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Colonization and Pathogenesis of *Cryptococcus neoformans* in Gnotobiotic Mice

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Congenitally immunodeficient nude (*nu/nu*) mice and their immunocompetent littermates (*nu/+*) were used to determine whether the absence of thymus-matured T cells would alter the capacity of *Cryptococcus neoformans* to colonize their mucosal surfaces or enhance their susceptibility to systemic cryptococcosis, or both, following oral challenge. We present data demonstrating that an encapsulated strain of *C. neoformans* serotype A colonized the alimentary tracts of germfree, conventional, and antibiotic-treated conventional *nu/nu* mice. Scanning electron microscopy showed that *C. neoformans* adhered to the epithelial surfaces of the oral cavities, esophagi, and gastrointestinal tracts of monoassociated *nu/nu* and *nu/+* mice, and culture data showed that there were more viable *C. neoformans* cells in the alimentary tracts of *nu/nu* mice than of *nu/+* mice. Tetracycline-treated conventional *nu/nu*, but not *nu/+*, mice were also colonized with *C. neoformans* following intragastric challenge. *C. neoformans*-monoassociated and tetracycline-treated conventional *nu/nu* mice succumbed to disseminated cryptococcosis with cerebral involvement 3 to 4 weeks after oral challenge, whereas no mortality was observed for similarily challenged *nu/+* mice. These results demonstrate that an encapsulated strain of *C. neoformans* can colonize mucosal surfaces and cause systemic cryptococcosis in immunodeficient *nu/nu* mice, suggesting that the alimentary tract can be a portal of entry for *C. neoformans* in an immunodeficient host. These data also indicate that functional T cells play an important role in resistance to systemic cryptococcosis of endogenous origin.

Cryptococcosis is a fungal infection that can be initiated by inhalation of the yeast *Cryptococcus neoformans*. Following establishment of a pulmonary infection, any organ can be infected; however, dissemination to the central nervous system is a serious clinical manifestation. Cryptococcal disease is most frequently encountered in immunocompromised individuals (e.g., patients with Hodgkin's disease, leukemia, lymphosarcoma, or acquired immunodeficiency syndrome, or patients undergoing steroid treatment [4, 13, 18, 21–23]). In addition, subtle defects in cell-mediated immunity have been reported for patients with cryptococcosis when an overt immunodeficient state was not readily apparent (2, 5, 6, 17). Although the true incidence is not known, evidence indicates that clinical disease produced by *C. neoformans* is increasing (4, 10) and is likely to increase further as the use of immunosuppressive regimens becomes more widespread (10).

Because *C. neoformans* is generally considered an environmental saprophyte, isolation of this organism from human clinical specimens is often considered indicative of the presence of cryptococcal disease. However, several investigators have isolated *C. neoformans* from the throat, nose, and sputum samples of patients with bronchopulmonary disease or nonpulmonary malignant disease in the absence of any clinical, X-ray, or serological evidence of cryptococcal disease (9, 14, 19, 21). In some cases this mucosal association persisted for 1 to 3 years (19). These studies suggest that *C. neoformans* can on occasion persist in the human oropharynx (9, 19) and may be an endogenous source of lung (aspiration) or alimentary tract (swallowing) infections. Little is known about the interactions between *C. neoformans* and the host at mucosal surfaces, the mechanism of *C. neoformans* adherence, the factor(s) which predisposes the host to colonization, and whether *C. neoformans* can switch from a commensal to a pathogen, especially in an immunocompromised host undergoing treatment with oral antibiotics that decrease the populations of normal flora. Previous studies with immunocompetent conventional animals have demonstrated that *C. neoformans* does not readily colonize the alimentary tract following intragastric challenge with large numbers of cryptococci (1, 7).

In this study we subjected immunodeficient nude (*nu/nu*) and immunocompetent (*nu/+*) germfree and conventional mice, as well as conventional mice maintained on oral antibiotics, to oral challenge with *C. neoformans* to assess the ability of *C. neoformans* to colonize murine mucosal surfaces and cause systemic cryptococcosis following an oral challenge.

