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Cecal Colonization and Systemic Spread of *Candida albicans* in Mice Treated with Antibiotics and Dexamethasone

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ABSTRACT

Infections with *Candida albicans* have become a significant problem among very low birth weight infants in the neonatal intensive care unit. Risk factors are multiple and include administration of antibiotics and glucocorticoids, such as dexamethasone. Experiments were designed to study the combined effect of oral broad-spectrum antibiotics and parenteral dexamethasone on cecal colonization and extraintestinal dissemination of *C. albicans* in separate groups of mice that were orally inoculated with one of four *C. albicans* strains that were either wild-type *INT1/INT1* or had one or more disruptions of the *INT1* gene. Intestinal colonization was monitored by quantitative culture of the mouse cecum, and extraintestinal invasion was monitored by quantitative culture of the draining mesenteric lymph nodes and kidneys. At sacrifice, the average numbers of cecal *C. albicans* differed from 7.7 $\log_{10}$/g to 6.7 $\log_{10}$/g ($p < 0.01$) in mice orally inoculated with *C. albicans* containing two functional copies of *INT1* and no functional copies of *INT1*, respectively. The incidence of extraintestinal dissemination to mesenteric lymph nodes and kidneys correspondingly varied from 57 to 13% ($p < 0.01$) and 83 to 4% ($p < 0.01$) in mice inoculated with these two *C. albicans* strains. Mice orally inoculated with *C. albicans* containing one functional copy of *INT1* had intermediate levels of cecal colonization and extraintestinal dissemination. Thus, cecal colonization and extraintestinal dissemination of *C. albicans* was facilitated in antibiotic-treated mice given dexamethasone. In addition, the presence of two functional copies of the *INT1* gene was associated with the greatest levels of cecal colonization and extraintestinal dissemination of *C. albicans*. **(*Pediatr Res* 51: 290–295, 2002)**

Abbreviations
VLBW, very low birth weight
NICU, neonatal intensive care unit
MLN, mesenteric lymph nodes

Candida albicans is the most prevalent fungal pathogen in clinical disease (1), particularly among VLBW infants in the NICU (2–5). Among NICU patients, the incidence of systemic candidiasis appears to be rising dramatically (6). More than a decade ago, between 1.5 and 4.5% of VLBW infants developed a systemic *Candida* infection (2, 3, 7, 8). However, a recent study found an 11-fold increase in one nursery between 1981 and 1995 (9). Among hospitals participating in the United States National Nosocomial Infection Survey, *Candida* species represented the sixth most common nosocomial pathogen, accounting for 7% of all bloodstream infections, with *C. albicans* the most frequent species isolated (1, 10). In the general patient population, overall mortality caused by systemic candidiasis remains high, ranging from 63 to 85% in untreated patients and from 33 to 54% in those receiving appropriate antifungal therapy [reviewed by Todischini (11)]. Morbidity is also high, and multiple extensive complications often accompany candidemia, including meningitis, renal insufficiency or failure, endophthalmitis, pulmonary abscesses, endocarditis, and osteomyelitis (2, 3, 7, 12, 13).

C. albicans is a commensal in the human gastrointestinal tract (14, 15), and it is generally accepted that increased intestinal colonization is a major factor predisposing high-risk patients to systemic candidiasis (12, 14, 16). Other risk factors for systemic candidiasis include prematurity, broad-spectrum antibiotics, abdominal surgery, neutropenia, vascular catheters, mesenteric ischemia, damage to the gastrointestinal mucosa, total parenteral nutrition, and corticosteroids (1, 12, 16). In the past decade, there has been a significant increase in the use of glucocorticoids (particularly i.v. dexamethasone) in the NICU (17, 18), and this practice is coincident with the increase in *C. albicans* infections (7, 19). Although dramatic physiologic improvement in pulmonary function can been seen almost immediately with the use of steroids in premature infants, both

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short-term and long-term adverse affects, including increased risk of infection, are now being documented [(20), also reviewed by Jobe (21)].

