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Enhanced Oxidative Killing of Azole-Resistant *Candida glabrata* Strains with *ERG11* Deletion

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The susceptibility of genetically defined *Candida glabrata* strains to killing by H_2O_2 and neutrophils was assessed. Fluconazole-susceptible L5L and L5D strains demonstrated survival rates higher than those of two fluconazole-resistant strains lacking the *ERG11* gene coding for 14α-demethylase. Fluconazole resistance can occur by mechanisms which increase fungal susceptibility to oxidative killing by H_2O_2 and neutrophils.

Mucosal candidiasis is a significant infection among human immunodeficiency virus-infected patients (2) and may portend clinical progression to AIDS (11). Although *Candida albicans* remains the most prevalent infecting yeast, non-*C. albicans* species have increased as mucosal commensals (15) and pathogens (18), particularly among the immunocompromised. Fluconazole, an antifungal drug with clinical efficacy against *Candida* species (6), is a bis-triazole which acts primarily by inhibiting 14α-demethylation of ergosterol, leading to the accumulation of 14α-methylated sterols in fungal membranes (8, 9). However, infections by fluconazole-resistant *C. albicans* (17) and *Candida glabrata* (22) have developed because of the widespread use of this drug.

Despite the high prevalence of severe mucosal candidiasis among human immunodeficiency virus patients and the potential spread of azole-resistant yeasts to other immunocompromised patients, deeply invasive or systemic candidiasis remains an uncommon infection among human immunodeficiency virus patients. Host defenses must thus play a pivotal in controlling *Candida* invasion (13). One such defense is the generation of H_2O_2 and other microbicidal oxidants generated by neutrophils (PMN) (10, 12). We investigated the killing by hydrogen peroxide (H_2O_2) and PMN of four strains of *C. glabrata* with defined *ERG3* and *ERG11* gene deletions, as these deletion mutants lack $\Delta^{5,6}$-desaturase and 14α-demethylase enzyme activities, resulting in altered yeast membrane sterol synthesis and fluconazole resistance (7).

***C. glabrata* yeasts.** The characteristics of the four strains (7) are briefly summarized as follows.

(i) L5L. L5L has intact *ERG11* and *ERG3* genes and is susceptible to fluconazole (MIC, 6.25 μg/ml).

(ii) L5D. L5D is an *ERG3* gene deletion mutant, lacking $\Delta^{5,6}$-desaturase activity, which synthesizes fecosterol and is susceptible to fluconazole (MIC, 3.12 μg/ml).

(iii) L5LUD40R. L5LUD40R is an *ERG11* gene deletion mutant which arose as a spontaneous aerobically viable mutant from a lawn of L5LUD40 deletion mutants; it lacks 14α-demethylase activity, synthesizes lanosterol as its major sterol, and is resistant to fluconazole (MIC, >100 μg/ml).

(iv) L5DUD61. L5DUD61 is a double mutant, obtained by deleting the *ERG11* gene following deletion of the *ERG3* gene, which lacks both $\Delta^{5,6}$-desaturase and 14α-demethylase activities and synthesizes 14α-*methyl*-fecosterol; it is resistant to fluconazole (MIC, >100 μg/ml).

Prior to each experiment, *C. glabrata* strains were grown in broth containing 1% yeast extract, 2% peptone, and 2% dextrose (YEPD broth) for 16 h at 30°C and washed thrice in phosphate-buffered saline (PBS) (pH 7.4).

PMN. Heparinized whole blood from healthy donors was separated (3) with lymphocyte separation medium (Organon-Teknika Corporation, Durham, N.C.) gradient with centrifugation at 300 × *g* at room temperature for 30 min. The resultant erythrocyte-PMN pellet was then subjected to gravity sedimentation with 3% dextran (T500; Pharmacia, Uppsala, Sweden) at room temperature for 15 min followed by hypotonic lysis. This procedure yielded ≥95% PMN and <5% eosinophils; viability was >99% by trypan blue exclusion.

Oxidative killing. For H_2O_2 experiments, 10^7 *C. glabrata* yeasts were incubated in PBS with and without 10 mM H_2O_2 for 1 h at 37°C. In cell experiments, 10^7 yeasts were tumbled at 12 rpm with or without 10^7 human PMN in 10% fresh or heat-inactivated autologous serum for 1 h at 37°C. An incubation of 1 h represented 0.27 to 0.81 generation times under optimal growth conditions (7). Phagocytosis was assessed by counting the number of ingested *C. glabrata* yeasts within 200 PMN observed in random oil immersion fields. The phagocytic index was calculated as the mean number of ingested *C. glabrata* yeasts per PMN from three experiments. CFU were enumerated on two to three replicate Sabouraud plates for each experiment. Killing was calculated by using the formula 100%(control CFU − oxidative CFU)/control CFU. Data were analyzed by using a two-tailed paired-sample *t* test (5).

