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Cryptococcus neoformans Isolates from Transplant Recipients Are Not Selected for Resistance to Calcineurin Inhibitors by Current Immunosuppressive Regimens

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The immunosuppressants tacrolimus (FK506) and cyclosporine A inhibit calcineurin and have potent antifungal activity. In this study, 24% of *Cryptococcus neoformans* isolates from solid-organ transplant patients exhibited altered sensitivity to these drugs, which may have an impact on the infectious course but does not appear to be the consequence of immunosuppressive therapy.

Invasive fungal infections occur in up to 40% of organ transplant patients and are often difficult to treat (28). Advancements in therapy such as fluconazole prophylaxis have decreased fungal infections (33), but fluconazole-resistant fungal species and *Cryptococcus neoformans* infections, which often occur late after discontinuation of fluconazole prophylaxis (11), remain problematic. *C. neoformans* risk factors in transplant patients are poorly understood, and prognosis is difficult to assess (7, 30, 37). This may be attributable to the low relative risk of cryptococcosis in transplant recipients (1 to 3%), nonstandardized treatment regimens, and prolonged intervals between onset and diagnosis (17, 20).

Data concerning well-known risk factors for cryptococcosis from the human immunodeficiency virus (HIV) field may provide some insights relevant to transplant settings; however, determining the effect of therapy-induced immunosuppression on this pathogen is essential to understanding pathogenesis and epidemiology in transplant populations. Although the relative risk of *C. neoformans* infection in transplant patients is low, the attributable mortality rate of up to 45% (16–18) highlights a need to better understand and control this infectious malady.

The immunosuppressants cyclosporine A (CsA) and tacrolimus (FK506) are mainstays of immunosuppressive therapy. CsA and FK506 bind to the immunophilins cyclophilin A and FKBP12, respectively, and these complexes inhibit the protein phosphatase calcineurin (5, 13, 15, 20, 25, 26). In humans, calcineurin is required for T-cell activation in response to antigen presentation, and its inhibition suppresses immune responses and reduces transplanted organ rejection (8, 14, 27). Calcineurin is also an important signaling molecule in fungal cell wall maintenance, responses to cation stress, pheromone response, and, in *C. neoformans*, growth at 37°C and virulence (4, 9, 13, 23, 24, 25, 34, 36). CsA and FK506 inhibit fungal calcineurin and exert broad antifungal effects, either alone or in combination with other antifungal drugs (3, 32).

Immunosuppressive treatment alters the course of cryptococcal infection in animal models and may also affect infection of human transplant recipients (17). CsA can clear *C. neoformans* pulmonary infections in a murine model of infection, although CsA exacerbates cryptococcal meningitis in rabbits via immunosuppression and failure to cross the blood-brain barrier (22, 29). A retrospective study showed cryptococcal infections in patients receiving FK506 less commonly involve the central nervous system, a hallmark of cryptococcosis (17). This may be attributable to both FK506 antifungal activity and its unique ability to cross the blood-brain barrier (25, 32). Cryptococcosis in solid-organ transplant recipients provides an ideal system in which to test if immunosuppressive treatments also have antifungal activity in vivo. Here we examined what effect immunosuppressive treatment has on *C. neoformans* strains infecting solid-organ transplant recipients. As a control, isolates from transplant recipients were compared to 17 isolates from HIV-infected patients not exposed to immunosuppressants.

Thirty-three clinical isolates typed as *C. neoformans* were collected from 30 organ transplant recipients at several transplant centers around the world (Table 1). Based on carbohydrate assimilation profiles (bioMérieux), all 33 strains and the control strain H99 typed as *C. neoformans*. Analysis of the *MAT*-encoded *STE20* gene (primers available upon request) and mating assays identified most isolates as serotype A *MAT*α (Table 1). By serotyping of capsular antigen with a cell agglutination kit (Iatron), all isolates (except PAT8ISO1 and PAT12ISO1) were serotype A, confirming the PCR results. PAT12ISO1 typed as serotype B, and PAT8ISO1 was untypeable. In summary, all clinical strains tested were serotype A *MAT*α with the exception of PAT12ISO1, a serotype B *MAT*α variety *C. gattii* isolate.

