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ARTICLES

A Murine Model for Disseminated Candidiasis in Neonates

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ABSTRACT: *Candida albicans* is the leading fungal pathogen causing invasive disease in immunocompromised patients including the neonate. A reliable animal model for disseminated candidiasis in the neonate is needed to study the unique aspects of this host-pathogen interaction. To establish such a model, 2-d-old BALB/c mouse pups were given i.p. injections with varied inocula of *C. albicans* or saline control. Pups were examined every 3–8 h for death. Surviving pups were killed at 72 h. Kidney, lung, spleen, liver, and brain were homogenized and plated for colony counts and/or fixed for histological staining. The i.p. injection of *C. albicans* led to mortality in a dose-dependent fashion. Disseminated infection was confirmed by colony counts of homogenized kidney, lung, and brain, as well as by histological examination. Infection with a *C. albicans* mutant lacking the cell surface adhesin, Als3p, led to significant reduction in mortality relative to WT ($p = 0.03$). This model will be useful to study the unique aspects of antifungal defense in a neonatal host and will provide a means to test novel therapeutic strategies. **(*Pediatr Res* 69: 189–193, 2011)**

Candida albicans is the leading fungal pathogen in immunocompromised patients (1) and the third most common pathogen overall causing late-onset sepsis in premature infants (2). Well-described risk factors for disseminated disease in this population include gastrointestinal (GI) colonization, prolonged hospitalization, broad-spectrum antibiotic use, central venous catheters, and parenteral nutrition (3–5). Colonization of preterm infants has been documented to occur through both vertical (from mother to infant) and horizontal routes (6). Even with antifungal therapy, candidiasis is often fatal among premature infants and is associated with neurodevelopmental impairment among survivors. Follow-up examinations at 18–22 mo corrected age show significant increases in rates of CP, blindness, deafness, and mental retardation (7). The severity of these infections has led to development of prophylactic strategies to reduce colonization and limit invasive disease (8–11). Although effective, these strategies require prolonged exposure to antifungal agents with their associated risks (12,13). Novel therapeutic strategies are needed to improve these outcomes. However, the mechanisms leading to immune compromise in the neonate are likely different from other patient populations at risk (14). An animal model of disseminated candidiasis in a neonatal host is therefore needed to recapitulate unique aspects of this host-pathogen interaction.

Murine models have been a feasible and reliable method to study the pathogenesis of candidiasis but have their limitations. Unlike humans, the mouse is not naturally colonized in the GI tract with *C. albicans*. To achieve persistent colonization, the animals must be treated with antibiotics and/or immunosuppressive agents, and dissemination is uncommon (15,16). Two strategies have been used to circumvent these issues. The most widely used model involves i.v. injection of adult animals *via* the lateral tail vein. This model has been used extensively to study virulence properties of the organism and immunological adaptations of the animal in response to hematogenous infection (15,17). A second strategy is gastric inoculation of neonatal mice, which leads to persistent, albeit decreasing colonization over time with some dissemination and mortality. However, mortality was strain dependent and amounted to ~50% or less in these studies (18,19).

In this study, we sought to develop a model that would result in more reliable disease burden while still maintaining some clinical relevance. The goal was to avoid pharmacological immunosuppression so as to allow inquiry into inherent immune status in infected neonates and to obviate the variability and technical challenges inherent to GI colonization. GI pathology including abdominal surgery is an independent risk factor for disseminated disease (3,5). When the integrity of the bowel mucosa is compromised, translocation of the organism is likely facilitated with spread *via* the enteric or lymphatic circulation. Direct inoculation of the peritoneum can also occur in the setting of bowel perforation with similar routes to dissemination. This study was structured to test the hypothesis that disseminated disease and subsequent mortality could be induced in a reliable and reproducible fashion by the i.p. route in neonatal mice without additional immunosuppression. This model provides the framework to study the unique host-pathogen interface in neonatal candidiasis and the development of novel therapeutic strategies.

