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Decontamination of Gnotobiotic Mice Experimentally Monoassociated with *Candida albicans*

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Gnotobiotic AKR mice, experimentally monoassociated with *Candida albicans*, were successfully decontaminated by oral treatment with amphotericin B incorporated in the drinking water. Germfree mice first were swabbed orally with viable *C. albicans* and then were allowed to acclimatize for 4 weeks. The $\log_{10}$ of number of *C. albicans* per gram of organ (with luminal contents) was 7.9 and 7.7 in the stomach and cecum, respectively. Direct fecal smears, as well as impression smears of stomach and cecum mucosal surfaces, revealed yeast-phase cells, many with germ tubes, but no hyphal forms. No illness or mortality was observed over this period. The mice then were given amphotericin B dissolved in the drinking water and offered ad libitum. At levels of 0.1 and 0.2 mg/ml, the number of fecal *C. albicans* was decreased but not eliminated completely. However, 0.3 mg/ml was sufficient to decontaminate the mice completely and return them to the germfree state. Residual amphotericin B was detected in the feces of the mice only while they were receiving the 0.3 mg/ml dose level. These mice remained germfree until the termination of the experiment, 10 weeks after the antibiotic had been discontinued and replaced by plain drinking water.

Candidiasis, in recent years, has become a common health hazard in human patients undergoing physical and/or chemical treatment associated with organ transplantation (12), leukemia (3), lymphoma (1), and other neoplastic diseases. In addition, it has been known for quite some time that prolonged treatment of patients with broad-spectrum antibacterial antibiotics may lead to severe infection by *Candida* when the normal flora has been suppressed. Seelig (13, 14) has reported and reviewed the mechanisms by which candidiasis may become a hazard in such cases.

The occurrence of *Candida albicans* as a commensal or as a pathogen in the gastrointestinal tract of animals and man has been reviewed by Winner and Hurley (16). Several investigators have employed laboratory mice as experimental models to study host relationships to *C. albicans*. Huppert and Cazin (4) were unable to isolate *Candida* from conventionally reared mice. These same authors reported that antibiotic administration was necessary for the survival of *Candida* in the intestinal tract of such animals. Mourad and Friedman (7) and Rao and Sirsi (11) were unable to infect conventional mice with *Candida* administered via the oral route. By contrast, Phillips and Balish (8) were able to establish *C. albicans* in conventional mice but they found that no pathogenesis was associated with the colonization of the gastrointestinal tract of these mice. However, they did observe pathogenesis in one of three gnotobiotic mouse strains subjected to monoassociation with *C. albicans*. Clark (2), working with gnotobiotic and pathogen-free mice, concluded that, although mice do not have an innate resistance to colonization by *Candida* per se (based on the gnotobiotic mice), ecological mechanisms are operative in pathogen-free mice that affect colonization by *Candida*. Taking the work of Phillips and Balish (8) and of Clark (2) together, it appears that the strain of mouse, the diet fed, and the composition of the intestinal flora of the host may be factors that affect colonization.

The present investigation was undertaken (i) to establish *C. albicans* in previously germfree mice via oral inoculation and (ii) to determine whether such mice can be decontaminated successfully and returned to their previous germfree state by treatment with an appropriate anti-fungal agent such as amphotericin B.

MATERIALS AND METHODS

Animals. Sixteen germfree female AKR mice, 7 to 8 weeks old and weighing 18 to 25 g, were used in this study. They were fed autoclaved L-485 pellets (Teklad Mills, Winfield, Iowa) and either plain drinking water or water containing antibiotics as

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described below. The germfree AKR mouse colony has been maintained in Trexler-type isolators (Standard Safety Equipment Co., Palatine, Ill.) and has been inbred at Lobund Laboratory for more than 25 generations. The derivation and maintenance of these animals has been previously described by Pollard (9). Germfree mice are free of horizontally transmitted microorganisms (15), including many viruses (10), but are carriers of one or more vertically transmitted viruses (9, 10).

Inoculation of mice. A culture of *C. albicans*, Type B #792, was kindly supplied by E. Balish, Department of Medical Microbiology, University of Wisconsin, Madison. The culture was entered into the Notre Dame Culture Library as ND-803 and is being maintained on Sabouraud dextrose agar (SDA, BBL) slants by transfer at monthly intervals and storage at room temperature.