In addition to host risk factors, a variety of microbial factors have been implicated in *C. albicans* virulence, including variable phenotypic expression, morphologic switching, molecular mimicry, expression of various surface molecules (*e.g.* receptors, adhesins), hydrophobicity, thigmotropism, and production of proteinase and phospholipase (14, 15, 22–24). Using a method for sequential disruption of the *INT1* gene (25, 26), mutant strains have been generated and used to show that the *INT1* gene product contributes to *C. albicans* adherence to HeLa cells, mortality in i.v. inoculated mice, and filamentous growth on specific agar media known to facilitate filamentation (27–29). We have also shown that the presence of two functional copies of the *INT1* gene facilitates intestinal colonization in the orally inoculated mouse, but no dissemination (translocation) to normally sterile extraintestinal sites was observed (30). This observation is likely not related to differences in filamentation because these *C. albicans INT1* mutant strains appear to filament normally *in vivo* (27, 31) and in serum-containing liquid media (28, 29). In the present study, we used *C. albicans INT1* mutant strains to investigate the effect of dexamethasone on extraintestinal spread of *C. albicans* in antibiotic-treated mice.

METHODS

C. albicans *strains and cultivation conditions.* The parent *C. albicans* strain CAF2 (*INT1/INT1 URA3/ura3::imm434*) was obtained from W.A. Fonzi (Georgetown University, Washington, DC, U.S.A.) (26). Using the urablaster method, *C. albicans* strains were constructed with disruptions and reintegrations in the *INT1* gene, and *C. albicans* CAG1 (*INT1/int1::hisG-URA3-hisG ura3::imm434/ura3::imm434*), *C. albicans* CAG3 (*int1::hisG/int1::hisG-URA3-hisG ura3::imm434/ura3::imm434*), and *C. albicans* CAG5 (*int1::hisG/int1::hisG::INT1-URA3*) have been previously described (28, 29). The parent CAF2 (*INT1/INT1*) demonstrates extensive hyphal development on agar media known to stimulate filamentation, maximal adhesion to cultured epithelial cells, and maximal mortality in i.v. inoculated mice (28). The null mutation CAG3 (*int1/int1*) has markedly reduced hyphal formation on these agar media; minimal adherence to cultured epithelial cells, and minimal mortality in i.v. inoculated mice (28). The heterozygous deletion strain CAG1 (*INT1/int1*) and the heterozygous reintegrant CAG5 (*int1/int1 + INT1*) have an intermediate phenotype compared with CAF2 (*INT1/INT1*) and CAG3 (*int1/int1*) with respect to both adhesion to cultured epithelium and virulence. Compared with CAF2, all three mutant strains (CAG1, CAG3, CAG5) have decreased ability to colonize the gastrointestinal tract of antibiotic-treated mice (30, 31). It should be noted that CAF2, CAG1, CAG3, and CAG5 have similar growth rates, *i.e.* generation times, in broth medium, and that *URA3/ura3* strains were used in experiments (29). For clarity in this manuscript, *C. albicans* CAF2 (*INT1/INT1)*, CAG1 (*INT1/int1*), CAG3 (*int1/int1*), and CAG5 (*int1/int1 + INT1*) are referred to as *C. albicans* +/+, −/+, −/−, and −/−/+ reflecting two functional copies of *INT1*, a single gene disruption, a double gene disruption, and a double gene disruption with subsequent reintegration of one functional copy of *INT1*, respectively.

C. albicans stock cultures were maintained at −80°C in Sabouraud's dextrose broth (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 15% glycerol. For use in experiments, stock cultures were plated on minimal medium agar supplemented with 2% dextrose, incubated at 30°C for 48 h, inoculated into minimal medium dextrose broth, and incubated at 30°C with shaking for 18 h (28, 29). The *C. albicans* strains used in this study grew exclusively as yeast cells (blastoconidia) under these conditions. Yeast cells were washed and resuspended in sterile saline at 10^8/mL for oral inoculation into mice. Inoculum concentration was determined by hemocytometer and verified by quantitative culture on Sabouraud's dextrose agar incubated 48 h at 30°C.

Experimental treatment of mice. Adult, female (18 to 22 g) Swiss Webster mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN, U.S.A.). In each of two replicate experiments, 48 mice were divided into four groups of 12 mice and orally inoculated (feeding needle) with 0.1 mL containing 10^7 *C. albicans* +/+, −/+, −/−, or −/−/+. To facilitate intestinal colonization, mice were given 1 mg/mL bacitracin (Sigma Chemical Co., St. Louis, MO, U.S.A.), 2 mg/mL streptomycin sulfate (Sigma Chemical Co.), and 0.1 mg/mL gentamicin sulfate (Sigma Chemical Co.) in the drinking water for 3 d before oral inoculation with *C. albicans* (31, 32). Mice were also injected i.p. twice daily with 0.5 mL of 4 mg/mL (2 mg BID or approximately 100 mg/kg BID) dexamethasone sodium phosphate (Elkins-Sinn, Cherry Hill, NJ, U.S.A.) on the day of oral inoculation with *C. albicans*, and for three subsequent days. Mice were killed on the fourth day after oral *C. albicans* for quantitative determination of cecal microbes, and extraintestinal dissemination (translocation) of *C. albicans* to the MLN and kidneys. Antibiotics were continued for the duration of the experiment. A preliminary experiment showed that BID i.p. injections of 0.5 mL of saline, rather than dexamethasone, resulted in no extraintestinal dissemination of *C. albicans* +/+ in this model. Also, we have previously noted that in mice similarly treated with antibiotics and oral *C. albicans* (but not dexamethasone), extraintestinal dissemination of *C. albicans* occurs only in occasional (three of 124) mice (30).

