ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2006, p. 1573–1577 Vol. 50, No. 4
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.4.1573–1577.2006

Immunotherapy with Tacrolimus (FK506) Does Not Select for Resistance to Calcineurin Inhibitors in *Candida albicans* Isolates from Liver Transplant Patients

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Received 30 September 2005/Returned for modification 23 November 2005/Accepted 11 January 2006

In *Candida albicans*, calcineurin mediates tolerance to azole antifungal drugs, survival in serum, and virulence. In this study, we examined 24 *Candida* isolates from liver transplant recipients receiving a calcineurin inhibitor as a component of their immunosuppressive therapy. We were unable to detect a difference in susceptibility to calcineurin inhibitors in combination with fluconazole, serum, or calcium in these isolates.

Invasive fungal infections are a significant complication of organ transplantation occurring in up to 42% of liver transplant recipients (5, 8, 23–25). *Candida* species are the causative agent in 62% to 91% of invasive fungal infections after liver transplantation (23). Manipulation of the gastrointestinal tract during surgery allows translocation of endogenous organisms across the intestinal epithelium, resulting in a unique susceptibility to invasive candidiasis with most infections occurring within the first month posttransplantation.

Although *Candida albicans* is the predominant species with fluconazole therapy, up to one-third of isolates are non-*C. albicans Candida* spp., including *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* (11, 16). Although the incidence of fungal disease in liver transplantation has declined largely due to advancements in surgical techniques (27), the high associated mortality (25% to 67%) (14, 23, 25–27) highlights the continued need to understand the pathogenesis of these infections and to develop new treatment strategies.

Two mainstay immunosuppressants in liver transplantation, tacrolimus (FK506) and cyclosporine A (CsA), inhibit the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin that is required for T-cell activation in response to antigen presentation (6, 7, 9a, 13, 21). CsA and tacrolimus enter the cell and bind to the immunophilins cyclophilin A and FKBP12, respectively, and the resulting drug-protein complexes bind calcineurin (4, 15), preventing T-cell proliferation and suppressing immune responses involved in transplant rejection (2, 3, 6, 9).

In addition to their roles in human immunotherapy, these drugs also inhibit calcineurin function in several human fungal pathogens, including *C. albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (1, 3, 9, 12, 20, 22, 28). In *C. albicans* inhibition of calcineurin results in enhanced susceptibility to azole antifungal drugs, susceptibility to cation stresses (including Ca^{2+}, Na^{+}, and Li^{+}), decreased survival in serum, and avirulence in a murine systemic candidiasis model (1, 3, 22). Thus, calcineurin inhibitors could have two potential roles in antifungal therapy, either through use in a combination with azole antifungals or via an intrinsic ability to decrease serum survival.

A previous proof-of-principle study used a rat endocarditis model to show that the combination of fluconazole and cyclosporine A was more effective than either drug alone at treating both primary heart vegetative lesions and kidney lesions formed via hematogenous dissemination (18), suggesting that combination therapy could be effective in an in vivo setting. Although liver transplant patients receive a calcineurin inhibitor, which previous studies suggest could protect against invasive candidiasis (1, 2, 3), a substantial proportion of patients still develop disease. Therefore, we investigated whether *Candida* isolates from patients immunosuppressed with tacrolimus exhibited altered susceptibility to these drugs with respect to azole tolerance, serum survival, and Ca^{2+} stress.

Twenty-four *Candida* isolates were collected from 22 liver transplant recipients receiving tacrolimus. The median duration of immunosuppression prior to isolate collection was 26 days and ranged from 5 days to 11 years. The strains were identified using API carbohydrate assimilation strips (bioMérieux) (16) and CHROMagar Candida (Hardy Diagnostics). Isolates consisted of 18 *Candida albicans*, 3 *Candida glabrata*, 2 *Candida parapsilosis*, and 1 *Candida tropicalis* (Table 1). The strains were further classified as either invasive or colonizing. Invasive candidiasis was defined as isolation of *Candida* from at least one blood culture or from normally sterile body fluids either intraoperatively or by percutaneous needle aspiration in patients with signs and symptoms indicative of infection (16). Isolation of *Candida* from nonsterile samples in patients who did not fulfill the above criteria and for whom antifungal therapy was not employed as treatment was considered to represent colonization. Eleven strains were considered invasive, while 13 were classified as colonizing (17).