C. albicans, for oral inoculation into germfree mice, was grown at 37 C for 24 h in ampoules of Sabouraud dextrose broth (SDB, BBL). The ampoule was hermetically sealed and passed aseptically via peracetic acid spray through a double-entry lock into a Trexler-type plastic isolator housing the germfree mice. The sealed ampoule was opened inside the isolator, and each mouse was swabbed orally with a cotton-tipped applicator dipped into the culture.

Microbiological monitoring. The germfree status of the mice, prior to oral inoculation with *C. albicans*, was established by methods previously described by Wagner (15). After oral inoculation, the mice were monitored specifically for *C. albicans* at weekly intervals prior to, during, and after cessation of antibiotic administration. Briefly, a freshly voided fecal pellet was subdivided: one portion was inoculated into SDB and incubated at 37 C for 2 days; the other portion was smeared and fixed on a slide, stained with Hucker Gram stain, and observed with a microscope.

Quantitative counts of *C. albicans* were performed on mice 2 weeks after the original oral inoculum had been administered and prior to the onset of antibiotic treatment. The mouse to be tested was killed by cervical dislocation and the stomach and cecum, with contents, were ground separately in 1.0 ml of SDB with a sterile mortar and pestle. The homogenates were serially diluted and plated in triplicate on SDA. Colonies were counted after incubation at 37 C for 48 h. Mice from the same group were killed for the preparation of direct impression smears taken from the mucosal surfaces of the stomach and cecum walls after removal of luminal contents. The smears were Gram stained and examined with a microscope for direct evidence of colonization of the mucosal surfaces.

Terminal whole body cultures were run 5 to 10 weeks after antibiotic treatment had been discontinued in order to determine the final status of decontamination in these mice. The mouse to be tested was killed, and the total carcass was aseptically homogenized in a blender containing SDB. The homogenate was transferred to large tubes of SDB and incubated at 37 C for 4 weeks. Subtransfers from these original cultures were also made and similarly incubated.

Treatment of mice with amphotericin B. Amphotericin B (Fungizone, E. R. Squibb and Sons, Inc., N.Y.) was prepared fresh each week as a concentrated solution in sterile water. The concentrate was diluted as needed to desired concentrations, 0.1, 0.2, or 0.3 mg/ml of sterile drinking water, and was given ad libitum as the sole source of drinking fluid.

Two weeks after the initiation of antibiotic treatment, all mice were given a total body immersion in aqueous 1:2,000 Zephiran chloride (Winthrop Laboratories, N.Y.) and were immediately transferred to a new presterilized isolator. Amphotericin B treatment was continued after transfer, and the effect on the *C. albicans* population was noted. The dip and transfer procedure was repeated each time that a change in the concentration of antibiotic was made (see protocol in Table 1).

The antibiotic was discontinued after the results of the microbiological monitoring showed that *C. albicans* could no longer be detected. The mice were put back on plain drinking water and the experiment was continued for an additional 10 weeks to verify their decontaminated status in the absence of antibiotic.

Sensitivity of C. albicans to amphotericin B. A simplified test for the sensitivity of *C. albicans* to amphotericin B was run on the original stock culture as well as on the fecal organisms cultured directly from the inoculated mice prior to and during antibiotic feeding. A freshly voided fecal pellet was uniformly suspended in 0.5 ml of SDB and distributed evenly over the surface of an SDA plate. Paper disks (Schleicher and Schuell, Keene, N. H.) were prepared with different known concentrations of amphotericin B and placed on the seeded lawns. The plates were incubated at 37 C and observed for the appearance of growth inhibition zones.

Drug excretion in feces and urine. Qualitative tests for the presence of amphotericin B in feces and in urine were run. Sterile paper disks were saturated directly with either freshly voided urine or with a suspension of freshly voided pellets (2 pellets in 0.5 ml of sterile distilled water). Observation for growth inhibition zones was made on SDA plates freshly seeded with a stock culture of *C. albicans*.

RESULTS

Establishment of C. albicans in gnotobiotic mice. *C. albicans* became established in all gnotobiotic mice after oral inoculation. The data on the enumeration of gastrointestinal yeast revealed that both the stomach and cecum (including contents) had approximately equal numbers of the yeast: the $\log_{10}$ of number per gram of organ was 7.89 and 7.66, respectively. All gnotobiotic mice appeared healthy and remained asymptomatic during an acclimatization and observation period of 4 weeks. Gram-stained impression smears made from the stomach mucosal surface showed the presence of yeast on the glandular portion whereas cecal impression smears revealed the presence

of yeast throughout the cecum. These stained mucosal surface smears, as well as fecal smears, were characterized by the presence of abundant yeast-like cells, many with germ tubes. No hyphal forms were detected in any of the smears examined.

