormant, per-

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sister state. The HipA7 allele, which causes the high persistence of the *hip* mutant, apparently has decreased binding to the antitoxin HipB, which leads to increased persister production.

We reasoned that a similar selection for high-persister mutants is likely to occur in vivo, especially in cases of recalcitrant infections, where pathogens are periodically exposed to high levels of antimicrobial compounds. With this in mind, we tested a collection of 150 clinical isolates of *Candida* species for their persister levels. The strains were obtained from cancer patients who received daily topical chlorhexidine (CHX) treatment. We report that the strains isolated from patients with long-term *Candida* carriage had increased levels of surviving persisters.

MATERIALS AND METHODS

Patient selection. Forty-six cancer patients, including 4 patients with hematological malignancies and 42 patients with solid tumors, from two affiliated teaching hospitals and a cancer hospital of the Shandong University Medical School were selected for this study and sampled for oral *Candida* carriage. The patients' ages ranged from 21 to 76 years, and the average age was 48.8 years. There were 29 male patients and 17 female patients. The inclusion criteria for the study were a previous diagnosis of advanced cancer for more than 1 year and treatment by chemotherapy. The exclusion criteria for this study were significant physical defects, the wearing of dentures, uncontrolled diabetes mellitus, and the use of systemic antifungal medication or steroids within the previous 2 weeks. The patients with head and neck cancer receiving radiation therapy were excluded. This study was approved by the Ethics Committee of Shandong University (approval no. 2008-03) and an institutional review board at the Northeastern University Division of Research Integrity (institutional review board no. 09-01-02).

Sampling methods. All patients received regular oral hygiene care that included 0.2% CHX daily. The oral cavities of the patients were sampled for *Candida* carriage, and the specimens were obtained 8 h following regular oral hygiene care. The specimens were collected by passing a sterile swab across mucosal surface of the inner cheek five times. The procedure was repeated a total of three times within a half-hour interval. The swabs were transferred to a sterile test tube containing 500 μ l sterile phosphate-buffered saline (PBS). The tubes containing the swabs were vigorously vortexed for 30 s to suspend the samples. After 30 s, the swabs were removed and the samples were pelleted by centrifugation and concentrated by resuspension in 50 μ l buffer. This suspension was spread plated on CHROMagar *Candida* differential medium and incubated for 24 to 48 h (24). Colonies were identified as *Candida* by CHROMagar on the basis of a color change and the colony morphology on this selective and differential medium (23). The *Candida* strains were confirmed to be members of the genus *Candida* by use of the API 20C system (BioMerieux, Marcy l'Etoile, France), based on carbon source utilization and other differential biochemical assays, according to the manufacturer's directions (33). *Candida albicans* species were additionally confirmed by observing by microscopy the induction of germ tube formation by serum (19). If multiple *Candida* colonies were present, a single isolate was chosen for further analysis for each patient and time point. Patients were sampled for *Candida* carriage once per week, until an oral *Candida* yeast was not isolated or the patient left the hospital after chemotherapy.

Antifungal drug susceptibility testing. Stock solutions (10 mg/ml) of AMB (Fisher Scientific) and CHX (Sigma-Aldrich) were prepared in dimethyl sulfoxide. The MICs for the clinical isolates were determined by broth microdilution on the basis of the CLSI guidelines described in document M27-A2 (29). Briefly, cells from an overnight culture grown in yeast-peptone-dextrose (YPD) medium were harvested by centrifugation, washed twice in sterile PBS, and resuspended to 0.5×10^3 to 2.5×10^3 cells/ml in RPMI 1640 medium (Gibco) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich). The cells were dispensed into a 96-well microtiter plate at 100 μ l per well, and the antifungals were added by making a series of twofold dilutions at 100 μ l per well in RPMI 1640 medium. The plates were incubated at 37°C for 48 h. The MICs of AMB and CHX were determined visually and were defined as the lowest concentration of drug that resulted in no growth.