According to the package insert, the LD_{50} for this preparation of dexamethasone (injected parenterally) is approximately 800 mg/kg in adult female mice. Thus, mice were given a high dose of dexamethasone in this study, *i.e.* 100 mg/kg BID. Others have used similarly high doses to facilitate systemic infection with *C. albicans* in rodents (33, 34). Our primary aim was to use dexamethasone to induce systemic spread of *C. albicans*, and lower doses were not tested. Mice and humans have different pharmacokinetics and often much higher concentrations (mg/kg) of an agent must be used to achieve a blood level comparable to that obtained in a human. However, it should be noted that we cannot assume that the dose of dexamethasone used in this study has clinical relevance for humans.

Mice were housed under controlled conditions and handled by specially trained personnel. Using PCR to identify the genotype of *C. albicans* strains recovered from mouse ceca, these conditions have been shown to eliminate cross-contamination of inoculated strains among mouse treatment groups (30, 31). Experiments were performed according to the National Institutes of Health guidelines on the use of experimental animals. The University of Minnesota Institutional Animal Care and Use Committee approved all protocols.

Recovery of* C. albicans *from mouse ceca, MLN, and kidneys. Although *C. albicans* can colonize all portions of the gastrointestinal tract, colonization is typically maximal in the cecum of the adult mouse, and the cecum is most often used to monitor candida colonization in mice (32, 35–39). Mice were killed by cervical dislocation, ceca were aseptically excised from four mice (randomly chosen) in each treatment group in each of the two replicate experiments. Each cecum was weighed, homogenized, serially diluted, plated on agar media, and incubated at 35°C for 48 h (33). Agar media included colistin-nalidixic acid agar supplemented with 5% sheep red blood cells for selective isolation of Gram-positive bacteria and yeast, MacConkey agar for selective isolation of Gram-negative bacilli, and synthetic minimal medium agar (40) without uracil for cultivation of yeast containing the *URA3* gene. The lower limit of assay detection was 3.0 $\log_{10}$/g of cecum. Cecal tissue was not processed for strictly anaerobic bacteria because this antibiotic regimen reliably eliminates all detectable cecal anaerobic bacteria (41, 42).

To visualize *in vivo* morphology of *C. albicans* strains, cecal contents were analyzed from all mice. Cecal contents were rinsed from tissue with a minimal volume (1–2 mL) of sterile saline solution, and a minimal volume (100–200 μL) was stained with calcofluor according to manufacturer's directions (Fungi-Fluor Kit, Polysciences, Inc., Warrington, PA, U.S.A.). (The remaining tissue and contents were used for quantitative culture as described above.) Specimens were examined under epifluorescent microscopy. Fungal elements (100/mouse from each of eight mice per treatment group) were identified as either yeast or filamentous forms, the latter defined as an outgrowth four times the width of the mother cell or a chain of four or more elongated yeast cells.

All surviving mice were monitored for extraintestinal dissemination of intestinal microbes by culturing the draining MLN, as well as the kidneys. Before excision of the cecum, the MLN and kidneys were aseptically excised, homogenized, and quantitatively cultured as described (33, 39). Tissue homogenates were plated on tryptic soy agar supplemented with 5% sheep red blood cells and minimal medium agar without uracil. The lower limits of microbial detection were 5 per MLN (entire tissue) and 1.3 $\log_{10}$/g of kidney.

Microbes were identified by standard techniques (43). Identification of *C. albicans* was confirmed by Gram's stain, coupled with observation of characteristic hyphal formation after incubation of yeast cells in rabbit serum incubated for 3 h at 37°C. All *C. albicans* strains used in this study form hyphal elements, including germ tubes, in serum after 3 h incubation at 37°C (29). Using specific primers for the *INT1* locus (28), PCR was performed on two colonies from each cecum and one colony from each MLN, and the genotype of the recovered *C. albicans* consistently corresponded to the inoculated strain.