Elimination of C. albicans from gnotobiotic mice. Ad libitum consumption of drinking water containing amphotericin B at the 0.1 or 0.2 mg/ml level for 9 and 8 weeks, respectively, was not adequate to completely eliminate *C. albicans* from these mice (Table 1), although at these dose levels, the number of yeasts recovered from feces was markedly reduced. Both the parent stock culture and the isolates from mice receiving the antibiotic displayed well defined zones of inhibition around the paper disks that had been impregnated with 3 μg of the antibiotic. Disks treated with 1.5 μg of antibiotic gave minimal zones against these same inocula.

Mice receiving the antibiotic at either the 0.1 or 0.2 mg/ml level excreted the drug in the urine in detectable quantities, but no residual drug was found in the feces (Table 1). These doses were apparently insufficient to completely eliminate the yeast from the gastrointestinal tract.

At the dosage of 0.3 mg of amphotericin B per ml of drinking water over an 8-week period, *C. albicans* was recovered from the mice after 1, 2, and 3 weeks of treatment, but by the fourth week and thereafter the yeast could no longer be isolated (Table 1, time period 4). The antibiotic was detected in both the urine and feces when administered at this higher level.

No fungicidal activity was detected in the feces and urine samples of normal germfree or conventional AKR mice.

Finally, antibiotic was discontinued and replaced by plain drinking water over a period of 10 weeks. The mice remained yeast-free throughout this period. Terminally, cultures of the total carcass also tested sterile.

The above results indicate that amphotericin B, administered in the drinking water ad libitum at 0.3 mg/ml, was effective in decontaminating mice monoassociated with *C. albicans*, thus effectively returning them to their original germfree state.

TABLE 1. *Decontamination of C. albicans-monoassociated gnotobiotic mice by amphotericin B administered in the drinking water*

Sequential time periods	Concn of antibiotic (mg/ml of water)	Detection of *C. albicans*	Detection of antibiotic in:	
			Feces	Urine
1 (4 weeks)	None	+	ND[a]	ND
2 (9 weeks)	0.1	+	−	+
3 (8 weeks)	0.2	+	−	+
4 (8 weeks)	0.3	+ then −[b]	+	+
5 (10 weeks)	None	−	ND	ND
Controls				
Germfree mice	None	−	−	−
Conventional mice	None	−	−	−

[a] ND, Not done.

[b] *C. albicans* was recovered from these animals after 1, 2, and 3 weeks of treatment at this dose level. All subsequent cultures were uniformly negative.

DISCUSSION

The data reported herein confirm the findings of others (2, 8) that *C. albicans* can be established readily in gnotobiotic mice by oral inoculation. Quantitative counts of approximately 10^8 *C. albicans* per g (wet weight) of stomach and cecum (wall plus luminal contents) indicated a well established *Candida* population in these mice, yet no pathology or evidence of illness was observed. *C. albicans* grew in the mouse in vivo in the yeast phase, with many of the cells showing germ tubes, but no hyphae were observed.

Phillips and Balish (8) have reported that the infective stage of *Candida* is the hyphal form. These workers observed pathogenesis in only one of three mouse strains gnotobiotically monoassociated with *C. albicans*. Hyphal forms were seen only in the strain of mice that developed lesions. The other two mouse strains remained asymptomatic and harbored only yeast-phase cells. Due to variation in the diet fed these mouse strains, these authors were not able to determine whether the differences in pathogenicity were due to genetic differences of the host or due to dietary effects.

In the present experiments, the hyphal stage was never detected in vivo. This probably explains the absence of symptoms and pathology over a 4-week observation period prior to the onset of amphotericin B treatment. Since the AKR strain of mouse and the L-485 Teklad diet fed were different than those employed by Phillips and Balish (8), we have no explanation for the lack of pathogenesis in our animals. Presumably the culture, the mouse strain, or the diet could have affected this parameter.

The main objective of this work was to decontaminate the mice and return them to their previous germfree state after gnotobiotic monoassociation with *C. albicans* had been established. This was successfully accomplished by feeding amphotericin B ad libitum in the drinking water at the level of 0.3 mg/ml over an 8-

week period. Previous levels of 0.1 or 0.2 mg/ml were insufficient to eliminate the organism. It is believed that, at these lower dosages, the antibiotic was mainly absorbed higher up in the gastrointestinal tract so that little or no residual antibiotic accumulated to inactivate the organisms residing in the lower bowel. The presence of antibiotic in the urine but not in the feces of these animals (Table 1) supports this hypothesis. However, at 0.3 mg/ml, both the urine and the feces were positive for residual antibiotic. Under these conditions, *C. albicans* was completely eliminated and the animals continued to test germfree for 10 weeks after all antibiotic treatment had been discontinued.