The clinical strains were assayed for production of known virulence traits. Each strain tested, including strains from both HIV-infected subjects and transplant recipients, produced both urease and melanin, with some modest differences in melanin production. Urease production was assayed by sus-

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TABLE 1. Clinical strain data

Strain[a]	Genotype	Organ transplanted	Immunotherapy[b]	Site(s) infected[c]	Sensitivity[d]	Relative capsule size[e]
PAT2ISO1	Aα	Liver	MMF, FK506	Blood	S	−
PAT2ISO2	Aα	Liver	MMF, FK506	Abdominal abscess	S	−
PAT3ISO1	Aα	Lung	CsA	Cutaneous	S	+
PAT4ISO1	Aα	Liver	FK506	Pulmonary/cutaneous	S	−
PAT5ISO1	Aα	Liver	FK506	CSF	S	=
PAT6ISO1	Aα	Kidney/pancreas	MMF, FK506	Lung biopsy	HS	=
PAT7ISO1	Aα	Liver	MMF, FK506	CSF	S	=
PAT7ISO2	Aα	Liver	MMF, FK506	CSF	S	=
PAT8ISO1	Aα	Liver		Leg biopsy	HS	+
PAT9ISO1	Aα	Kidney		Abscess	S	+
PAT10ISO1	Aα	Heart	FK506	CSF	HS	+
PAT11ISO1	Aα	Kidney	FK506	CSF	S	+
PAT12ISO1	Bα	Liver	FK506	Pulmonary	S	=
PAT13ISO1	Aα	Liver	FK506	CSF	S	−
PAT14ISO1	Aα	Kidney	Azathioprine	Cutaneous	S	+
PAT15ISO1	Aα	Kidney	Azathioprine	CSF/pulmonary	S	+
PAT16ISO1	Aα	Kidney	Azathioprine	CSF	S	−
PAT17ISO1	Aα	Kidney	Azathioprine	CSF	S	=
PAT18ISO1	Aα	Kidney	Azathioprine	CSF	S	−
PAT19ISO1	Aα	Heart	MMF, CsA	CSF	S	−
PAT20ISO1	Aα	Liver	FK506	Pulmonary	S	=
PAT21ISO1	Aα	Kidney	CsA	Pulmonary/skin/CSF	S	−
PAT22ISO1	Aα	Kidney	FK506	Pulmonary	HS	=
PAT23ISO1	Aα	Kidney	FK506	Pulmonary	S	−
PAT24ISO1	Aα	Liver	FK506	CSF/pulmonary	R	−
PAT25ISO1	Aα	Kidney	Azathioprine	Pulmonary	S	−
PAT26ISO1	Aα	Kidney	Azathioprine	CSF	R	−
PAT27ISO1	Aα	Liver	FK506	Pulmonary	R	=
PAT28ISO1	Aα	Liver	FK506	Pulmonary	HS	=
PAT29ISO1	Aα	Kidney/pancreas	FK506	CSF/pulmonary	S	=
PAT30ISO1	Aα	Kidney	MMF, FK506, pred	CSF/blood	S	+
PAT30ISO2	Aα	Kidney	MMF, FK506, pred	CSF/blood	S	+
PAT31ISO1	Aα	Kidney	MMF, FK506, pred	CSF	S	−

[a] Multiple isolates from the same patient are given the same PAT number with successive ISO numbers (i.e., PAT2ISO1 and PAT2ISO2 are from the same patient).
[b] Immunosuppressive therapy: data not available for PAT8ISO1 and PAT9ISO1. MMF, mycophenolate mofetil; pred, prednisone.
[c] CSF, cerebrospinal fluid.
[d] S, sensitive; HS, hypersensitive; R, resistant.
[e] Relative capsule size is shown as significantly larger than (+), smaller than (−), or equal to (=) that of H99 (unpaired two-tailed Student *t* test with 25 samples each).