In vitro fluconazole MIC testing was performed in triplicate for all isolates using E-test strips (AB Biodisk) on RPMI me-

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TABLE 1. *Candida* isolates used in this study

Isolate[a]	Source[b]	Species[c]	Antifungal prophylaxis	Fluconazole MIC[d] (μg/ml)	Susceptibility[e]	
					Fluconazole + tacrolimus	Serum + tacrolimus[f]
PAT1 ISO1	Eye	*C. albicans*	None	2.0–3.0	S	S
PAT2 ISO1	Peritoneal fluid	*C. albicans*	None	0.25	S	S
PAT3 ISO1	Peritoneal fluid	*C. albicans*	None	0.25–0.38	S	S
PAT4 ISO1	Peritoneal fluid	*C. albicans*	None	0.25–0.38	S	S
PAT5 ISO1	Blood	*C. albicans*	None	0.25–0.38	S	S
PAT6 ISO1	Hematoma	*C. albicans*	None	0.75	S	S
PAT7 ISO1	BAL	*C. albicans*	None	0.38	S	S
PAT8 ISO1	Sputum	*C. albicans*	None	1.5–2.0	S	S
PAT9 ISO1	BAL	*C. albicans*	Fluconazole	0.25	S	S
PAT9 ISO2	Urine	*C. albicans*	Fluconazole	0.5	S	S
PAT9 ISO3	Sputum	*C. albicans*	Fluconazole	0.39–0.50	S	S
PAT10 ISO1	Urine	*C. albicans*	None	0.38–0.50	S	S
PAT11 ISO1	BAL	*C. albicans*	Fluconazole	1.5–2.0	S	S
PAT12 ISO1	BAL	*C. albicans*	None	1.5	S	S
PAT12 ISO2	Sputum	*C. albicans*	None	1.5–2.0	S	S
PAT13 ISO1	Urine	*C. albicans*	Voriconazole	0.25–0.50	S	S
PAT14 ISO1	Blood	*C. albicans*	Fluconazole	0.25–0.38	S	S
PAT15 ISO1	Pleural fluid	*C. albicans*	Fluconazole	0.38–0.50	S	S
PAT16 ISO1	Peritoneal fluid	*C. tropicalis*	None	0.25–0.38	S	S
PAT17 ISO1	Urine	*C. parapsilosis*	None	1.5–2.0	S	S
PAT18 ISO1	BAL	*C. glabrata*	Voriconazole	**>256**	S	**R**
PAT19 ISO1	Urine	*C. glabrata*	Voriconazole	**>256**	S	**R**
PAT20 ISO1	Blood	*C. parapsilosis*	Fluconazole	0.75–1.5	S	S
PAT21 ISO1	Blood	*C. glabrata*	Fluconazole	32–48	S	S

[a] Strains in bold are invasive isolates.
[b] BAL, broncheal alveolar lavage; eye, endophthalmitis.
[c] Species was determined by API testing.
[d] Fluconazole MICs were determined by E-Test; resistance, MIC ≥ 64 μg/ml; sensitive-dose dependent, MIC = 16 to 32 μg/ml; sensitive, MIC ≤ 8 μg/ml.
[e] S, susceptible; R, resistant.
[f] Susceptibility as measured in liquid culture. S, sensitive (population change < onefold); R, resistant (population change > onefold).

dium and confirmed by NCCLS microdilution testing (19). Only two isolates, both *Candida glabrata*, were resistant to fluconazole (MIC > 64 μg/ml). All other isolates were susceptible to fluconazole, except *C. glabrata* isolate PAT21 ISO1, which was sensitive-dose dependent (Table 1). All three *C. glabrata* isolates with decreased susceptibility to fluconazole were collected from patients who had received an azole as antifungal prophylaxis (Table 1); however, azole prophylaxis was not always associated with fluconazole resistance.