MATERIALS AND METHODS

Mice. Germfree and conventional athymic nude (*nu/nu*) and heterozygous (*nu/+*) BALB/c mice were used in this study. BALB/c mice were originally obtained from Norman Reed, Montana State University, Bozeman, Mont. Germfree BALB/c mice were housed in polycarbonate cages with corncob bedding in flexible-film isolators at the University of Wisconsin Gnotobiotic Laboratory, Madison, Wis.; established procedures were followed (15). Conventional BALB/c mice were obtained by inoculating the drinking water of germfree mice with 1 ml of gastrointestinal homogenate from a conventional mouse reared in a standard clean animal room environment. Conventional mice were associated with their microflora for 2 weeks before being used in this study. In some experiments, conventional BALB/c mice were maintained on orally administered antibiotics to decrease the numbers of bacteria in the gastrointestinal (GI) tract. Con-

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TABLE 1. Colonization of gnotobiotic nude (*nu/nu*) and heterozygous (*nu/+*) BALB/c mice with an encapsulated strain of *C. neoformans*

Days after challenge	No. of mice		No. of *C. neoformans*[a] in:			
			Stomach		Cecum	
	nu/nu	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*
1	6	6	1.7 ± 1.7	3.2 ± 0.6	3.7 ± 0.5	4.2 ± 0.7
3	11	12	2.4 ± 0.8	0.5 ± 0.5	3.4 ± 0.8	1.0 ± 0.5
7	6	6	2.4 ± 0.3	2.0 ± 2.0	3.3 ± 0.4	4.3 ± 1.1
14	12	12	5.2 ± 1.3[b,c]	1.8 ± 0.6	3.8 ± 1.4	3.8 ± 0.5
19	2	2	6.2	4.8	8.1	4.9
21	8	9	5.8 ± 0.3[b,c]	3.6 ± 0.6	6.1 ± 0.4[b]	4.3 ± 0.9
28	0	6	—[d]	2.4 ± 2.4	—	4.5 ± 1.6

[a] Mean $\log_{10}$ *C. neoformans* CFU per gram (dry weight) ± standard error of the mean. Counts represent data from pooled organs of two to three mice killed at each time interval. Except for day 19, two to four repeat experiments were carried out at each time interval.

[b] The numbers of *C. neoformans* organisms cultured from the stomachs and ceca of nude (*nu/nu*) mice were significantly increased ($P < 0.05$) on days 14 and 21 after oral challenge (compared to day 7).

[c] Nude (*nu/nu*) mice had significantly higher levels ($P < 0.05$) of *C. neoformans* A cultured from their stomachs on days 14 and 21 after oral challenge than their heterozygous (*nu/+*) littermates did.

[d] —, All nude mice died 19 to 28 days after oral challenge.

ventional BALB/c mice were given 660 μg of tetracycline per ml in their drinking water 2 days before being used in this study. Fecal pellets obtained from mice prior to and after antibiotic treatment were serially diluted, plated on brain heart infusion agar, and incubated aerobically for 48 h at 37°C. Administration of antibiotics to conventional mice reduced the number of aerobes and facultative anaerobes from between 10^{10} and 10^{11} to between 10^7 and 10^8/g of feces. Mice ranged from 6 to 10 weeks of age at the start of each experiment. Sterilized food (5010C; Ralston Purina Co., St. Louis, Mo.) and water were consumed by all mice ad libitum. The microbial status of germfree mice was monitored weekly by established microbiological procedures (20).

Organism and animal inoculations. An encapsulated strain of *C. neoformans* serotype A was used in this study. Cultures were maintained on Sabouraud dextrose agar. Before being inoculated into animals, the yeast cells were transferred to Sabouraud dextrose broth and incubated at 37°C for 48 h on a rotary shaker. Cryptococci were harvested, washed three times by centrifugation (1,000 × *g* for 15 min), and suspended in phosphate-buffered saline (PBS). We subjected germfree BALB/c mice to oral challenge (monoassociated) by swabbing the mouth of each mouse in the experiment with a cotton swab that had been inserted into a suspension containing 10^7 *C. neoformans* organisms per ml of PBS. Both conventional and tetracycline-treated conventional BALB/c mice were gavaged (18-gauge animal feeding needle) with 10^6 viable *C. neoformans* organisms in 0.2 ml of PBS. The gavage inoculum was prepared by counting yeast cells on a hemacytometer and appropriately adjusting the concentration. To verify the number of viable cells, the inoculum was serially diluted in PBS, portions (0.05 ml) were plated on Sabouraud dextrose agar, and CFU were determined.

