INFECTION AND IMMUNITY, Sept. 1985, p. 654–663
0019-9567/85/090654-10$02.00/0
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Vol. 49, No. 3

Ecology of *Candida albicans* Gut Colonization: Inhibition of *Candida* Adhesion, Colonization, and Dissemination from the Gastrointestinal Tract by Bacterial Antagonism

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Received 19 December 1984/Accepted 20 May 1985

Antibiotic-treated and untreated Syrian hamsters were inoculated intragastrically with *Candida albicans* to determine whether *C. albicans* could opportunistically colonize the gastrointestinal tract and disseminate to visceral organs. Antibiotic treatment decreased the total population levels of the indigenous bacterial flora and predisposed hamsters to gastrointestinal overgrowth and subsequent systemic dissemination by *C. albicans* in 86% of the animals. Both control hamsters not given antibiotics and antibiotic-treated animals reconventionalized with an indigenous microflora showed significantly lower gut populations of *C. albicans*, and *C. albicans* organisms were cultured from the visceral organs of 0 and 10% of the animals, respectively. Conversely, non-antibiotic-treated hamsters inoculated repeatedly with *C. albicans* had high numbers of *C. albicans* in the gut, and viable *C. albicans* was recovered from the visceral organs of 53% of the animals. Examination of the mucosal surfaces from test and control animals indicated further that animals which contained a complex indigenous microflora had significantly lower numbers of *C. albicans* associated with their gut walls than did antibiotic-treated animals. The ability of *C. albicans* to associate with intestinal mucosal surfaces also was tested by an in vitro adhesion assay. The results indicate that the indigenous microflora reduced the mucosal association of *C. albicans* by forming a dense layer of bacteria in the mucus gel, out-competing yeast cells for adhesion sites, and producing inhibitor substances (possibly volatile fatty acids, secondary bile acids, or both) that reduced *C. albicans* adhesion. It is suggested, therefore, that the indigenous intestinal microflora suppresses *C. albicans* colonization and dissemination from the gut by inhibiting *Candida*-mucosal association and reducing *C. albicans* population levels in the gut.

Systemic *Candida* infections are important causes of morbidity and mortality among patients who are compromised immunologically or who are undergoing prolonged antibiotic therapy (41, 52, 69). The passage of viable *Candida albicans* through the gastrointestinal (GI) mucosa into the host bloodstream is believed to be an important mechanism leading to systemic candidosis (38, 61, 62), particularly in patients with acute leukemia (43). Patient and animal studies support this hypothesis and indicate that a number of opportunistic fungal pathogens can spread systemically from the intestinal lumen to invade visceral organs (28, 29, 67, 68). We recently reported, for instance, that *C. albicans*, *Candida parapsilosis*, *Candida pseudotropicalis*, *Candida tropicalis*, and *Torulopsis glabrata* could opportunistically colonize the gut and disseminate to visceral organs after intragastic challenge of antibiotic-treated mice (37). The feeding of *C. albicans* to animals not given antibiotics, in contrast, does not lead to *Candida* dissemination from the GI tract (13, 37, 67) unless extremely high numbers of yeasts are ingested (38, 61, 62), suggesting that large numbers of fungi may be a determinant of fungal dissemination (45).

Since antibiotic treatment predisposes animals to GI overgrowth and subsequent dissemination by *Candida* organisms, it has been suggested that members of the indigenous intestinal microflora suppress the growth of *C. albicans* within the gut, thereby preventing systemic invasion (43, 61, 62). Several studies have shown that certain intestinal bacteria are inhibitory to both in vitro growth and GI colonization by *C. albicans* (4, 32–34, 44, 47, 48). It is important to note, however, that these studies do not reflect interactions as they normally occur in the intestinal tract (17, 21). Most, if not all, in vitro studies have reported on *C. albicans* suppression by a single bacterial species (45), which is hardly representative of the 400 to 500 different bacterial species that normally inhabit the intestinal tract (54). Likewise, studies in which a monoflora of *Escherichia coli* (or any other single bacterial species) antagonized *C. albicans* growth in the gut of gnotobiotic animals (4, 32, 33, 44) cannot be expected to reflect interactions of a complex indigenous microflora (17, 21). Bacteria under the former condition reach abnormally high numbers in the gut (31). *E. coli* itself is suppressed by the strict anaerobes which dominate the intestinal microflora (22, 63). Thus, it is still not known whether *E. coli* can exert an inhibitory mechanism(s) over *C. albicans* under normal in vivo conditions, as has so often been suggested (4, 32–34, 44, 45). In fact, Clark (10) showed that *C. albicans* grew unchecked for several weeks in the GI tracts of gnotobiotic mice containing an intestinal flora of *Bacteroids* sp., *Lactobacillus* sp., *Streptococcus faecalis*, *Streptococcus lactis*, and *E. coli*. Recent studies also indicate that enteric bacilli (including *E. coli*) do not inhibit the growth of *C. albicans* in the GI tracts of conventional mice (2, 37a). The present studies were initiated to examine the mechanisms by which a complex indigenous microflora inhibits *C. albicans* colonization and dissemination from the GI tract.