Previous studies with *Candida* have shown that treatment with a calcineurin inhibitor results in enhanced susceptibility to azole antifungals (1, 3, 9a, 22). Eleven of the strains were from patients who had received prophylactic treatment with an azole concurrently with their tacrolimus immunosuppressive therapy. The average duration of azole therapy was 86 days and ranged from 3 to 397 days.

We tested whether the clinical isolates were susceptible to the combination of fluconazole and tacrolimus (Fig. 1) or CsA (data not shown) by serial spot dilution assays. All strains were counted with a hemacytometer, normalized to an initial concentration of 10^7 cells/ml, and 1:10 serial dilutions were spotted onto YPD or YPD containing 10 μg/ml fluconazole (Diflucan; Pfizer) with or without 1 μg of tacrolimus (Prograf; Astellas Pharma US, Inc.) per ml. All plates were incubated at 30°C for 24 to 48 h and observed for growth. Controls included *C. albicans* strains SC5314 (wild-type strain), a homozygous calcineurin B deletion mutant (JRB64, *cnb1*/*cnb1*), and an *rbp1*/*rbp1* deletion mutant (YAG171) that lacks FKBP12 and is therefore unresponsive to tacrolimus. Due to their resistance to fluconazole alone, the *C. glabrata* isolates were also tested on solid medium containing 256 μg per ml fluconazole with and without 1 μg per ml tacrolimus. At the higher concentration of fluconazole all three *C. glabrata* isolates demonstrated susceptibility to the combination of fluconazole and tacrolimus. Therefore, despite previous exposure to tacrolimus, there was no selection for resistance to the combination of calcineurin inhibitors and fluconazole in any of the isolates.

Calcineurin is also required for *Candida albicans* survival in serum (3, 22). Wild-type *C. albicans* strains grow robustly in fetal bovine serum (FBS) at 30°C, but lose viability under the same conditions when CsA or tacrolimus is added. Therefore it is possible that calcineurin inhibitors alone would exert an intrinsic antifungal activity in vivo when present in serum, which would hinder hematogenous dissemination.

We hypothesized that successfully invading isolates may have a decreased susceptibility to the combination of calcineurin inhibitors and serum. We measured the fold population change of all isolates after 24 h growth in FBS (Gibco certified FBS) with or without 1 μg per ml tacrolimus (Fig. 2). Cells were grown overnight in YPD at 30°C, washed twice in phosphate-buffered saline, inoculated into FBS at a concentration of approximately 2,000 cells per ml, and incubated for 24 h at 30°C. Dilutions were plated onto YPD plates for CFU counts at 0 and 24 h. The population change was determined by dividing the CFU at 24 h by the CFU at 0 h. Strains were also streaked onto serum plates (50% FBS,