Statistical analysis. Statistical analyses were performed using StatView 5.0.1 (SAS Institute, Cary, NC, U.S.A.). To verify reproducibility, the experimental protocol was repeated on two separate days. Each experiment contained 12 mice per treatment group inoculated with *C. albicans* +/+, −/+, −/−, or −/−/+, for a total of 48 mice per experiment. Cecal flora was analyzed from four mice per treatment group in each replicate experiment for a total of eight mice per treatment group. Microbial dissemination to MLN and kidneys was analyzed in all surviving mice. Numbers of tissue (cecal, MLN, kidney) *C. albicans* were analyzed by the Kruskal-Wallis test followed by *post hoc* testing using the unpaired Mann-Whitney *U* test. Fractional data were analyzed by χ^2 with continuity correction. P values <0.05 were considered significant.

RESULTS

Mortality and cecal colonization. A total of 24 mice (12 mice in each of two experiments) were orally inoculated with *C. albicans* +/+, −/+, −/−, or −/−/+ followed by dexamethasone treatment for 4 d. Occasional mortality was observed, *i.e.* one mouse inoculated with −/− and three mice inoculated with −/+, but there were no significant differences in mortality in mice inoculated with *C. albicans* +/+, −/+, −/−, or −/−/+. All mortality took place during the night, and these mice were unavailable for study because of the timing of their deaths. All surviving mice appeared healthy for the duration of these experiments, and there was no gross pathology at autopsy other than the cecal enlargement typical of mice given broad-spectrum antibiotics (41).

At sacrifice, mice had no detectable cecal Gram-negative bacilli, and only two mice (both inoculated with −/+) had cecal Gram-positive bacteria, identified as *Micrococcus* sp. in one mouse and *Bacillus* sp. in the other. The average concentration of cecal *C. albicans* +/+, −/+, −/−, and −/−/+ varied 10-fold from 7.7 $\log_{10}$/g to 6.7 $\log_{10}$/g for *C. albicans* +/+ and −/−, respectively (Fig. 1). The numbers of *C. albicans* recovered on colistin nalidixic agar were similar to those recovered on minimal medium lacking uracil, indicating that the *URA3* locus (and thus the *INT1* gene construct) remained stable *in vivo*. Data reported in Figure 1 reflect the numbers of cecal *C. albicans* recovered on minimal medium lacking uracil. The numbers of cecal *C. albicans* +/+ were consistently greater than each of the other three mutant strains, which did not differ from each other, except *C. albicans* −/+ was greater than −/− at $p = 0.05$. The numbers of cecal *C. albicans* −/−/+ were also greater than −/−, but this difference was not significant ($p = 0.3$). Although the *C. albicans* inocula was exclusively yeast cells, all strains formed filaments in cecal contents (Fig. 2), for which the percent filamentation (average ± SE from eight mice per group) for *C. albicans* +/+, −/+, −/−, and −/−/+ was noted to be 46 ± 4%, 19 ± 5%, 19 ± 6%, and 43 ± 5%, respectively.

Extraintestinal dissemination. Extraintestinal dissemination of *C. albicans* was monitored by culturing the MLN and kidneys of all surviving mice. *C. albicans* was the only mi-

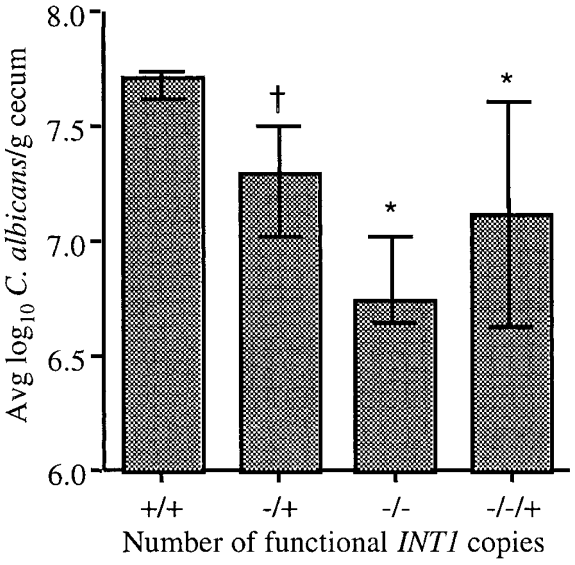

Figure 1. Effect of the number of functional *INT1* copies on the concentration of cecal *C. albicans* in antibiotic-treated mice orally inoculated with *C. albicans* +/+, −/+, −/−, or −/−/+ and treated with dexamethasone. Data represent eight mice per group. *$p < 0.01$; †$p < 0.05$, decreased compared with mice orally inoculated with *C. albicans* +/+. Error bars indicate the range of data from the 25th to the 75th quartiles, and are therefore not symmetrical above and below the average value.