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MATERIALS AND METHODS

Animals. Male Syrian hamsters (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing 100 to 120 g each were used in all experiments and maintained on Purina mouse chow 5015.

Fungi. A clinical isolate of *C. albicans* (CA34) recovered from the blood of a patient with systemic candidosis was maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) slants at 22 to 25°C. For intragastric challenge of hamsters, *C. albicans* cells were grown aerobically in 100 ml of Sabouraud dextrose broth (BBL Microbiology Systems, Cockeysville, Md.) for 18 h at 37°C. For mucosal association studies, *C. albicans* cells were grown in Sabouraud dextrose broth as above, harvested by centrifugation, and washed three times in phosphate-buffered saline (PBS; pH 7.0).

Bacteria. The indigenous cecal microflora for recolonization of antibiotic-decontaminated animals was obtained from Syrian hamsters. Animals were sacrificed by cervical dislocation and placed immediately into an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) that was maintained at less than 5 parts of oxygen per 10^6 parts of an atmosphere consisting of 5% CO_2, 10% H_2, and 85% N_2 (3). The hamster ceca then were removed aseptically and homogenized for 10 s in a Waring blender vessel containing 30 ml of sterile, prereduced tryptic soy broth (Difco) with 0.04% (wt/vol) Na_2CO_3 to compensate for 5% CO_2 in the anaerobic chamber (65). These cecal homogenates were placed into 50-ml sterile tubes, mixed vigorously, and allowed to settle briefly. The resulting supernatants containing the indigenous cecal microflora were transferred inside the anaerobic chamber to 10-ml glass ampoules, stoppered, and placed on ice for transport to rigid plastic germfree isolators (Germfree Laboratories, Inc., Miami, Fla.) containing antibiotic-treated hamsters. Cecal homogenates were administered to hamsters within 15 min of preparation.

Filtrates of cecal contents were prepared by a modification of a previously described method (53). Briefly, cecal homogenates (prepared as described above) were removed from the anaerobic chamber and centrifuged twice at 10,000 × g for 15 min. The remaining supernatants then were passed through 0.20-μm filters (Millipore Corp., Bedford, Mass.) to remove any remaining bacteria and placed in 10-ml glass ampoules for transport to a separate group of antibiotic-treated hamsters.

Animal preparation and inoculation. Hamsters were housed in sterile, covered plastic cages and supplied with sterile Purina mouse chow and sterile distilled water containing vancomycin (500 μg ml^{-1}), ampicillin (1 mg ml^{-1}), and gentamicin (100 μg ml^{-1}) (VAG) ad libitum for 3 days (36, 37). Control animals were housed identically but without the addition of VAG to the drinking water. After animal decontamination, hamsters were transferred to germfree isolators as described previously (37).

To test the ability of indigenous cecal bacteria to protect against *C. albicans* colonization and dissemination, cecal homogenates (or filtrates as controls) were administered to antibiotic-decontaminated hamsters (by both oral and rectal routes) once daily for 5 consecutive days, beginning 5 days after stopping antibiotic treatment. For oral administration, hamsters were lightly anesthetized with methoxyflurane (Pittman-Moore Co., Washington Crossing, N.J.) and cecal homogenates or filtrates administered by carefully inserting a 5-cm 18-gauge plastic catheter through the mouth into the stomach and injecting 1 ml of the material. For rectal administration, the same catheter was placed approximately 5 mm into the rectum, the skin was pinched tightly around the catheter, and 2 ml of the material was injected. The latter dose has been shown to be an amount adequate to reach the cecum (66).

On the day after the last administration of cecal homogenates, or filtrates, the animals were challenged intragastrically (as above) with 0.5 ml of Sabouraud dextrose broth containing 10^7 CFU of *C. albicans*. Another group of hamsters not treated with antibiotics also was inoculated with 10^7 CFU of *C. albicans* intragastrically at 0, 8, and 16 h after the first yeast challenge, to maintain high numbers of *C. albicans* organisms in the gut without antibiotic treatment.