All the mice drank and tolerated the antibiotic well. None displayed evidence of drug toxicity and all appeared healthy during and after treatment. However, the procedures used in mice may have limited value when applied to treatment of candidiasis in man. Amphotericin B (solubilized in sodium deoxycholate) has been reported by Kravetz et al. (5) to be too bitter in taste and to produce severe diarrhea upon oral administration to human patients. These side effects were ascribed to the deoxycholate solubilizer rather than the amphotericin B itself. These observations have been confirmed recently by us using squirrel monkeys (unpublished observation). Oral nonsolubilized amphotericin B is well tolerated in humans but is unsatisfactory in treatment of systemic candidiasis because of the low blood levels attainable with the noncolloidal antibiotic (6). Perhaps the use of other solubilizing agents could make oral treatment adaptable to man.

It is concluded from these studies that experimental *C. albicans* "infection" in gnotobiotic mice can be successfully eliminated by administration of amphotericin B ad libitum per os for a sufficient time period and at a dose level high enough to maintain inhibitory concentrations in the lower bowel and in voided feces.

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LITERATURE CITED

1. Bodey, G. P. 1966. Fungal infections complicating acute leukemia. J. Chronic Dis. 19:667-687.
2. Clark, J. D. 1971. Influence of antibiotics or certain intestinal bacteria on orally administered *Candida albicans* in germ-free and conventional mice. Infect. Immunity 4:731-737.
3. Hart, P. D., E. Russell, Jr., and J. S. Remington. 1969. The compromised host and infection. II. Deep fungal infection. J. Infect. Dis. 120:169-191.
4. Huppert, M., and J. Cazin, Jr. 1955. Pathogenesis of *Candida albicans* infections following antibiotic therapy. II. Further studies of the effect of antibiotics on the *in vitro* growth of *Candida albicans*. J. Bacteriol. 70:435-439.
5. Kravetz, H. M., V. T. Andriole, M. A. Huber, and J. P. Utz. 1961. Oral administration of solubilized amphotericin B. N. Engl. J. Med. 265:183-184.
6. Louria, D. G. 1958. Some aspects of absorption, distribution, and excretion of amphotericin B in man. Antibiot. Med. 5:295-301 (cited by W. T. Butler, J. Am. Med. Assoc. 195:371-375, 1966).
7. Mourad, S., and L. Friedman. 1961. Active immunization of mice against *Candida albicans*. Proc. Soc. Exp. Biol. Med. 106:570-572.
8. Phillips, A. W., and E. Balish. 1966. Growth and invasiveness of *Candida albicans* in the germ-free and conventional mouse after oral challenge. Appl. Microbiol. 14:737-741.
9. Pollard, M. 1973. Research animals in medicine, p. 1005-1013. *In* L. T. Harmison (ed.), Publication No. (N.I.H.) 72-233, Department of Health, Education, and Welfare, Washington, D.C.
10. Pollard, M., M. Kajima, and B. A. Teah. 1965. Spontaneous leukemia in germfree AK mice. Proc. Soc. Exp. Biol. Med. 120:72-75.
11. Rao, R. G., and M. Sirsi. 1962. The pathogenicity of *Candida albicans* and the effect of Nystatin on experimental candidiasis. Indian J. Med. Res. 50:1-7.
12. Rifkind, D., T. L. Marchioro, S. A. Schneck, and R. B. Hill, Jr. 1967. Systemic fungal infections complicating renal transplantation and immunosuppressive therapy. Clinical, microbiologic, neurologic, and pathologic features. Am. J. Med. 43:28-38.
13. Seelig, M. S. 1966. The role of antibiotics in the pathogenesis of *Candida* infections. Am. J. Med. 40:887-917.
14. Seelig, M. S. 1966. Mechanisms by which antibiotics increase incidence of severity of candidiasis and alter the immunologic defenses. Bacteriol. Rev. 30:442-459.
15. Wagner, M. 1970. Gnotobiotes: standards and guidelines for the breeding, care, and management of laboratory animals, p. 28-39. National Academy of Science, Washington, D.C.
16. Winner, H. I., and R. Hurley, 1964. *Candida albicans*. J. and A. Churchill Ltd., London, England.