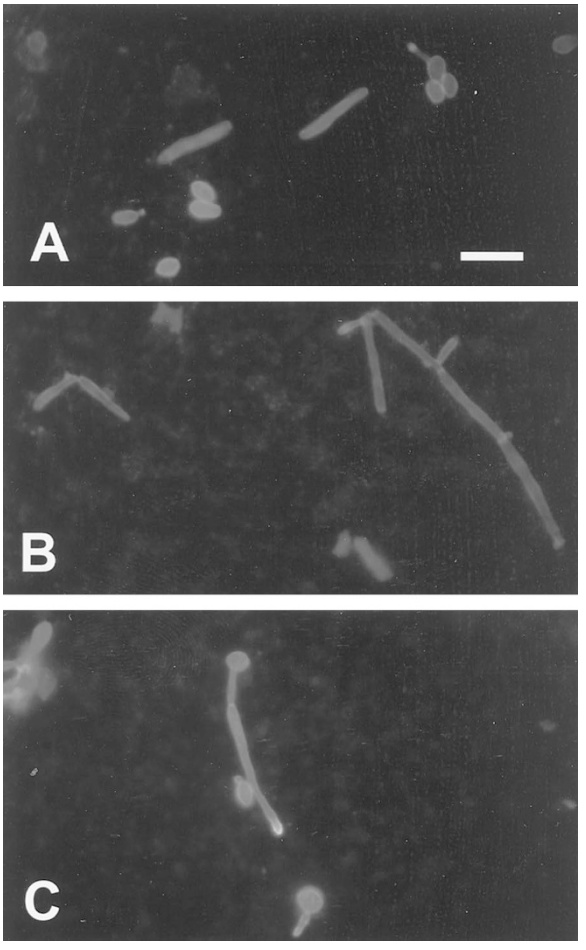

Figure 2. Calcofluor staining of cecal contents of mice colonized with *C. albicans* +/+ (*A*), −/− (*B*), and −/−/+ (*C*) showing presence of filamentous forms *in vivo*. Bar = 10 μm.

crobe recovered from MLN and kidneys. The incidence of *C. albicans* translocation to the MLN was highest for *C. albicans* +/+ and lowest for *C. albicans* −/−, with significant differences noted between +/+ and −/+ and between +/+ and −/− (Table 1). Although the average number of viable *C. albicans* +/+ recovered from MLN was greater than the numbers of *C. albicans* −/+, −/−, or −/−/+ recovered from MLN, these differences were not significant.

Similar to the data for MLN, the numbers of mice with *C. albicans* +/+ recovered from kidneys was also greater than each of the three mutant strains (−/+, −/−, −/−/+), which were not significantly different from each other (Table 2). Although the average number of *C. albicans* recovered from kidney tissue was greatest for *C. albicans* −/−, there was only one mouse with *C. albicans* −/− detected in kidney tissue (reflecting the lowest incidence of *C. albicans* translocation among the four *C. albicans* strains tested), so this information was difficult to interpret. Compared with *C. albicans* +/+, there were fewer numbers of *C. albicans* −/+ and −/−/+ recovered from kidney tissue, with statistical differences of $p = 0.07$ and $p < 0.01$, respectively.

DISCUSSION

Two of the most prominent and prevalent risk factors for *Candida* sepsis among VLBW infants on the NICU are broad-spectrum antibiotics and glucocorticoid therapy. We have previously shown that gastrointestinal colonization of the mouse by *C. albicans* was facilitated by administration of broad-spectrum antibiotics (30–32). However, despite the fact that increased intestinal colonization is generally accepted as a risk factor for systemic candidiasis (12, 14, 16), we noted only occasional extraintestinal dissemination of intestinal *C. albicans* in previous studies (30). In the current study, dexamethasone appeared to facilitate cecal colonization as well as systemic spread of orally inoculated *C. albicans*.