Enumeration of *C. albicans* in tissues. Counts of viable *C. albicans* were performed on cecal contents and visceral organs 24 h after administration of *C. albicans*. Animals receiving multiple inoculations of *C. albicans* were cultured for *C. albicans* 24 h after the first yeast challenge. Hamsters were sacrificed by cervical dislocation, their abdomens were soaked with 70% ethanol, and skin coverings and abdominal walls were reflected in layers to avoid contamination of the viscera. Spleens, kidneys, and livers were aseptically excised and placed into separate Waring blender vessels, each containing 20 ml of sterile tryptic soy broth. Ceca then were removed and placed into similar vessels containing 30 ml of tryptic soy broth. Organs were homogenized for 10 s at high speed. Serial 10-fold dilutions of organ homogenates were plated on Sabouraud dextrose agar supplemented with vancomycin (7.5 μg ml^{-1}) and ampicilin (100 μg ml^{-1}) for the enumeration of *C. albicans*. This medium yielded colony counts from *C. albicans* broth cultures that were comparable to blood agar, or Sabouraud dextrose agar without antibiotics, but it inhibited the growth of the indigenous microflora (36, 37). Plates were incubated aerobically for 48 h at 37°C to determine the number of CFU per organ.

Enumeration of indigenous cecal bacteria. Cecal population levels of indigenous bacteria were determined in antibiotic-treated and control animals in the anaerobic chamber. The ceca were removed aseptically and homogenized in prereduced tryptic soy broth as desribed above. Serial 10-fold dilutions were then plated on blood agar and AII agar (3) supplemented with 0.05% maltose (R. Freter, personal communication) without palladium chloride (46). For some experiments, indigenous microflora population levels were determined separately for cecal contents and cecal walls. The AII agar plates were incubated in the anaerobic chamber for 5 days at 37°C. Blood agar plates were incubated aerobically and anaerobically at 37°C for 48 h.

Mucosal association studies. The ability of *C. albicans* to associate with intestinal mucosal surfaces was tested both in vitro and in vivo. For the in vitro studies, a modification of a previously described adhesion assay (16) was performed. Briefly, animals were sacrificed by cervical dislocation and placed in the anaerobic chamber, and then the small bowel and ceca were aseptically removed from either antibiotic-treated or untreated hamsters. These were opened by a longitudinal incision, washed gently with cold PBS, and cut into square sections (approximately 1 by 1 cm). Each Erylenmeyer flask (25 ml) containing 10 ml of test solution (see below) received five intestinal slices from either small bowel or cecum. Yeast cells in PBS were added to each assay flask to give a final concentration of 10^6 CFU/ml, and the mixtures were incubated (with occasional agitation) in the anaerobic chamber at 37°C. Counts of the numbers of viable *C. albicans* associating with intestinal slices were performed 2 h after inoculation. Each slice was removed,

TABLE 1. Association of *C. albicans* with intestinal slices

Assay		Source of intestinal slices[b]	Log_{10} mean no. of *C. albicans*/slice	Association index[c]	Log_{10} mean no. of *C. albicans*/slice	Association index[c]
System	Solution[a]					
1	IC	Antibiotic-treated hamsters	2.4	0.13	2.5	0.08
2	IF	Antibiotic-treated hamsters	4.2	2.62	4.0	1.82
3	PBS	Antibiotic-treated hamsters	5.0	13.41	4.8	13.04
4	IC	Untreated hamsters	2.5	0.09	2.4	0.06
5	IF	Untreated hamsters	3.8	0.60	3.4	0.25
6	PBS	Untreated hamsters	3.9	0.70	3.7	0.73
7	PBS + BA	Antibiotic-treated hamsters	NT[d]	NT	4.3	4.19
8	PBS + VFA	Antibiotic-treated hamsters	4.1	1.17	4.1	1.86

[a] PBS + BA, PBS containing bile acids (lithocholic acid, 3.0 mM; deoxycholic acid, 2.6 mM); PBS + VFA, PBS containing VFA (valeric acid, 1.2 mM; isovaleric acid, 2.2 mM; butyric acid, 12.4 mM; isobutyric acid, 1.4 mM; propionic acid, 20.1 mM; acetic acid, 49.3 mM).

[b] Antibitoic-treated hamsters given VAG for 3 days as described in the text.

[c] The association index is described in the text.