***Candida* persister determination.** Twenty-four-hour biofilms were formed by standard methods, as described by Ramage et al. (25). Briefly, cells were obtained from an overnight culture grown at 30°C in YPD liquid medium. Cells

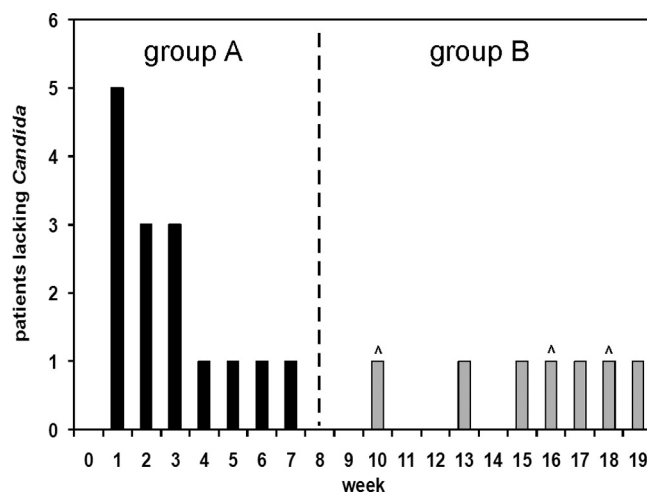


FIG. 1. Oral *Candida* carriage in patients undergoing chemotherapy. Cancer patients were sampled weekly for oral *Candida* carriage, and the first week in which *Candida* was no longer isolated was plotted. Carets above the bars indicate patients who left the study after chemotherapy concluded. The patients were divided into two groups. In group A, *Candida* was isolated for less than 8 consecutive weeks, and in group B, *Candida* was isolated for more than 8 weeks.

were harvested by centrifugation, washed twice in sterile PBS (pH 7.4), and resuspended in RPMI 1640 medium with L-glutamine and 0.165 M MOPS (Bio-Whittaker). The optical density at 600 nm of the suspension was adjusted to 0.1, or approximately 1×10^6 cells/ml. One hundred microliters of this suspension was aliquoted into the wells of a flat-bottom microtiter plate (Costar 3370; Corning). The plates were incubated for 24 h at 37°C on a microtiter plate shaker (model 4625; Lab-Line Instruments) at approximately 100 rpm. For persister quantification, the method of LaFleur et al. (16) was followed. Briefly, the biofilms were washed twice with sterile PBS to remove nonadherent cells and were challenged with 100 μ g/ml AMB or 200 μ g/ml CHX dissolved in fresh RPMI 1640 medium for 48 h at 37°C. At these concentrations, regular cells are killed and only persisters survive. The biofilms were then washed twice with PBS; and the surviving persisters were quantified by scraping the biofilm, vigorously vortexing the biofilm, serially diluting the suspension, and plating the suspension. The percentage of persisters was calculated by comparing the number of persisters with the total number of cells in an untreated 24-h biofilm control.

RESULTS

Candida carriage in the oral cavities of cancer patients.

Candida was isolated from the oral cavities of patients receiving chemotherapy for cancer treatment in 22 of 46 cases. The frequency of oral *Candida* carriage was 47.8%, slightly less than in studies which estimated the occurrence of *Candida* in the oral cavity of cancer patients to be from 66% to 70% (4, 5). Patients with *Candida* carriage were sampled once per week until an oral yeast was not isolated. For each patient, the week in which *Candida* was no longer isolated is shown in Fig. 1. The patients were divided into two groups: those with long-term carriage and those with transient carriage. Fifteen patients and 42 isolates were classified as group A, in which *Candida* was isolated for less than 8 weeks. *Candida* carriage was present for an average of 2.8 weeks in group A patients. Seven patients and 108 isolates were classified as group B, in which *Candida* was isolated for more than 8 weeks. On average, *Candida* was isolated for 15.4 consecutive weeks from group B patients. Three group B patients left the study with active *Candida* carriage when chemotherapy had concluded, including one