Quantitative culture techniques. Mice were killed by ether inhalation at various intervals following oral challenge with *C. neoformans*. The kidneys, liver, lungs, spleen, brain, stomach, and cecum were aseptically removed from each mouse, and the same organs from three mice were pooled, homogenized in 5 ml of PBS, and serially diluted. Undiluted (1.0 ml) and diluted (0.05 ml) tissue homogenates were plated in duplicate on Sabouraud dextrose agar. Colonies were counted after incubation for 48 h at 37°C. Data are expressed as the number of *C. neoformans* CFU per gram (dry weight) of each pooled tissue homogenate. Dry weight was assessed by placing 1 ml of tissue homogenate in an aluminum pan and drying it at 60°C for 24 h. Generally, two to four repeat experiments were carried out at each sacrifice interval. At various intervals after gavage, fecal pellets from conventional mice and conventional mice maintained on tetracycline were collected (two or three per mouse), suspended in PBS (1.0 ml), homogenized, and serially diluted. Undiluted (0.5 ml) and diluted (0.1 ml) fecal homogenates were plated on Sabouraud dextrose agar containing 5 μg of gentamycin per ml and 100 U of penicillin G per ml. After 72 h at 37°C the number of *C. neoformans* CFU per gram of feces was determined. Significant differences in *C. neoformans* CFU were determined by using the Student *t* test.

SEM. Procedures for the preparation and fixing of mucosal tissues for scanning electron microscopy (SEM) have been described previously (8). Briefly, segments of selected tissues were quickly removed and placed in vials containing cold (4°C) 0.1 M cacodylate-buffered glutaraldehyde (2.5%). The glutaraldehyde-fixed samples were then rinsed in 0.1 M cacodylate buffer, postfixed in osmium tetroxide, rinsed again in cacodylate buffer, and dehydrated in increasing concentrations of ethanol. Tissues were then critical point dried, fixed to aluminum stubs, coated with gold-palladium, and examined under a JEM U3 or a JEOL 50A SEM at 15 to 20 kV.

RESULTS

Colonization of germfree BALB/c mice with encapsulated *C. neoformans*. Adult germfree athymic nude (*nu/nu*) and heterozygous (*nu/+*) BALB/c mice were orally challenged (monoassociated) with *C. neoformans* to determine whether this microorganism could colonize the alimentary tract. Viable cryptococci were isolated from the stomachs and ceca of BALB/c mice within 24 h of oral challenge (Table 1). On days 3 and 7 after oral challenge, *C. neoformans* was recovered from the stomachs and ceca of *nu/nu* and *nu/+* BALB/c mice in low numbers (10^1 to 10^4/g [dry weight]). On days 14, 19, and 21 after oral challenge, the number of cryptococci in the stomachs and ceca of *nu/nu* BALB/c mice had dramatically increased ($P < 0.05$) to 10^4 to 10^6/g (dry weight). In contrast, the number of *C. neoformans* cells isolated from the stomachs and ceca of *nu/+* BALB/c mice between 14 and 28 days after oral challenge remained

between 10^2 and 10^5/g (dry weight). The number of cryptococci isolated from the stomachs of *nu*/+ mice on days 14 and 21 was significantly lower ($P < 0.05$) than the number isolated from the stomachs of *nu*/*nu* mice.

SEM of mucosal surfaces of BALB/c mice. Various mucosal surfaces were viewed by SEM to determine the distribution of encapsulated *C. neoformans* along the alimentary tract of monoassociated BALB/c mice. SEM revealed *C. neoformans* adherent to the epithelial surface of the cheek (not shown), the ventral surface of the tongue (Fig. 1A), and the esophagus (Fig. 1B). SEM of the GI tract showed numerous *C. neoformans* cells associated with the keratinized portion of the stomach (Fig. 1C) and the mucous layer of the large intestine (Fig. 1D). Therefore *C. neoformans* adhered primarily to the epithelial surfaces of the oral cavity, esophagus, and GI tract of each BALB/c mouse.