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Abbreviations: CFU, colony forming units; **GI,** gastrointestinal; **scFv3,** single-chain variable fragment 3

METHODS

Strains and media. *C. albicans* strains used in this study include WT strain SC5314 (20) and strain 1843 containing a homozygous deletion in *ALS3* (*iro1-ura3*Δ*: :λimm*^{434}*/iro1-ura3*Δ*: :λimm*^{434} *als3la*Δ*/als3sa*Δ*-URA3*) (21) generously provided by Lois Hoyer. Starter cultures for injection were grown 16 h at 37°C with vigorous agitation in yeast extract peptone dextrose (YEPD) medium comprising 1% yeast extract, 2% peptone, and 2% dextrose (Difco Laboratories, Becton, Dickinson and Company, Franklin Lakes, NJ). Cultures were predominantly (>99%) yeast forms after this incubation. Before inoculation, overnight cultures of *C. albicans* were washed, enumerated on a hemacytometer, and resuspended in pyrogen-free saline (Hospira, Inc., Lake Forest, IL). The concentration was adjusted such that the desired unit dose per gram could be delivered in a volume of 10 μL.

Injection of neonatal mice. Timed pregnant BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). Pregnant dams were maintained in individual cages with unlimited access to food and water. Mice were monitored to determine the date of parturition. Pups were delivered in litters ranging from 3 to 9 pups and were randomized before inoculation to either sterile saline (control) or $10^{\times}$ colony forming units (CFU)/g of *C. albicans* yeast. Randomization was performed within cages rather than by litter to account for maternal and litter variations. Although cross-contamination of pups assigned to different experimental groups was theoretically possible, this risk was minimized by the short duration of the experiment. Furthermore, in experiments using an endpoint such as mortality that is influenced by many variables, the risk of cross-contamination was outweighed by the risk of confounding effects of maternal and litter variability inherent to a litter-based randomization scheme. Pups were injected on postpartum d 2. Just before injection, each pup was weighed to the nearest 10th of a gram. Weight of pups was closely clustered around 2 g, so each pup received a standard dose of 20 μL yeast or sterile saline injected i.p. in the lower half of the abdomen. After injection, pups were examined every 3–8 h for death or signs of illness. The pups were dissected at the time of natural death or killed for dissection when found moribund. All surviving animals were killed at 72 h after injection. A single kidney and lung were harvested and immediately homogenized for colony counts. If the time of natural death could not be accurately determined within 2 h, the organ colony counts were excluded from analysis. The remaining kidney, lung, spleen, liver, and brain were fixed in 10% buffered formalin (Fisher Scientific, Kalamazoo, MI) for subsequent histology. In selected studies, brain tissue was also collected for homogenization and colony counts.

Selected tissues underwent histological preparation and silver staining at the institutional core facility. Kidney, lung, and brain were homogenized with a FastPrep-24 Instrument (MP Biomedicals, Inc., Solon, OH) using Lysing Matrix D (Qbiogene; MP Biomedicals, Inc.) in 1-mL sterile saline, and appropriate dilutions were plated on YEPD. Colony counts were performed after an overnight incubation at 37°C. All animal studies were reviewed and approved by the Lifespan Institutional Animal Care and Use Committee, which oversees the animal care facility where animals were housed for this study.

RESULTS

C. albicans *infection in neonatal mice.* Mouse pups were injected i.p. on postpartum d 2 with concentrations of *C. albicans* strain SC5314 ranging from 10^4 to 10^8 CFU/g or with saline. Mice were followed closely for signs of illness and killed at 72 h after injection. Figure 1 shows the Kaplan-Meier survival curve summarizing these experiments. Doses of 10^6 CFU/g and below were not associated with mortality and caused no apparent clinical symptoms. Doses above 10^6 CFU/g caused increased mortality in a dose-dependent fashion. Because 10^7 CFU/g led to near complete mortality by study end point with a range of time of death throughout the observation period, this dose was selected for subsequent experiments.

Tissue sections of kidney, spleen, liver, and brain were silver stained to detect fungal elements (Fig. 2). Yeast and hyphal elements were scattered in the kidney and liver parenchyma with no specific anatomic relationship (Fig. 2*A* and *B*). There was no fungus detected in brain by histology (not shown). In the low power view of the spleen, abundant fungal elements were diffusely present in the capsular region (Fig. 2*C*). A high power view of the same region demonstrated prominent hyphae with penetration into the spleen parenchyma (Fig. 2*D*).

