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Avirulence of *Candida albicans FAS2* Mutants in a Mouse Model of Systemic Candidiasis

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Disruption of both alleles of the *Candida albicans FAS2* gene abolishes the ability of the organism to establish infection in a murine model of systemic candidiasis. Within 72 h all mice inoculated with 10^6 CFU of the parental *C. albicans* strain had died. In contrast, all animals inoculated with the mutant strain CFD2 survived for the course of the experiment (21 days). Animals infected with either mutant strain CFD1 or CFD3, in which only one *FAS2* allele was disrupted, also succumbed to infection, but mortality was not observed until 4 days postinfection and survivors remained for up to 20 days postinfection. The results demonstrate that *FAS2* is required for successful *C. albicans* infection.

The incidence of fungal infection has risen significantly, due in part to an increase in the number of individuals immunocompromised by disease or therapies. Unfortunately, the arsenal of antifungal drugs available has not expanded to meet the problem. Antifungal agents currently in use primarily exploit differences between fungal and mammalian cell surface structure (5, 7, 10); however, the overall similarity of the two cell types limits the number of obvious targets. In this regard, it has been demonstrated that certain auxotrophic mutant strains of *Candida albicans* are avirulent (4, 11, 14). These observations led to the suggestion that if prototrophy for a particular nutritional requirement is demonstrated to be necessary for pathogenicity, the corresponding biosynthetic enzyme and/or pathway might be utilized to identify potential therapeutic compounds for candidiasis (11).

Fatty acid synthase is a candidate that may fit these criteria. The enzyme is responsible for synthesis of fatty acids found in membrane-containing organelles and is essential for growth in the absence of a sufficient exogenous supply of fatty acids (15). Fungal fatty acid synthase is specified by two genes, designated *FAS1* and *FAS2* (12, 13, 16), which encode the polypeptides β and α, respectively. The two polypeptides contain the seven component activities necessary for fatty acid synthesis, and the active enzyme is an $\alpha_6\beta_6$ hexamer (17, 18). This is in contrast to the mammalian counterpart, in which all activities are present on a single polypeptide (15, 19). The active mammalian enzyme is a homodimer configured in a head-to-tail arrangement (15, 19). Because of the different structures of the fungal and mammalian fatty acid synthases, compounds with therapeutic value that differentially affect the enzymes might be found. In fact, compounds with the latter characteristic have recently been reported (2); however, it has not yet been demonstrated that fatty acid synthase is required for pathogenicity in models of systemic fungal infection.

In order to begin to address this issue, the ability of *C. albicans FAS2* mutant strains to establish infection in a murine model of systemic candidiasis has been investigated. *C. albicans* strains used in the study included a parental control (strain SC5314 [9]) and three strains in which either one (strains CFD1 [Δ*fas2*::*hisG-URA3-hisG*/*FAS2*;Δ*ura3*::*imm434*/Δ*ura3*::*imm434*] and CFD3 [*FAS2*/Δ*fas2*::*hisG-URA3-hisG*;Δ*ura3*::*imm434*/Δ*ura3*::*imm434*]) or both (CFD2 [Δ*fas2*::*hisG*/Δ*fas2*::*hisG-URA3-hisG*;Δ*ura3*::*imm434*;Δ*ura3*::*imm434*]) *FAS2* alleles had been disrupted or deleted. Mutant strains were derived from strain CAI4 by established protocols (6), and their construction has been described previously (20). CFD3 was derived from CFD2 and contained one reconstructed *FAS2* allele (20). CFD3 was included in all experiments to ensure that all phenotypic traits observed for CFD2 were due solely to the *FAS2* mutation rather than to unrelated mutations that may have occurred during CFD2 construction. It should also be noted that the immediate parent of *FAS2* mutants, strain CAI4, did not serve as a control in these experiments, since its Ura^- phenotype renders it avirulent (3, 4, 11). Instead, since CAI4 was derived directly from SC5314 (6), the latter strain was used as the parental control. CFD2 grows in vitro only in a medium supplemented with fatty acids (yeast extract-peptone-dextrose [YEPD] containing 1% [vol/vol] Tween 40, 0.01% [wt/vol] myristic acid, and 0.01% [wt/vol] stearic acid), while all other strains used grow without the addition of fatty acids. In addition, CFD2 is devoid of all fatty acid synthase activity, while CFD1 and CFD3 possess approximately 80% of wild-type activity (20). In vitro growth rates of all strains in fatty acid-supplemented liquid medium are comparable, and all strains undergo the yeast-to-hyphal-stage transition (20).