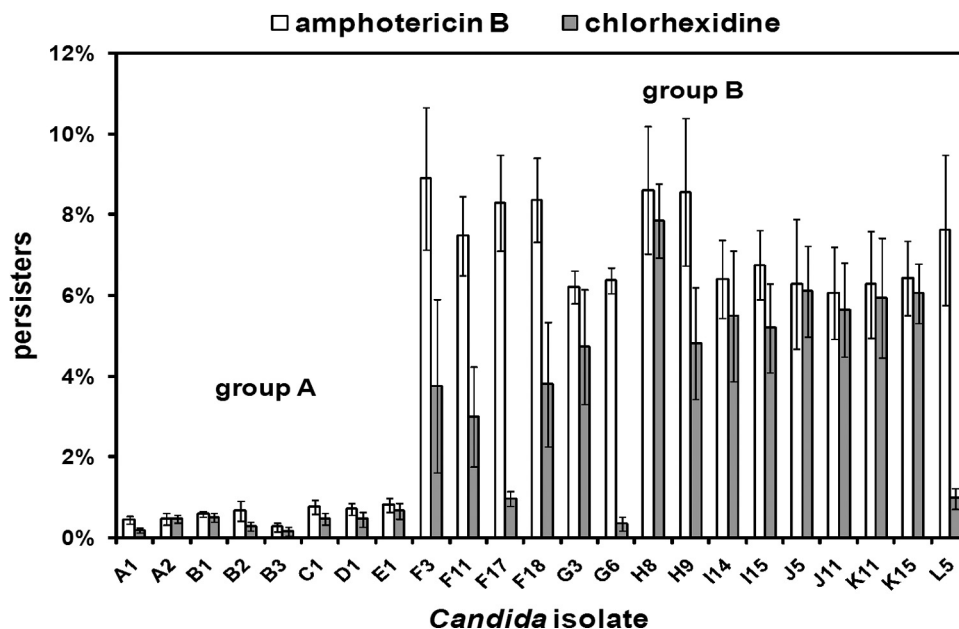


FIG. 3. *C. albicans* high-persister strains isolated from cancer patients. The level of surviving persister cells was determined by exposing the biofilms of a panel of clinical isolates to 100 $\mu\text{g/ml}$ AMB or 200 $\mu\text{g/ml}$ CHX for 48 h. After antifungal challenge, the biofilms were washed, disrupted, diluted, and plated for determination of colony counts. A total of 131 *C. albicans* strains were tested for survival in the presence of AMB, and representative strains were also tested for survival in the presence of CHX. Clinical isolates from patients A to E are representatives from group A in which *Candida* was isolated for less than 8 consecutive weeks ($n = 39$). Clinical isolates from patients F to L are *hip* mutants from group B patients, who had long-term *Candida* carriage ($n = 92$). The number following each patient indicates the week that each strain was isolated. The data represent the average of three independent experiments performed with biological duplicates, and the error bars are standard deviations.

increased levels of drug-tolerant persisters compared to the number among the susceptible isolates. These findings suggest that persisters contribute to the recalcitrance of pathogens to antimicrobials.

DISCUSSION

The discovery of persister cells in bacteria and fungi raises the possibility that drug tolerance may be an overlooked contributor to the therapeutic failure of antimicrobials (17). The present study suggests that *Candida* persisters are clinically relevant. High-persister *C. albicans* isolates were obtained exclusively from a group of cancer patients with chronic carriage. Persister cells apparently contribute to the maintenance of *Candida* carriage in the oral cavity of cancer patients treated with prophylactic CHX.

The high-persister *Candida* isolates formed increased levels of these drug-tolerant cells in biofilms, but they did not exhibit increased AMB or CHX MICs. Considering that resistance to high concentrations of AMB or CHX is extremely rare, this result was not surprising. Our results raise the intriguing possibility that persister cells may serve as an indicator of therapeutic failure for fungicidal agents. In contrast, the standard MIC detection does not differentiate between cases of rapidly resolving infections and chronic infections. Elevated MICs for azole antifungals are known to correlate with previous antifungal therapy and recurrent oropharyngeal candidiasis (11). However, the correlation between an increased MIC and recurrent infection does not appear to be true for fungicidal agents, such as AMB and CHX.

This study indicates the significance of persisters in oral candidiasis. The formation of *hip* mutants may be a general feature of recalcitrant fungal infections, although it is likely that persisters from both wild-type strains and *hip* mutants contribute to drug tolerance and the survival of the pathogen. It must be noted that a recent study reproducing our previous

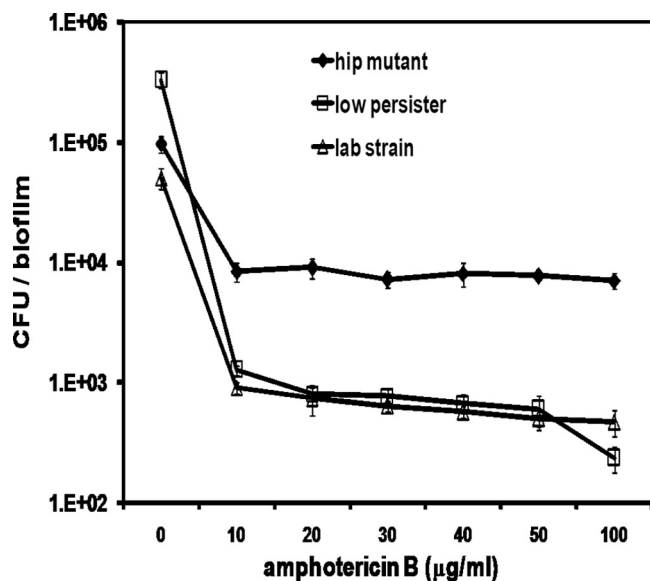


FIG. 4. Dose-dependent killing of *C. albicans* biofilms by AMB. Biofilms were cultured in RPMI 1640 medium for 24 h and exposed to AMB for 48 h. After the antifungal challenge, the biofilms were washed, disrupted, diluted, and plated for determination of colony counts. The data represent the average of three independent biological replicates for each strain, and the error bars are standard deviations.