For all *C. albicans* strains examined, the level of colonization was greater than that previously noted without the use of dexamethasone. In a previous study using the same *C. albicans* strains, oral antibiotics, and mouse strain, the average concentration of cecal *C. albicans* varied from 5.4 $\log_{10}$/g to 3.8 $\log_{10}$/g for *C. albicans* +/+ and −/−, respectively (31). In the current study, addition of dexamethasone resulted in a dramatic increase (at least 100-fold) in cecal colonization to an average of 7.7 $\log_{10}$/g and 6.7 $\log_{10}$/g for *C. albicans* +/+ and −/−, respectively. Dexamethasone was also associated with increased filamentation of *C. albicans* in the intestinal tract. Without dexamethasone, the percent filamentation in cecal contents (average ± SE) was previously reported to be 14.3 ± 4.3% and 3.7 ± 1.5% for *C. albicans* +/+ and −/−, respectively (32). However, in dexamethasone-treated mice, the percent filamentation was increased ($p < 0.01$, unpaired *t* test) to 46 ± 4% and 19 ± 6% for *C. albicans* +/+ and −/−, respectively. Thus, treatment with dexamethasone appeared to facilitate both cecal colonization and filamentation of *C. albicans* in mouse cecal contents.

Table 1. *Translocation of* C. albicans *to MLN of antibiotic-treated mice orally inoculated with* C. albicans *+/+, −/+, −/−, or −/−/+ and treated with parenteral dexamethasone*

C. albicans strain	No. mice with viable *C. albicans* recovered from MLN/Total no. surviving mice (%)	Average no. of *C. albicans* recovered from MLN of positive mice (range)
+/+	13/23 (57%)*	488 (5–4250)
−/+	5/21 (24%)†	104 (5–375)
−/−	3/23 (13%)‡	5 (5–5)
−/−/+	8/24 (33%)	51 (5–190)

* One MLN was not processed because of technical error.
† Decreased compared with *C. albicans* +/+, $p < 0.05$.
‡ Decreased compared with *C. albicans* +/+, $p < 0.01$.

Table 2. *Translocation of* C. albicans *to kidneys of antibiotic-treated mice orally inoculated with* C. albicans *+/+, −/+, −/−, or −/−/+ and treated with parenteral dexamethasone*

C. albicans strain	No. mice with viable *C. albicans* recovered from kidneys/Total no. surviving mice (%)	Average $\log_{10}$/g *C. albicans* recovered from kidneys of positive mice (range)
+/+	20/24 (83%)	3.9 (1.6–>5.0)
−/+	4/21 (19%)*	2.7 (1.5–4.3)†
−/−	1/23 (4 %)*	4.2
−/−/+	6/24 (25%)*	2.1 (1.3–2.9)*

* Decreased compared with *C. albicans* +/+, $p < 0.01$.
† Decreased compared with *C. albicans* +/+, $p = 0.07$.

In dexamethasone-treated mice, increased cecal colonization also was associated with systemic spread of *C. albicans*. Overall, *C. albicans* was recovered from the MLN of 32% of mice (57% of those inoculated with *C. albicans* +/+ and 13% of those receiving *C. albicans* −/−). Systemic spread of *C. albicans* to the kidneys paralleled these numbers, *i.e.* 83% of the mice colonized with *C. albicans* +/+ showing detectable kidney involvement whereas *C. albicans* was recovered from the kidneys of only 4% of those colonized with *C. albicans* −/−. These data support previous studies in which the presence of two functional copies of the *INT1* gene was associated with increased intestinal colonization with *C. albicans* in the orally inoculated, antibiotic-treated mouse (30–32). However, in our previous work we did not find dissemination of either the wild-type *C. albicans* or *INT1* mutant *C. albicans* beyond the intestinal tract. The addition of dexamethasone led to the spread of all *C. albicans* strains, in varying degrees, to both the MLN and kidneys. It is tempting to speculate that this comparatively low incidence of translocation of the mutant *C. albicans* strains −/+, −/−, and −/−/+, compared with *C. albicans* +/+, might have been related to differences in the number of functional copies of the *INT1* gene. However, significant differences in intestinal colonization among these strains could have been the overriding factor modulating extraintestinal dissemination of *C. albicans* +/+, −/+, −/−, and −/−/+.

In general, the data reported herein support the concerns of clinicians regarding multiple risk factors for systemic candidiasis in the VLBW infant. Even if not administered concurrently, the use of broad-spectrum antibiotics may lead to an increased level of intestinal colonization with *C. albicans*, which can be further augmented by glucocorticoid therapy, predisposing the premature infant (and other high-risk patient populations) to systemic candidiasis.

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