pending colonies in 1 ml of distilled H_2O in the presence of a BBL urease differentiation disk (Becton Dickinson & Co.) for 6 h at room temperature. Melanin production was assessed by spotting $\approx 10^6$ cells onto Niger seed agar medium at 30°C. Although some variation in melanin production was noted at 24 h, most strains appeared to approach wild-type levels of melanin production by 48 h. While it is clear that strains unable to produce melanin are less virulent (reviewed in reference 19), there is no indication that more subtle differences in melanin production have a clinical impact. There were also measurable differences in the ability of the clinical strains from transplant patients to produce capsule, measured by growing cells on solid Dulbecco's modified Eagle's medium (2), resuspending them in phosphate-buffered saline, and visualizing capsule with India ink (Table 1). Mutations in capsule genes attenuate virulence (6). However, modest variations in capsule production among nonisogenic strains are not correlated with altered virulence (10, 21).

Serotype A strains of *C. neoformans* are sensitive to growth inhibition by calcineurin inhibitors at 37 to 39°C (26). Here, we tested the susceptibility of the clinical isolates to CsA and FK506. Isolates from transplant patients and HIV-infected subjects were serially diluted onto YPD rich medium (31) with or without 100 μg of CsA or 1 μg of FK506 per ml at 35°C and observed for growth at each dilution. Most strains, including the control strain H99, proliferated in the presence of either calcineurin inhibitor at this temperature. However, several of the clinical strains, including PAT6ISO1, PAT8ISO1, PAT10ISO1, PAT22ISO1, and PAT28ISO1, were unable to grow on media containing FK506 or CsA at this temperature, indicating a requirement for calcineurin activity at lower temperatures in these clinical isolates (Fig. 1A and data not shown). This hypersensitivity was observed with clinical isolates from both transplant and HIV-infected patients, and thus the drug hypersensitivity represents natural variation in the population and is unrelated to drug exposure in vivo.

Mutations increasing drug susceptibility could occur in downstream calcineurin targets or parallel pathways that render calcineurin essential at lower temperatures. Strains that lack Cts1 or the small G protein Ras1 are temperature sensitive and hypersensitive to calcineurin inhibitors (1, 12). Mutations in these genes could contribute to calcineurin inhibitor hypersensitivity in the patient population. Indeed, three hyper-

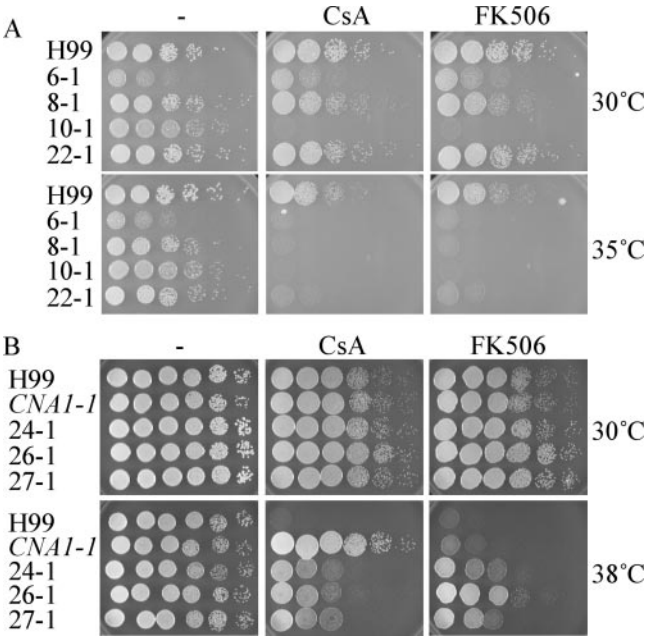

FIG. 1. Drug-hypersensitive and -resistant *C. neoformans* clinical isolates. (A) Some clinical isolates are hypersensitive to calcineurin inhibitors. Fivefold serial dilutions (starting at ≈10,000 cells) of wild-type strain H99, PAT6ISO1, PAT8ISO1, PAT10ISO1, and PAT22ISO1 were grown on YPD solid medium with no drug (−), 100 μg of CsA per ml, or 1 μg of FK506 per ml for 48 h at 30 or 35°C. (B) Several clinical isolates are resistant to calcineurin inhibitors. Wild-type strain H99, *CNA1-1* mutant strain MCC11, and clinical isolates PAT24ISO1, PAT26ISO1, and PAT27ISO1 were diluted as in panel A and grown on YPD solid medium with no drug (−), 100 μg of CsA per ml, or 1 μg of FK506 per ml for 48 h at 30 or 38°C.

sensitive strains (PAT6ISO1, PAT8ISO1, and PAT10ISO1) also exhibit temperature-sensitive growth (data not shown).