Pathogenesis of encapsulated *C. neoformans* in orally challenged BALB/c mice. To assess whether colonization with encapsulated *C. neoformans* could lead to systemic disease, the internal organs of orally challenged nude (*nu*/*nu*) and heterozygous (*nu*/+) BALB/c mice were cultured at various time intervals after monoassociation. On days 1, 3, and 7 after oral challenge, viable *C. neoformans* was not detected in the kidneys, livers, lungs, spleens, or brains of monoassociated *nu*/*nu* mice (Table 2). By day 14 after oral challenge, however, *C. neoformans* was recovered from the internal organs of monoassociated *nu*/*nu* mice (Table 2), coincident with increased levels of *C. neoformans* in the stomachs and ceca (Table 1). On day 21 after oral challenge, *nu*/*nu* mice had significantly higher ($P < 0.05$) levels of *C. neoformans* in their internal organs than their *nu*/+ littermates did. Nude mice succumbed to disseminated cryptococcosis with cerebral involvement 19 to 28 days after oral challenge. In contrast, no mortality was observed in *C. neoformans*-colonized *nu*/+ mice during a 12-week observation period, even though small numbers of viable cryptococci ($<10^5$/g [dry weight]) were isolated from their internal organs throughout the 28-day study (Table 2).

Recovery of encapsulated *C. neoformans* from the feces of conventional mice maintained on tetracycline. Conventional *nu*/*nu* and *nu*/+ BALB/c mice were given tetracycline in their drinking water 2 days before being subjected to intragastric challenge with 10^6 *C. neoformans* organisms. Tetracycline-treated conventional *nu*/*nu* BALB/c mice shed low but constant numbers of *C. neoformans* (ca. 10^3 organisms per g) in their feces (Table 3). By day 8 after intragastric challenge, 66% of tetracycline-treated conventional *nu*/*nu* BALB/c mice shed viable *C. neoformans* in their feces. Conversely, encapsulated cryptococci were not isolated from the feces of tetracycline-treated conventional *nu*/+ mice by day 3 after intragastric challenge. After tetracycline administration was discontinued (day 8), conventional *nu*/*nu*, but not *nu*/+, BALB/c mice continued intermittently to have constant low numbers of *C. neoformans* ($<10^3$ organisms per g) in their feces. At 2 weeks after tetracycline administration was discontinued, the interal organs of *nu*/*nu* and *nu*/+ conventional BALB/c mice were cultured to determine whether mice colonized with *C. neoformans* had developed disseminated disease. For *nu*/*nu* BALB/c mice, counts were 4.5 ± 1.0, 6.3 ± 0.8, 5.6 ± 0.5, 4.7 ± 1.1, and 5.8 ± 0.6 for kidney, liver, lung, spleen, and brain tissue, respectively. Nude mice died 17 to 22 days after intragastric challenge with *C. neoformans*. In contrast, no viable *C. neoformans* organisms were recovered from the internal organs or feces of conventional *nu*/+ BALB/c mice. Similarly, *C. neoformans* was isolated from the feces (10^3 to 10^4/g) of gavaged conventional *nu*/*nu* BALB/c mice given penicillin and streptomycin in their drinking water.

Recovery of encapsulated *C. neoformans* from the feces of conventional BALB/c mice. Conventional athymic nude (*nu*/*nu*) BALB/c mice were gavaged with 10^6 *C. neoformans* organisms to assess whether a complex GI microflora would alter the ability of *C. neoformans* to colonize immunodeficient mice. Of the seven intragastrically challenged immunodeficient conventional *nu*/*nu* mice used in this study, five (71%) shed viable *C. neoformans* in their feces (Table 3). Consistently low levels of viable *C. neoformans* organisms were cultured from feces (ca. 5×10^3 to 7×10^3 organisms per g of feces) during the 7-day observation period. Thus a complex GI microflora did not appear to prevent encapsulated cryptococci from colonizing the alimentary tract of immunodeficient conventional *nu*/*nu* mice in small numbers.