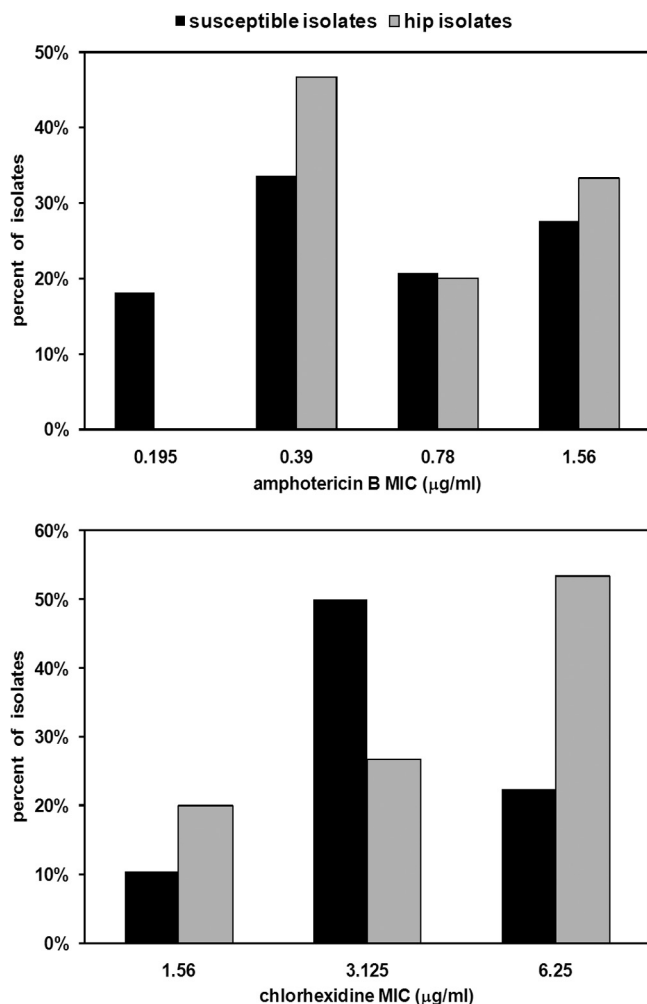


FIG. 5. Susceptibility of *Candida albicans* isolates to AMB and CHX. The MICs for the *hip* mutants and susceptible *C. albicans* strains isolated from cancer patients were determined by broth microdilution assay. The data for the isolates are expressed as the percentage of all strains in each group: $n = 116$ and $n = 15$ for susceptible strains and *hip* strains, respectively.

findings reported persisters in biofilms of some, but not all, isolates of *C. albicans* and *C. glabrata* (1). This conclusion was based on the complete eradication of the population of some strains by AMB at 100 µg/ml. However, the same experiments showed biphasic killing, suggesting the presence of cells with elevated tolerance, or persisters. Importantly, after initial killing of the bulk of the cells in the presence of low drug concentrations, surviving persisters of each strain were detected in the presence of AMB at concentrations as high as 50 µg/ml. Apparently, the level and the degree of drug tolerance of persisters vary among different strains, as well as among growth types (such as biofilm versus planktonic cell growth). In the present study, each clinical isolate produced measurable levels of persisters tolerant to 100 µg/ml AMB, although high-persister strains were identified only from the *C. albicans* isolates and not the *C. glabrata* isolates.

The strains in this study were isolated from patients with *Candida* carriage who were at high risk for the development of

candidiasis but who did not exhibit signs of infection. Thus, there is no direct evidence suggesting that persisters contribute to relapsing infections. However, *Candida* carriage has been shown to correlate with an increased incidence of systemic candidiasis in solid-organ transplant patients (7). Future studies will test whether the elevated levels of persister cells in clinical isolates of cancer patients contribute to relapsing infections. The isolation of *hip* mutants could lead to the identification of the *hip* genes by whole-genome sequencing or other comparative approaches. Additional studies are needed to determine the molecular basis and clinical relevance of persisters of pathogenic fungi.

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