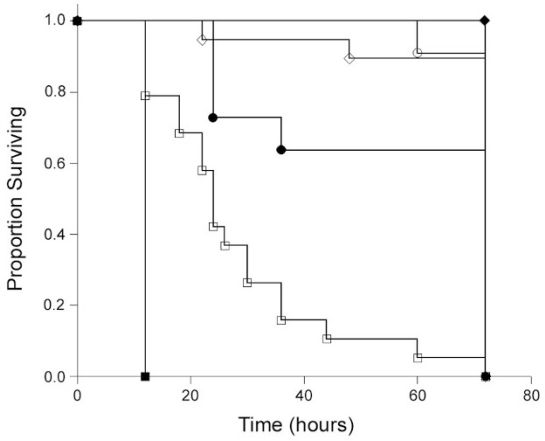

Figure 1. Kaplan-Meier survival curve by dose injected. Two-day-old mouse pups were injected i.p. with the following doses of WT *C. albicans*, and survival curves were plotted: ■, 1×10^8 CFU/g ($n = 3$); □, 1×10^7 CFU/g ($n = 19$); ●, 5×10^6 CFU/g ($n = 11$); ○, 1×10^6 CFU/g ($n = 11$); ◆, 1×10^4 CFU/g ($n = 3$); and ◇, saline ($n = 19$). Mortality occurred in a dose-dependent fashion at doses higher than 10^6 CFU/g.

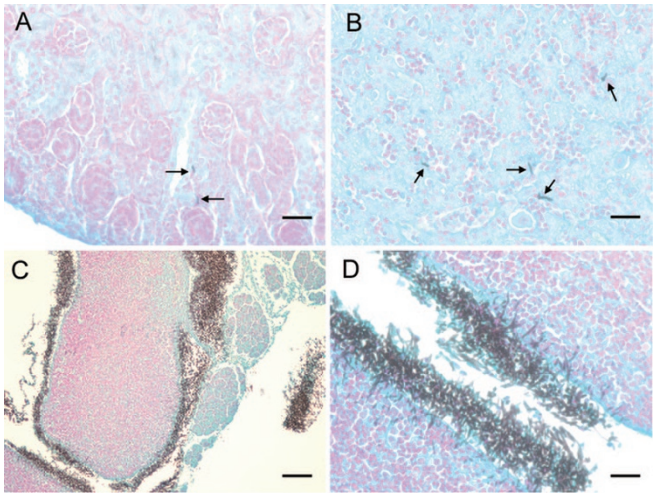

Figure 2. Tissue histology. Silver stain of representative sections from kidney (*A*), liver (*B*), and spleen (*C*, ×10 magnification and *D*, ×40 magnification) are depicted from an animal injected with 10^8 CFU/g WT *C. albicans*. *Arrows* indicate hyphal elements visible within the organ parenchyma. Heavy involvement of the capsular and subcapsular regions of the spleen was seen. *A*, *B*, and *D*: *bar* = 25 μm; *C*: *bar* = 100 μm.

Colony counts were obtained from homogenized kidney and lung and were highly variable (Table 1). In general, colony counts were higher in kidney compared with lung at a given dose, and extent of fungal burden was proportional to dose injected. Statistical analysis using a negative binomial model supported this dose-response relationship with $p = 0.0005$ for kidney and $p = 0.003$ for lung. Although no fungal elements were detected in histological studies of the brain, an additional experiment was conducted to examine brain involvement by colony counts, a more sensitive measure. Among 12 pups injected, all died by study end point, and nine

Table 1. *Tissue burden by dose of WT* Candida albicans *injected*

	Kidney (CFU/Organ)				Lung (CFU/Organ)				
Dose (CFU/g)	Mean	Median	Minimum	Maximum	Mean	Median	Minimum	Maximum	*n*
10^7	10,442	600	0	71,300	1446	500	0	6100	13
5×10^6	8734	70	0	66,000	1055	30	0	7800	8
10^6	806	20	0	8000	20	25	0	40	11
10^4	13	20	0	20	0	0	0	0	3

pups (75%) had colony counts ranging from 100 to 240 colonies per brain. Although a lower fungal burden was seen relative to kidney and lung, these data support some involvement of the CNS. No organisms were recovered from any organs taken from animals in the saline control groups, providing reassurance in regard to the possibility of cross-contamination among animals in the same litter.

Assessment of ALS3 mutant in neonatal model. To determine the utility of this model in assessing virulence determinants of *C. albicans*, a mutant (1843) carrying a homozygous deletion of the adhesin gene, *ALS3*, was evaluated in the neonatal mouse model. Previous work demonstrated that this strain had reduced adhesion to epithelial and endothelial cells *in vitro* (22). The *als3* mutant strain yielded a statistically significant reduction in mortality relative to WT (Fig. 3, $p = 0.03$). The median survival for the WT was 24 h compared with 44 h for the mutant. Tissue burden in kidney and lung was also compared in these animals (Table 2). Because colony count data were again highly disperse and not normally distributed, a negative binomial model was used for analysis. Once again, tissue burden was higher in kidney than in lung for both strains. Although trends toward higher colony counts in mice injected with WT *versus* mutant could be identified, there was no significant difference in tissue fungal burden in these animals.