DISCUSSION

In these studies we used quantitative culturing and SEM to directly assess the capacity of *C. neoformans* to colonize murine mucosal surfaces. These studies illustrate that *C. neoformans* had a greater capacity to colonize the alimentary tracts of T-cell-deficient *nu*/*nu* mice than the GI tracts of immunocompetent *nu*/+ mice. Following oral challenge of germfree BALB/c mice, consistently low numbers of *C. neoformans* were isolated from the GI tracts of *nu*/+ mice throughout the study, whereas the number of cryptococci in the GI tracts of *nu*/*nu* mice steadily increased. When conventional *nu*/*nu* and *nu*/+ BALB/c mice were given tetracycline orally to suppress the normal flora and then gavaged with *C. neoformans*, only conventional *nu*/*nu* mice shed viable encapsulated cryptococci in their feces. In addition, *C. neoformans* was capable of colonizing immunodeficient *nu*/*nu* mice even in the presence of a complex GI microflora, indicating that bacteria in the normal flora were not inhibiting colonization by *C. neoformans*. These data suggest that the increased capacity of *C. neoformans* to colonize mucosal surfaces may be related to defects in cell-mediated immunity.

Encapsulated cryptococcal yeast cells adhered to most mucosal surfaces throughout the murine alimentary tract. The ventral surface of the tongue, the keratinized section of the stomach, and the colon appeared to be the areas most heavily populated with *C. neoformans*. Investigators have demonstrated that other species of yeasts quickly colonize the alimentary tracts of orally challenged germfree mice (3, 16). Following oral association of germfree mice with *Candida albicans*, yeast cells colonized most mucosal surfaces, whereas hyphae penetrated the dorsal surfaces of the tongues and the cardial-atrium junctions of the stomachs of these mice (3). Histological sections from the stomachs of *Candida pintolopesii*-associated mice demonstrated layers of yeast cells confined to the secretory epithelium (16). In contrast, our study showed that the encapsulated *C. neoformans* adhered primarily to the keratinized portion of the murine stomach.

We observed that oral challenge of either germfree or tetracycline-treated conventional nude mice with *C. neoformans* can lead to disseminated cryptococcosis. Since *C. neoformans* is present in the environment of the *C. neoformans*-challenged mice, it is possible that cryptococci were inhaled into the lungs and then disseminated to the other internal organs. Investigations that established pulmonary cryptococcosis by either aerosol exposure or intranasal inoculation have shown that *C. neoformans* was present in