In order to investigate whether *FAS2* was required for *C. albicans* infection, experiments that examined the virulence of mutant strains in a mouse model of systemic candidiasis were performed (14). Each *C. albicans* strain was grown overnight in YEPD medium supplemented with fatty acids at 30°C. Cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS) prior to use. Subsequently, groups of seven BALB/c mice (18 to 20 g each; Charles River Laboratories) were injected with 0.5 ml of a cell suspension of either SC5314, CFD1, CFD2, or CFD3 at the concentrations given in the legend to Fig. 1. Mice were observed twice daily for signs of morbidity. Animals in a moribund state were euthanized by CO_2 inhalation. Concomitantly, each *C. albicans* strain was used to inoculate an additional 15 mice. Five members from

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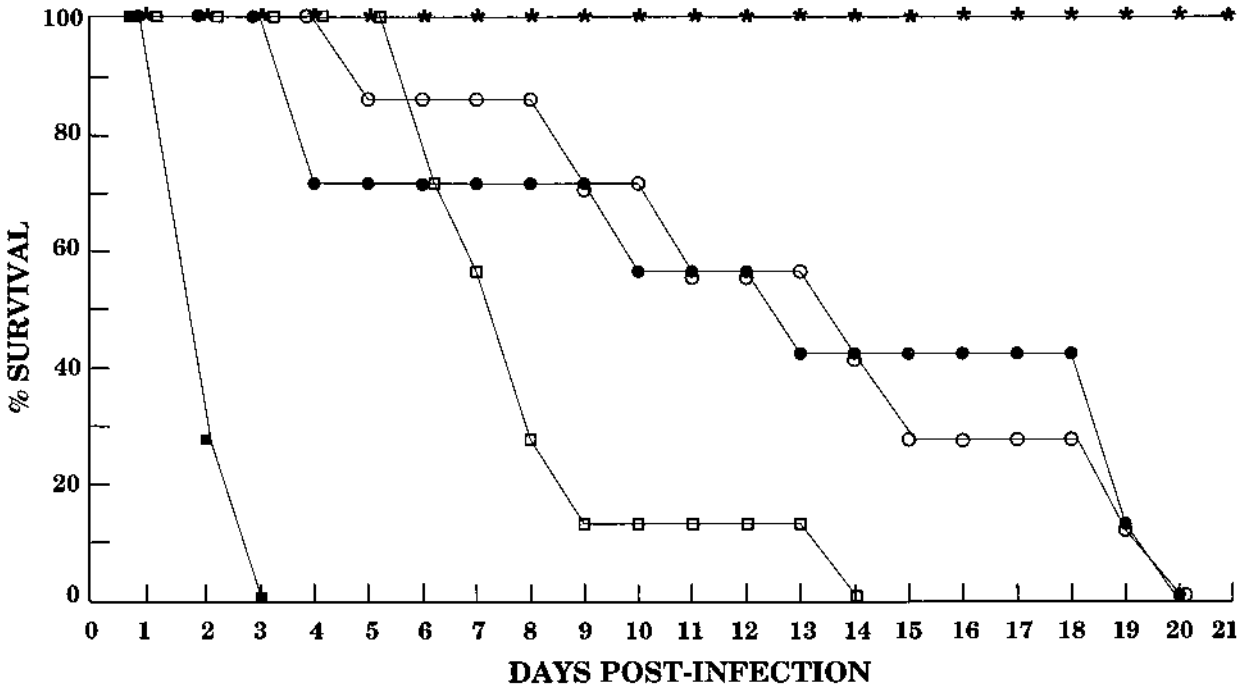

FIG. 1. Survival of mice following infection with *C. albicans FAS2* mutant strains. Mice were inoculated with 10^6 CFU of SC5314 (*FAS2*/*FAS2*) (■), 10^5 CFU of SC5314 (□), 10^6 CFU of CFD1 (●), 10^6 CFU of CFD3 (○), or 10^6 or 10^7 CFU of CFD2 (∗). Product-limit survival estimates were calculated by the Kaplan-Meier method, and the log rank test was employed to examine the homogeneity of survival curves among the strains. The overall differences in survival among strains were highly statistically significant ($P = 0.0001$). Individual comparisons did not vary from the overall pattern: CFD2 > SC5314 ($P = 0.0001$), CFD2 > CFD1 ($P = 0.0002$), CFD2 > CFD3 ($P = 0.0001$).