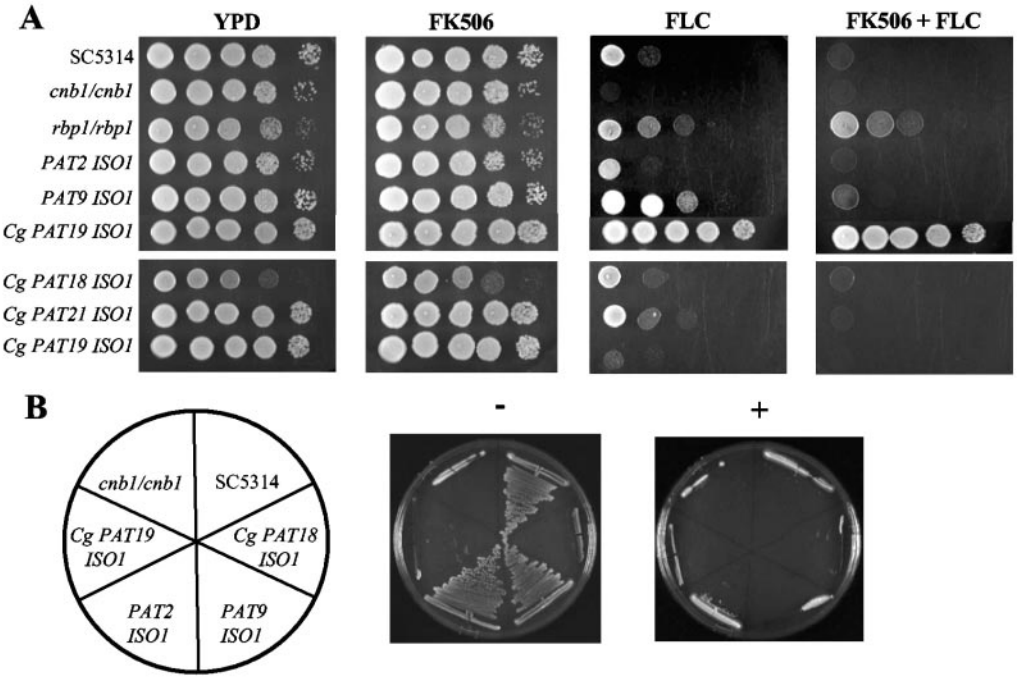

FIG. 1. Invasive and noninvasive clinical isolates. (A) Isolates were grown for 48 h at 30°C on YPD medium alone or containing tacrolimus (1 μg per ml), fluconazole (FLC; 10 μg per ml, upper panels; 256 μg per ml, lower panels), or both drugs. Control strains SC5314 (wild type), *cnb1*/*cnb1* (calcineurin deletion mutant), and *rbp1*/*rbp1* (FKBP12 deletion mutant) were tested along with representative clinical isolates. (B) Isolates were streaked on 50% FBS medium alone (−) or containing 1 μg per ml tacrolimus (+) and grown for 48 h at 30°C. *C. glabrata* isolates show reduced growth compared with *C. albicans* strains on solid serum-containing medium. All strains failed to grow in the presence of tacrolimus and serum.

distilled H_2O, and 2% Bacto agar) with or without 1 μg per ml tacrolimus (Fig. 1B).

All *C. albicans* strains were able to grow robustly in liquid serum culture, undergoing a 10,000- to 100,000-fold increase in population. By contrast, the *C. glabrata* and *C. parapsilosis* strains underwent more limited expansion in liquid serum, on the order of 10- to 100-fold ($P = 0.0005$ and $P = 0.0045$, respectively) (Fig. 2). In accordance, the *C. glabrata* isolates did not exhibit visible growth on solid serum medium as judged by the failure to form colonies (Fig. 1B). All isolates demonstrated reduced growth in serum containing tacrolimus. Only two isolates, *C. glabrata* PAT18 ISO1 and PAT19 ISO1, were resistant to the combination. Although these strains have a reduced growth rate in the presence of tacrolimus, the population change was still greater than or approximately onefold, indicating continued growth or stasis rather than a reduction in viability (population change < 1-fold) as seen with all other isolates. Interestingly, invasive and colonizing isolates did not differ in their ability to proliferate in serum, and invasive isolates did not show selection for resistance to the combination of serum and calcineurin inhibitor.

Recent studies have indicated that calcium is the component within serum that is toxic to *C. albicans* calcineurin mutants (2). Fetal bovine serum contains approximately 3.5 to 4.0 mM calcium, a concentration at which calcineurin mutants are unable to survive (2). Although there was no difference in susceptibility to serum and tacrolimus between the invasive and colonizing isolates, we determined whether any exhibited altered calcium susceptibility (Fig. 3). The clinical isolates were prepared as in the serum assay and inoculated into phosphate-buffered saline with or without 1 μg per ml tacrolimus and 0, 2, 6, or 10 mM $CaCl_2$ (Sigma). Cultures were incubated at 30°C and CFU were counted at 0 and 24 h.