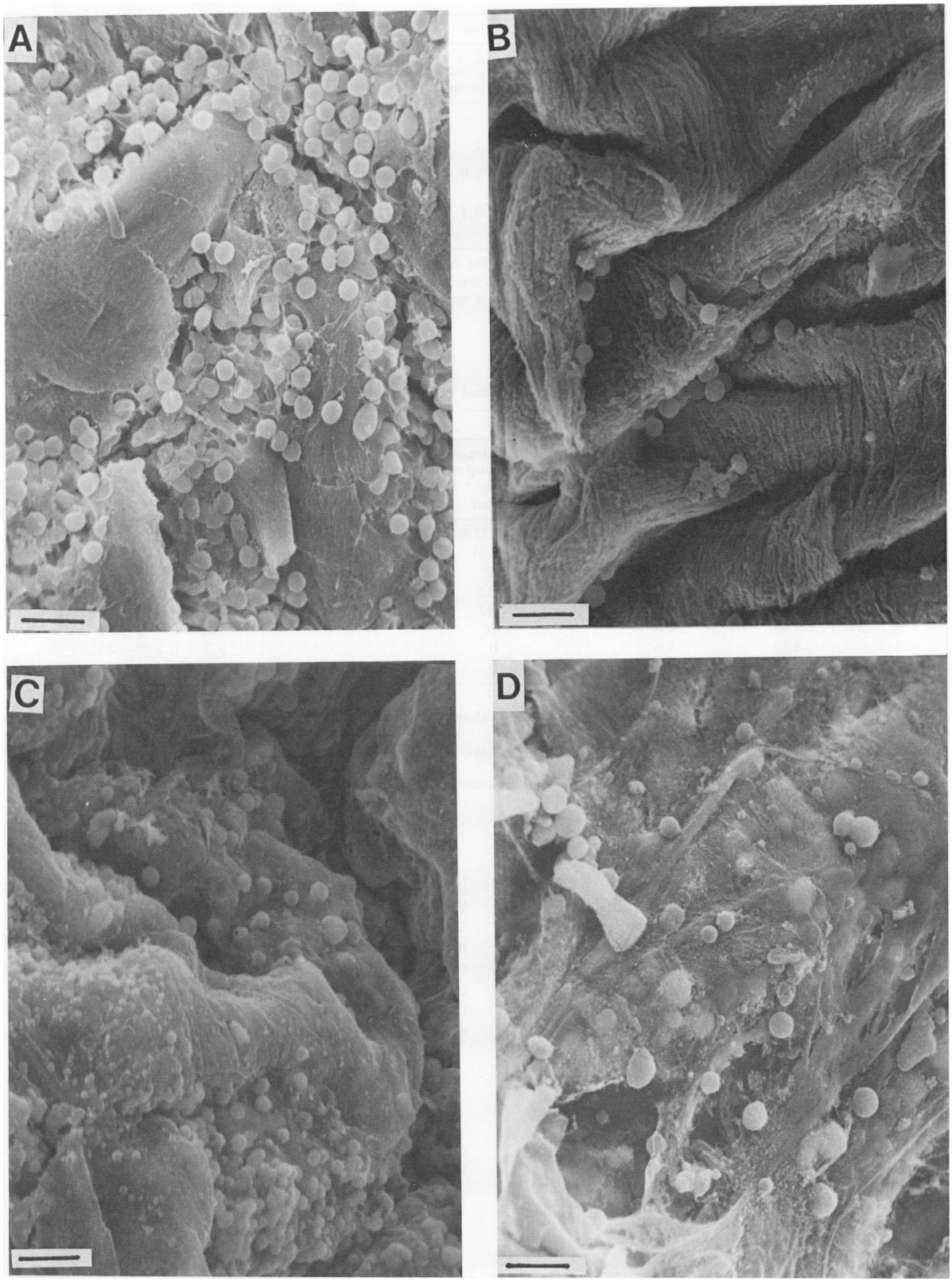

FIG. 1. The encapsulated strain of *C. neoformans* (serotype A) adhering to (A) the ventral surfaces of the tongues, (B) the esophagi, (C) the keratinized portions of the stomachs, and (D) the colons of monoassociated BALB/c mice. Bar, 10 μm.

TABLE 2. Pathogenesis of an encapsulated strain of *C. neoformans* in orally challenged gnotobiotic BALB/c mice

Days after challenge	No. of mice		No. of *C. neoformans*[a] in:									
			Kidney		Liver		Lung		Spleen		Brain	
	nu/nu	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*
1	6	6	0	0	0	0	0	1.0 ± 1.0[a]	0	0	0	0
3	11	12	0	0	0	0	0	0	0	0	0	2.5 ± 0.5
7	6	6	0	0	0	0.8 ± 0.8	0	3.4 ± 3.4	0	0	0	0
14	12	12	2.9 ± 1.7	0	3.4 ± 2.0	0.3 ± 0.3	3.8 ± 2.2	0	4.6 ± 2.4	0	4.2 ± 1.6[b]	0
19	2	2	5.8	0	7.8	0	7.7	7.6	7.3	ND[c]	6.8	0
21	8	9	5.0 ± 0.2[b]	1.3 ± 0.7	6.7 ± 0.2[b]	1.4 ± 0.7	8.1 ± 0.7[b]	1.9 ± 1.0	6.2 ± 0.1[b]	2.3 ± 0.4	6.0 ± 0.5[b]	0
28	0	6	—[d]	0.8 ± 0.8	—	0	—	1.5 ± 1.5	—	0	—	0

[a] Mean $\log_{10}$ *C. neoformans* CFU per gram (dry weight) ± standard error of the mean. Counts represent data from pools of two to three organs at each time interval after oral challenge. Except for day 19, two to four repeat experiments were carried out at each time interval.

[b] The number of *C. neoformans* organisms isolated from the internal organs of nude (*nu/nu*) mice was significantly higher ($P < 0.05$) than that isolated from their heterozygous (*nu/+*) littermates.