DISCUSSION

Invasive candidiasis portends a poor prognosis despite available antifungal agents. A sophisticated understanding of host-pathogen interactions will be required to make additional progress in treatment and prevention of these infections. The model described here will be useful in studies to explore the unique aspects of the neonatal host in this disease. Neonatal mouse models have been successfully used to study sepsis with other microorganisms including group B *Streptococcus* (GBS) (23), *Escherichia coli* (24), *Pseudomonas aeruginosa* (25), and *Listeria monocytogenes* (26,27). In some cases, these studies have uncovered significant differences that can be defined between the neonatal and adult host and demonstrate the importance of neonatal models to study invasive disease.

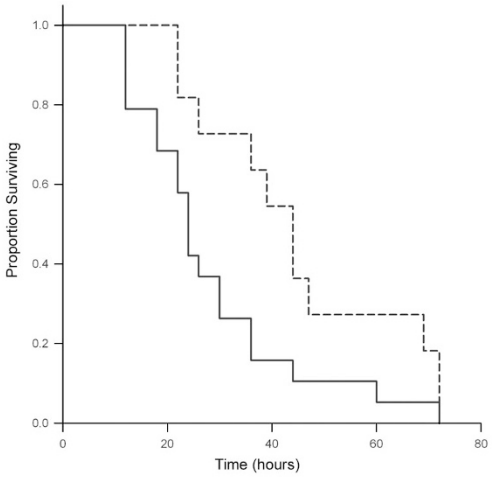

Figure 3. Kaplan-Meier survival curve comparing WT *C. albicans* and *als3* deletion mutant. Two-day-old mouse pups were injected i.p. with 10^7 CFU/g WT *C. albicans* (*solid line*, $n = 19$) or *als3* deletion mutant (*dashed line*, $n = 11$), and survival curves were plotted. Median survival was 24 h and 44 h, respectively ($p = 0.03$ by log-rank test).

Previous studies of *C. albicans* in the neonatal mouse used gastric inoculation as the route of infection. By using neonatal mice, Pope *et al.* (18) provided the first report of lethal candidiasis in an animal model after GI colonization without any additional measures to compromise immunity in the animals. In this study, 5- to 6-d-old mouse pups were inoculated *via* intragastric injection (5×10^8 CFU), and systemic spread of infection was seen in selected organs. Fungal invasion was seen in liver, kidney, and spleen within 6 h of inoculation, suggesting timely passage across the digestive tract wall or entry into the systemic circulation, possibly through the lymphatics. However, mortality was ~50% or less in this model and tissue burden decreased over time. A subsequent study using a lower dose (2×10^7 CFU) in 6-d-old pups by the same route also led to recovery of *C. albicans* from kidney, liver, lung, and spleen but in relatively smaller numbers and no mortality (19). A dose of 1×10^7 CFU led to still fewer or no recovery of fungi from these organs. However, long-term GI colonization was demonstrated in these animals and early colonization led to protective immune responses after later IV challenge with *C. albicans* as adults. In another study, 6-d-old mouse pups were orally inoculated with *C. albicans* after cortisone-induced immunosuppression. Histology of the entire GI tract showed highest frequency of invasion of mucosa by *Candida* in the jejunum. Only GI tract organs were studied in this model (28).

GI colonization models using adult mice show important differences from the neonatal models. Broad-spectrum antibiotic administration for 3 d before oral inoculation with *C. albicans* was necessary for GI colonization to develop (29). In addition, once colonized, extraintestinal dissemination in these animals was infrequent. However, when these animals were treated with dexamethasone, dissemination to kidney and mesenteric lymph nodes did occur but mortality remained low (16). Features of disease associated with infection by the i.p. route in adult mice are also available. Vonk *et al.* (30) studied the role of TNF-α and lymphotoxin (LT)-α by injecting *C.*

Table 2. *Tissue burden in mice infected with WT* vs *mutant (*als3−/−*)* C. albicans