Interestingly, *C. neoformans* strains hypersensitive to calcineurin inhibitors are negatively correlated with cerebrospinal fluid infection. Only one of the five hypersensitive strains was isolated from the cerebrospinal fluid, compared to a rate of over 60% cerebrospinal fluid involvement for all strains, and at least four of these five patients received FK506. Thus, strains naturally hypersensitive to FK506 and CsA appear less likely to establish a cerebrospinal fluid infection, and FK506 may have an even greater beneficial effect in patients infected with these strains.

The purpose of this study was to test if the calcineurin inhibitors used for immunosuppression exhibit enough antifungal activity in vivo to select resistant *C. neoformans* isolates. Strains from transplant and HIV patients were compared for sensitivity to CsA and FK506 at 38°C. Most strains were able to grow on medium lacking drugs at this temperature, but only three of the strains from transplant patients (PAT24ISO1, PAT26ISO1, and PAT27ISO1) (9%) grew on media containing either CsA (100 μg/ml) or FK506 (1 μg/ml) at 38°C (Fig. 1B). Mutant strains lacking FKBP12 (MCC1) or expressing a dominant calcineurin A mutation, Cna1-1, which fails to bind the cyclophilin A-CsA complex (strain MCC11), were resistant to FK506 and CsA, respectively, but the wild-type control strain, H99, was not able to grow in the presence of either drug at 38°C (Fig. 1B and data not shown). More than 35% (6 of 17) of the strains from HIV-positive patients who had not received any form of immunosuppressive treatment demonstrated some resistance to calcineurin inhibitors (data not shown). When the entire *CNB1* gene, encoding calcineurin B, and the CsA-cyclophilin/FK506-FKBP12 binding region of the *CNA1* gene, encoding calcineurin A, were amplified and sequenced from the resistant isolates from the transplant recipients, no missense mutations were detected.

Resistance to calcineurin inhibitors is understood at a molecular level. A loss-of-function mutation in the drug-binding immunophilin protein or a mutation in the immunophilin drug binding site of one of the immunophilins or calcineurin A or B confers CsA and FK506 resistance. The resistant isolates identified here are, however, resistant to both drugs, and it is unlikely that resistant strains harbor double mutations in the genes encoding FKBP12 and cyclophilin A. Also, no mutations were found in the drug binding regions of the known target proteins. Furthermore, these strains are sensitive to rapamycin and fluconazole (data not shown), suggesting that resistance to calcineurin inhibitors may not be due to drug efflux pump induction. Resistance could be due to induction of a downstream effector of calcineurin signaling or to changes in several genes, similar to the panel of fluconazole-resistant *C. albicans* strains described by White (35).

Why are resistant isolates not selected? Guidelines for therapy suggest a concentration that, at least in the bloodstream, is consistent with the concentration of FK506 and CsA necessary to block *C. neoformans* growth under some in vitro conditions. Furthermore, the fungi were exposed to various concentrations of drug in the human host, which should enhance the selection of resistant isolates. Thus, *C. neoformans* must somehow escape drug activity, possibly by residing in tissues where the concentrations are not high enough to inhibit growth or within cells, such as macrophages, where they could be protected from antifungal action. Although our observations suggest that the current levels of CsA and FK506 used for patients do not have a sufficient antifungal effect against *C. neoformans* to select resistance, future studies will address the role that these immunosuppressive and antifungal drugs may have in *Aspergillus* or *Candida* infections and in altering the spectrum of cryptococcal infection in the transplant population.

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