Killing experiments in the presence of H_2O_2 and PMN were performed for 1 h for all four mutant strains, with each mutant strain serving as its own control. As shown in Table 1, all *C. glabrata* strains were susceptible to 10 mM H_2O_2 in a cell-free system. However, the rates of killing of the L5LUD40R and L5DUD61 mutants were considerably greater than those of the L5L and L5D strains. PMN killing of *C. glabrata* mutants occurred in the presence of 10% heat-inactivated sera and was augmented with fresh autologous sera, as seen in Tables 2 and 3, respectively. Killing by PMN of L5LUD40R and L5DUD61 mutants was markedly enhanced compared with that of L5L, despite similar phagocytic indices and gross catalase activities (not shown) for all strains. In addition, both L5LUD40R and L5DUD61 mutants were significantly more susceptible to kill-

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TABLE 1. Killing of *C. glabrata* strains in the presence of 10 mM H_2O_2[a]

C. glabrata strain	% Killing (mean ± SD)
L5L	37.7 ± 2.6
L5D	28.4 ± 7.7
L5LUD40R	81.4 ± 0.7[b]
L5DUD61	60.4 ± 7.7[b]

[a] Data are expressed as means ± standard deviations from three separate experiments.
[b] Significant ($P < 0.01$ compared with values for L5L and L5D).

TABLE 3. Killing and phagocytosis of *C. glabrata* strains in the presence of PMN with 10% fresh autologous sera

C. glabrata strain	% Killing (mean ± SD)	Phagocytic index (mean ± SD)
L5L	62.5 ± 12.6	0.790 ± 0.156
L5D	63.5 ± 13.7	0.872 ± 0.200
L5LUD40R	96.8 ± 2.8[a]	0.877 ± 0.094
L5DUD61	95.4 ± 3.6[a]	0.862 ± 0.116

[a] Significant ($P < 0.00005$ compared with values for L5L and L5D from 10 experiments each).

ing than the L5D mutant. No differences in killing between L5LUD40R and L5DUD61 or between L5L and L5D strains were discerned.

The enhanced susceptibility of azole-treated *Candida* yeasts to killing by oxidants or phagocytes has been reported previously. The killing of *C. albicans* by rodent peritoneal exudative leukocytes and human PMN was reportedly enhanced in the presence of ketoconazole (4, 19). The rates of killing in the presence of H_2O_2 (0.4 to 3.5 mM) for both a clotrimazole-treated *C. albicans* (KD14) and a 14α-demethylase-deficient mutant (KD4900) were shown to be greater than those for the untreated strain and the 14α-demethylase-proficient revertant (KD4907), respectively (20). *C. albicans* pretreated with fluconazole (4 and 8 μg/ml) was more sensitive to killing by human PMN (16). *C. glabrata* preincubated with itraconazole (10^{-4} to 10^{-9} mM) was more vulnerable to killing by guinea pig PMN and sodium deoxycholate (3.12 g/liter) as shown by trypan blue staining of yeasts (24). The mechanisms for this susceptibility have not been elucidated.

For our study, all *C. glabrata* strains had specific characterizations of *ERG3* and *ERG11* gene deletions, 14α-demethylase and $\Delta^{5,6}$-desaturase enzyme activities, and specific growth rates and sterol profiles (7). All strains were susceptible to oxidative killing by H_2O_2 in a cell-free system at a biologically relevant concentration achievable within PMN vacuoles (10, 12). The L5LUD40R and L5DUD61 mutants were the most susceptible of the strains tested. Killing by human PMN of these *C. glabrata* strains was shown to be consistent with that of various *Candida* species (14, 21, 24). The presence of fresh serum enhanced both phagocytosis and killing by PMN because of better opsonization of yeasts with serum complement (21) and other heat-labile components (13). However, this enhanced killing by both H_2O_2 and PMN was shown to be specifically due to the *ERG11* and not the *ERG3* deletion among these four genetically defined *C. glabrata* strains.

We found the fluconazole-susceptible L5D mutant to have a susceptibility to killing by H_2O_2 and PMN similar to that of *C. glabrata* L5L. This finding correlates with the azole susceptibility profiles of the two strains. Mutations of the *ERG3* gene in the closely related yeast *Saccharomyces cerevisiae* have been reported to be associated with azole resistance (23). Although *erg3* deletion mutants of *S.* cerevisiae have been constructed, the susceptibilities to azole agents and to oxidative killing of these genetically defined *erg3* deletion mutants have not yet been tested (1).

Membrane perturbations associated with the absence of 14α-demethylase activity and sterol alterations in the *C. glabrata* L5LUD40R and L5DUD61 mutants could enhance the entry of oxidants into these yeasts. The mechanisms by which these deletion mutants are rendered more vulnerable to oxidative killing are currently being studied.

In summary, fluconazole-resistant *C. glabrata* strains lacking 14α-demethylase activity because of *ERG11* gene deletions were highly susceptible to killing by 10 mM H_2O_2 and human PMN.

TABLE 2. Killing and phagocytosis of *C. glabrata* strains in the presence of PMN with 10% heat-inactivated sera

C. glabrata strain	% Killing (mean ± SD)	Phagocytic index (mean ± SD)
L5L	43.9 ± 27.1	0.623 ± 0.140
L5D	53.8 ± 24.0	0.662 ± 0.157
L5LUD40R	80.8 ± 15.1[a]	0.633 ± 0.113
L5DUD61	80.8 ± 14.0[a]	0.692 ± 0.129

[a] Significant ($P < 0.0005$ and $P < 0.01$ compared with values for L5L and L5D, respectively, from nine experiments).

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