each group were later sacrificed by CO_2 inhalation at 24, 48, or 72 h postinfection. One kidney from each animal was removed and prepared for histological examination. After embedding in paraffin blocks, 6-μm-thick sections were cut and affixed to slides. Material was then stained with periodic acid-Schiff stain and examined by light microscopy. The other kidney, as well as the liver, from each animal was also excised, weighed, and finally homogenized in 5.0 ml of PBS. The homogenate was diluted in PBS, and aliquots were plated on Sabouraud dextrose agar, either without supplements or with 1% Tween 40 (vol/vol), 0.01% myristic acid (wt/vol), and 0.01% stearic acid. Plates were incubated at 30°C for 24 to 48 h, after which time CFU were quantitated.

The data in Fig. 1 demonstrate that all mice infected with CFD2 survived throughout the experiment, and animals injected with 10^7 CFU (10 times the control inoculum) of CFD2 also showed no signs of infection. In contrast, all mice infected with the parental control at 10^6 CFU succumbed to infection within 3 days. In addition, six of seven mice inoculated with 10^5 CFU of SC5314 succumbed to infection after 9 days, and one mouse survived for 14 days postinfection. Survival times for mice injected with either of the strains disrupted in only one *FAS2* allele (CFD1 and CFD3) were intermediate between that observed for mice inoculated with SC5314 and that observed for mice inoculated with CFD2.

Determination of the level of each *C. albicans* strain associated with host tissues suggests that CFD2 is rapidly cleared from the kidneys and liver (Table 1). Levels of both CFD1 and CFD3 are significantly lower than that observed for parental controls, but clearly, organisms persist. In order to determine the significance of differences observed among strains for each target organ, a general linear models procedure was used. A multiple comparison test was employed to hold the type I error (α) constant at 0.01. In terms of kidney infection, several statistically significant differences were seen at a P value of ≤0.01 for $\log_{10}$ CFU per gram at each time interval. At 24 h, all strains differed from each other, and the following order was established: SC534 > CFD1 > CFD3 > CFD2. At 48 h, SC5314 differed from all other strains. CFD1 and CFD3 differed from CFD2 but not from one another. Identical results were observed at 72 h, except that no data were obtained for SC5314 since there were no surviving animals at this time. Analysis of the data from infected liver tissue yielded similar results, with a P value of ≤0.01 for $\log_{10}$ CFU per gram at each time interval. At 24 h, SC5314, CFD1, and CFD3 differed from

TABLE 1. Recovery of *C. albicans* from infected tissues

Time postinfection (h)	Strain	$\log_{10}$ CFU/g (mean ± SD) in:	
		Kidney	Liver
24	SC5314	6.14 ± 0.13	4.38 ± 0.23
	CFD1	4.88 ± 0.15	4.15 ± 0.08
	CFD3	4.36 ± 0.20	4.19 ± 0.10
	CFD2	3.46 ± 0.25	3.45 ± 0.17
48	SC5314	6.20 ± 0.15	4.38 ± 0.16
	CFD1	4.04 ± 0.38	3.17 ± 0.30
	CFD3	3.42 ± 0.37	3.65 ± 0.29
	CFD2	0	0
72	SC5314	—[a]	—
	CFD1	3.75 ± 1.01	3.32 ± 0.42
	CFD3	3.94 ± 0.42	3.00 ± 0.20
	CFD2	0	0

[a] —, all mice succumbed to SC5314 infection by 72 h.