For each concentration of calcium tested we compared the population change of the tacrolimus-treated and untreated samples. Overall, there was no difference in the calcium susceptibility patterns of invasive and colonizing isolates. In the presence of tacrolimus all of the C. *albicans* isolates had significant reductions in viability at the 6 mM and 10 mM calcium concentrations ($P < 0.05$, two-tailed, t test). Additionally, at 2 mM calcium 7 out of the 18 *C. albicans* strains had significantly reduced viability ($P < 0.05$), and the remaining strains showed a modest decrease that was not statistically significant. The *C. glabrata* isolates demonstrated an increase in viability as the concentration of calcium in the medium increased (population change in 0 mM or 2 mM calcium versus 10 mM calcium, $P < 0.05$). This is similar to the situation in *Saccharomyces cerevisiae*, where inhibition of calcineurin results in decreased survival in low-calcium environments (10).

The purpose of this study was to determine if tacrolimus used for immunosuppression could exert enough antifungal activity in vivo in combination with fluconazole or serum to select resistant isolates. Analysis of isolates from liver transplant recipients did not reveal any differences in responsiveness to tacrolimus combined with serum, fluconazole, or calcium.

Previous studies have shown that in combination with fluconazole, tacrolimus has a MIC of ≤40 ng/ml (9). Tacrolimus therapeutic blood levels clinically can range from 5 to 20 ng/ml