[c] ND, Not done.

[d] —, Nude mice died 19 to 28 days after oral challenge.

TABLE 3. Effect of antibiotics on colonization of conventional BALB/c mice gavaged with encapsulated *C. neoformans*

Days after gavage[a]	Tetracycline-treated mice[b]				Conventional mice	
	Mean $\log_{10}$ *C. neoformans*/g of *nu/nu* mouse feces ± SEM (range)[c]	No. of shedders[d]	Mean $\log_{10}$ *C. neoformans*/g of *nu/+* mouse feces ± SEM (range)[c]	No. of shedders[d]	Mean $\log_{10}$ *C. neoformans*/g of *nu*/nu mouse feces ± SEM (range)[c]	No. of shedders[d]
1	4.2 ± 0.1 (4.0–4.8)	6/6	3.6 ± 0.3 (2.4–4.6)	6/6	4.1 ± 0.3 (3.3–5.1)	7/7
2	2.5 ± 0.3 (0–4.1)	5/6	3.0 ± 0.1 (0–3.4)	4/6	3.7 ± 0.4 (0–4.8)	6/7
3	3.2 ± 0.2 (0–3.4)	2/6	0[e]	0/6	3.7 ± 0.4 (0–4.7)	5/7
4	ND[f]	ND	ND	ND	3.6 ± 0.5 (0–4.2)	5/7
5	ND	ND	ND	ND	3.9 ± 0.3 (0–4.3)	4/7
6	2.7 ± 0.3 (0–3.4)	4/6	0	0/6	3.6 ± 0.1 (0–3.9)	5/7
7	3.2 ± 0.8 (0–4.6)	4/6	0	0/6	3.7 ± 0.2 (0–3.8)	5/7
8	2.8 ± 0.7 (0–4.1)	4/6	0	0/6	ND	ND

[a] BALB/c mice were gavaged with 10^6 *C. neoformans* organisms.

[b] Mice were given 660 μg of tetracycline per ml in their drinking water 2 days before being gavaged with *C. neoformans*. Mice were maintained on this antibiotic regimen for the duration of the study.

[c] Mean $\log_{10}$ *C. neoformans* CFU per gram of feces from mice shedding *C. neoformans* in their feces ± standard error of the mean. The range is the $\log_{10}$ *C. neoformans* CFU per gram of feces from all mice used in study.

[d] Number of mice with *C. neoformans* in their feces/number of mice in the study.

[e] No *C. neoformans* CFU detected.

[f] ND, Not done.

the lungs for 2 to 4 weeks before extrapulmonary dissemination occurred (11, 12). In our study, no buildup of viable encapsulated *C. neoformans* cells was detected in the lungs of nude mice prior to their dissemination to internal organs, making it unlikely that any massive dissemination occurred from the lungs during this period. Conversely, development of systemic disease in nude mice was coincident with increased viable populations of encapsulated cryptococci in the stomachs and ceca of orally challenged germfree *nu/nu* mice, suggesting that *C. neoformans* transmigrated from the GI tract into the internal organs. In contrast to *C. neoformans*-monoassociated and tetracycline-treated *nu/nu* mice, immunocompetent *nu/+* mice apparently controlled the dissemination of *C. neoformans* from the GI tract, indicating the importance of cell-mediated immunity in resistance to cryptococcal disease.

Our data on the inability of *C. neoformans* to colonize tetracycline-treated conventional *nu/+* mice is consistent with other studies which demonstrated that *C. neoformans* did not readily colonize conventional animals. Pigeons, whose environment often contains large numbers of *C. neoformans*, eliminated *C. neoformans* from their GI tracts within 24 h after intragastric challenge (1). Green and Bulmer (7) reported that only 24% of intragastrically challenged immunocompetent conventional mice shed *C. neoformans* in their feces and that mortality due to disseminated cryptococcosis occurred in only 3% of intragastrically challenged mice. In contrast, our data demonstrated that *C. neoformans* can colonize mucosal surfaces and cause systemic cryptococcosis in immunodeficient *nu/nu* mice, suggesting that the alimentary tract can be a portal of entry for *C. neoformans* in an immunodeficient host.

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