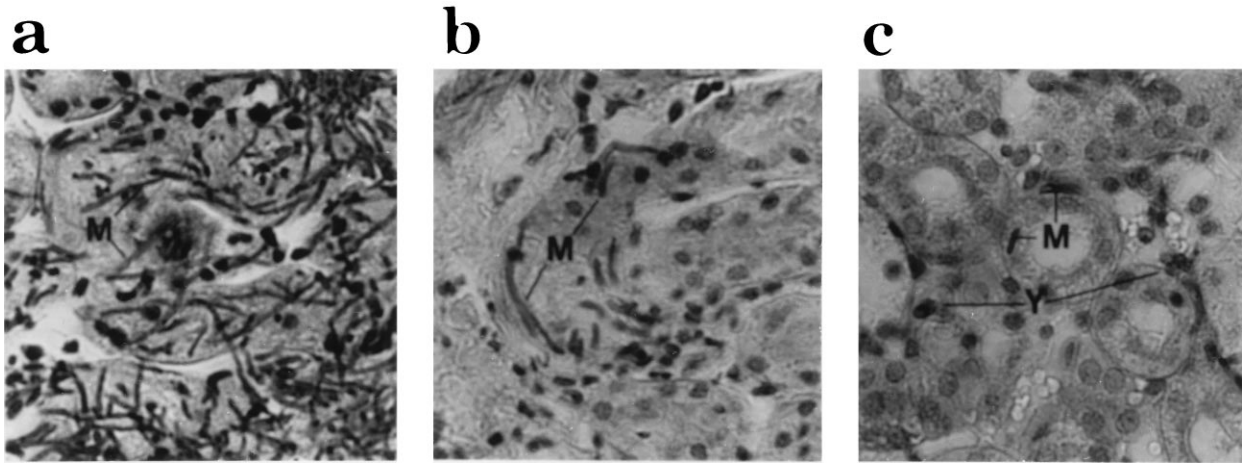

FIG. 2. Histological examination of kidneys from mice infected with *C. albicans* SC5314 (a), CFD3 (b), or CFD2 (c). Kidneys were excised 24 h postinfection, sectioned, and stained with periodic acid-Schiff stain prior to microscopic examination. Y, yeast cells; M, mycelia. A total of 100 fields were examined from each section for each strain. SC5314 was found in 100% of fields; CFD3 and CFD1 were found in 18 and 12% of fields, respectively. Magnification, ×400.

CFD2 but not from one another. At 48 h, SC5314 differed from CFD1, CFD3, and CFD2. CFD1 and CFD3 differed from CFD2 but not from each other. Identical results were noted at 72 h, except that no data was available for SC5314.

Histological examination of kidney tissue from animals infected with the individual strains supports these contentions (Fig. 2). While SC5314 showed extensive mycelial growth in infected tissue (Fig. 2a), only a small number of primarily yeast cells and/or stunted hyphae were observed in tissue from CFD2-infected cells (Fig. 2c). In comparison to SC5314, mycelia of smaller size and in smaller amounts were observed in kidney tissue infected with CFD3 (Fig. 2b) and CFD1 (data not shown).

The avirulence of strain CFD2 indicates that *FAS2* is necessary for pathogenesis of *C. albicans* in the mouse model of systemic candidiasis. Furthermore, since virulence of CFD3 was restored by reintroduction of a parental copy of *FAS2* into CFD2, it is clear that avirulence of CFD2 is directly associated with loss of fatty acid synthase activity. It is likely that failure of strain CFD2 to cause disease is related to an inability to grow in vivo in the absence of endogenous fatty acid synthase activity, suggesting that concentrations of exogenous fatty acids at the sites assayed are not high enough to support growth. Despite a reported 20% reduction in fatty acid synthase activity in CFD1 and CFD3 (20), both strains remained virulent, although signs of infection developed more slowly than for the wild type. The rapid clearance of CFD2 from the liver and kidneys, as well as histological observations, also supports the contention that avirulence of the *FAS2* null mutant strain is strictly a function of its auxotrophy and inability to grow in the host. This observation is in contrast to other studies, which showed attenuated virulence of *C. albicans CaMDR* (1) and *PHR1* (8) null mutants but in which the mutant strains persisted in the kidney.

Most important, the demonstration that nutritional sufficiency for fatty acids is a requirement for infectivity, coupled with the fact that mammalian and fungal fatty acid synthases are structurally distinct, suggests that fatty acid synthase might be an appropriate target for the development of new antifungals. In addition to the work presented here, it has been demonstrated recently that *C. albicans FAS2* null mutants are also avirulent in a rat model of oropharyngeal candidiasis (20). Thus, fatty acid synthase activity is necessary for infectivity in both a systemic and a mucosal model (20) of *C. albicans* infection. Furthermore, the results imply that exogenous fatty acids at disparate anatomical sites are not sufficient to overcome the defect. Clearly, no conclusions concerning whether fatty acid synthase is required in infections mediated by other fungi can be drawn, as such information must be obtained on a case-by-case basis.

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