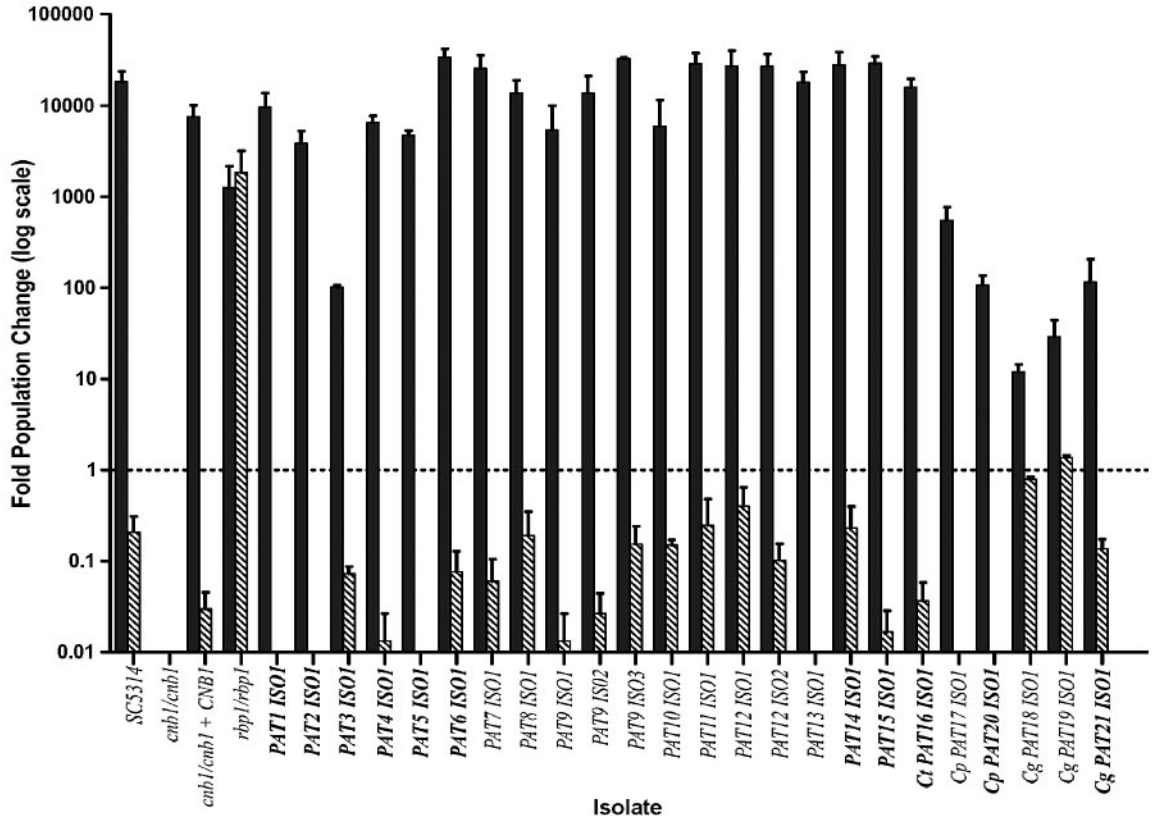

FIG. 2. Clinical *Candida* isolates are not resistant to the combination of serum and tacrolimus. The population change after 24 h growth in liquid FBS in the presence (diagonal hatched bars) or absence (solid bars) of 1 μg per ml of tacrolimus is shown. Invasive isolates are in bold. Error bars represent the standard error calculated from three independent experiments. Ct, *Candida tropicalis*; Cp, *Candida parapsilosis*; Cg, *Candida glabrata*.

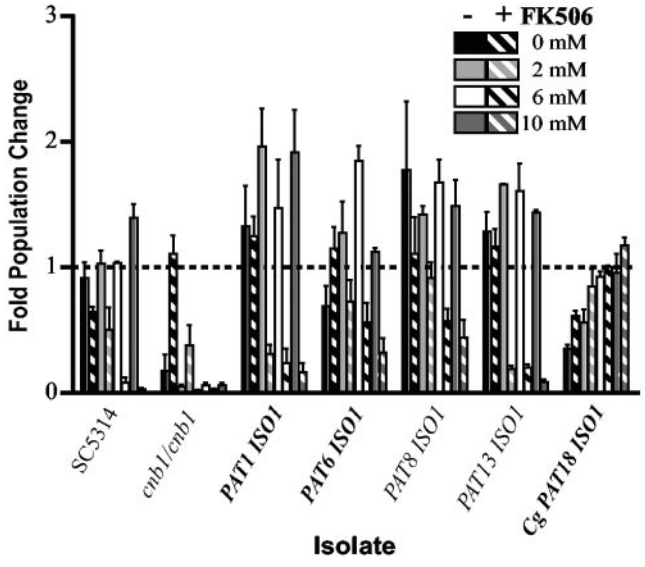

FIG. 3. Calcium susceptibility profile of representative invasive and colonizing isolates. Population change was determined after 24 h incubation in phosphate-buffered saline containing 0, 2, 6, or 10 mM $CaCl_2$ alone (solid bars) or with 1 μg per ml tacrolimus (FK506) (diagonally striped bars). All *C. albicans* isolates had significantly decreased viability in the presence of tacrolimus at 6 mM and 10 mM ($P < 0.05$, two-tailed t test). PAT1 ISO1 and PAT13 ISO1 also showed significant reduction in viability at 2 mM ($P < 0.05$). Invasive isolates are in bold. Error bars represent the standard error calculated from three independent experiments. Cg, *Candida glabrata*.

(trough level) to 68.5 ± 30 ng/ml (peak level). Although the levels obtained in the blood could be high enough to exert an effect, the local tissue concentrations that cells are exposed to in vivo is not known. One hypothesis for why resistance to calcineurin inhibitors may not develop in invasive isolates is that the local concentration of drug to which fungal cells are exposed is less than that needed to exert antifungal action. This could be a result of a lower therapeutic dose than optimal for antifungal activity with serum or fluconazole or sequestration of drug through binding to plasma proteins, or *Candida* may be sheltered from drug exposure by residing within tissues or within cells where drug concentrations may be lower.

The use of current clinical formulations of calcineurin inhibitors to augment antifungal therapy is hindered by their immunosuppressive effects, effects which likely outweigh the antifungal properties. Thus, nonimmunosuppressive analogs that retain the ability to target fungal calcineurin could have greater potential as therapeutic drugs. Alternatively, the combination of calcineurin inhibitors with other agents that augment their intrinsic antifungal activity may be a viable therapeutic approach.

We are grateful to Chiatogu Onyewu and Jill Blankenship for discussions and helpful suggestions.

These studies were supported by RO1 grants AI50438 (J.H.), AI42159 (J.H.), and AI054719 (N.S.).

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