	WT (CFU/organ), $n = 13$				*als3*−/− (CFU/organ), $n = 10$			
	Mean	Median	Minimum	Maximum	Mean	Median	Minimum	Maximum
Kidney*	10,442	600	0	71,300	3950	1100	0	13,500
Lung†	1446	500	0	6100	518	350	0	2200

* $p = 0.29$ for WT *vs* mutant.
† $p = 0.14$ for WT *vs* mutant.

albicans i.p. in adult TNF-LT double knockout mice and their WT littermates. Unlike this study in neonates, disseminated disease only occurred if immunosuppression was induced with cyclosporine before infection. Otherwise, the adult mice formed local abscesses that were cleared without disseminated disease. Taken together, these studies demonstrate that features of candidiasis in mouse models differ dramatically in the neonate compared with the adult, likely because of the relative immaturity of host defenses in the neonatal period. Such differences support the notion that features of disease unique to the neonatal host can be manifested by such an approach. However, there are additional factors placing preterm infants at risk for disseminated candidiasis that will be difficult to emulate in a murine model. Interventions such as indwelling catheters, parenteral nutrition, lack of enteral nutrition/breast milk, and many others that increase risk in the NICU are difficult to model and somewhat limit the applicability of host defense studies at this stage of mouse development to that of the preterm human.

In this study, the heavy involvement of the spleen with scattered foci in other organs supports a hematogenous route of dissemination, perhaps initiating in the spleen. Although involvement of the spleen was detected in the neonatal gastric inoculation model, colonization was similar or less than other organs (18). Presumably, the i.p. route of infection is responsible for the heavy spleen involvement in our model, either by direct contact with the organ or through lymphatic channels. Brieland *et al.* (31) inoculated *C. albicans* (5×10^6 CFU/mouse) *via* lateral tail vein injections into adult, immunocompetent mice and reported growth of *C. albicans* in various organs. The kidney was noted to have logarithmic growth in fungal burden. However, the liver and heart fungal burden declined quickly over time. The brain, lung, and spleen were all noted to have steady fungal loads with no significant change over the duration of the infection. Consistent with these data, the kidney counts in this study were higher than in lung tissue. The study by Brieland *et al*. collected data over a 21-d postinfection time course. In our model, mortality occurred within 72 h, and any surviving pups had generally cleared the infection by the 72-h time point. The kinetics of infection were therefore quite different from the model of Brieland *et al*.

The study by Brieland *et al*. described multiple foci of hyphal invasion in kidneys, hearts, brains, and spleens of infected mice, with the largest fungal burden in the kidney. We found the largest foci of hyphae around the splenic capsule. We also did not visualize hyphal elements in brain of neonatal mice by histology, whereas the adult model showed brain involvement within 48 h postinfection. Brain colony counts yielded consistent but reduced fungal burdens when compared with lung and kidney, suggesting that the fungal burden of the brain is not high enough to be detected by histology. Because tissue homogenates were the only way to assess fungal burden, involvement of vascular structures in the brain rather than the parenchyma itself is also possible. The differences in tissue distribution between these models likely relate to the route of infection and/or the dose of inoculation but may also be influenced by developmental stage of the animal.

We have previously described a single-chain variable fragment 3 (scFv3), which is specific to Als3p (22). Als3p is a cell wall protein expressed on *C. albicans* hyphae, which belongs to the Als family of adhesins (32). Als3p enables adherence to both epithelial and endothelial host cells through interaction with E-cadherin and N-cadherin, respectively, (33). Strain 1843, carrying a homozygous deletion of *ALS3*, demonstrates reduced adhesion to human epithelial and endothelial cells. Treatment of WT *C. albicans* with scFv3 resulted in reduced adherence, similar to the *als3* mutant (22). In our model, the *als3* deletion mutant showed somewhat attenuated mortality. Antibodies against Als3p such as scFv3 may therefore be useful to confer protection from disseminated candidiasis. This model provides fertile grounds to test this and other therapeutic strategies. Experiments are underway to evaluate scFv3 and other Als3p-specific antibodies for their capacity to provide protection. Novel chemotherapeutic agents against fungi could also be tested in this model for efficacy and to assess any unique toxicity that may arise in a neonatal setting. Studies of pathogenesis with *C. albicans* frequently find differences among strains. This model can be used to extend these observations and make comparisons among isolates that are presumed to be different in pathogenic potential. In addition, as non-*albicans* species increase in prevalence in the NICU, this model will have utility to compare the pathogenic features of the different *Candida* species and potentially tailor appropriate therapies.

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