[d] NT, Not tested.

washed three times with sterile PBS, and homogenized for 10 s at high speed in a Waring blender vessel containing 30 ml of sterile PBS. Serial 10-fold dilutions of either small bowel or cecal wall homogenates were plated on Sabouraud dextrose agar supplemented with vancomycin and ampicillin for the enumeration of *C. albicans*. Plates were incubated aerobically for 48 h at 37°C to determine the number of *C. albicans* per slice. Assay solutions also were quantitatively cultured. Counts of the rinsed intestinal slices were related to the count of 1 ml of test solution and defined as the association index. The association index = $(t/t + k) \times 100$, where t is the number of *C. albicans* associating with intestinal slices after rinsing, and k is the number of viable *C. albicans* per ml of assay solution after 2 h of incubation (16, 51).

To test the ability of *C. albicans* to associate with intestinal mucosal surfaces in vivo, untreated and antibiotic-treated (given penicillin G [500 U/ml] for 3 days as described above) animals were injected with 0.5 ml of Sabouraud dextrose broth containing 10^7 CFU of *C. albicans*. Twenty-four hours later, counts of the numbers of viable *C. albicans* were determined for cecal contents, cecal walls, and visceral organs. In addition, counts of the numbers of indigenous anaerobic bacteria and enteric bacilli per cecal contents or per gram of cecal wall were determined by plating 0.1 ml from serial 10-fold dilutions on AII agar and Tergitol-7 agar (Difco; 7) as described above. Several animals from each group also were sacrificed, and their intestinal tissues were examined by scanning electron microscopy (SEM) as described below.

The rate of *C. albicans* disassociation from intestinal tissues also was measured from ceca of antibiotic-treated and untreated animals challenged with *C. albicans*. Ceca were removed aseptically and opened, and nonadherent fungi and digesta were removed by washing with sterile PBS. Cecal tissues then were placed in 10 ml of sterile PBS and incubated on a Gyrotory shaker (at 10 rpm; New-Brunswick Scientific Co., Inc., Edison, N.J.) at 37°C. The amount of viable *C. albicans* cells in the surrounding PBS solution was determined by plating 0.1 ml from serial 10-fold dilutions at various intervals.

Variation in mucosal association assay parameters. *C. albicans* adhesion to intestinal mucosal surfaces was examined by performing the above adhesion assay in various test solutions, with intestinal slices from untreated or antibiotic-treated hamsters (Table 1). Antibiotic-treated animals were given sterile distilled water containing VAG as described above. To prepare hamster intestinal contents (IC), untreated animals were sacrificed and placed directly in an anaerobic chamber. Small bowel and ceca then were removed aseptically, and their contents were emptied into separate sterile test tubes, which were brought to 10 ml with PBS and mixed vigorously for 20 s. Indigenous microbes were removed from similar preparations by filtration (X2) through 0.20-μm Millipore filters and termed intestinal filtrates (IF). PBS alone, or mixed with secondary bile acids or volatile fatty acids (VFA), was used as the assay solution (Table 1).

SEM. Intestinal tissues were fixed overnight at 4°C by immersion in 2.5% gluteraldehyde in 0.2 M cacodylate buffer (pH 7.4), then washed in two changes of buffer, and postfixed in 1% osmium tetroxide in the same buffer for 1 h. The tissue then was washed in distilled water, dehydrated in acidified 2,2-dimethoxypropane (42), infiltrated with absolute alcohol, and critical point dried with a Polaron E-5100 critical-point dryer. Specimens were coated under vacuum with gold-palladium (60:40) in a Polaron sputter coater equipped with an omnirotary stage and examined in an ISI Super II scanning electron microscope at 15 kV.

Cecal VFA. The concentrations of cecal VFA were determined chromatographically (11; Supelco, Inc., Bellefonte, Pa., GC separation of VFA C2-C5, bulletin no. 749D, 1975) by a modification of an internal standard (2-methylpentanoic acid) method (40). The cecal contents of sacrificed antibiotic-treated and control animals were expelled into sterile preweighed tubes and mixed with 1 ml of 25% (wt/vol) metaphosphoric acid (Sigma Chemical Co., St. Louis, Mo.) and 1 ml of 2-methylpentanoic acid (11.2 mM Pfaltz and Bauer, Stamford, Conn.) for every 2 g of cecal content. The samples then were stoppered, mixed, and held at −70°C overnight. Samples were brought to room temperature, mixed, and centrifuged for 15 min at 17,500 × *g* at 10°C. After centrifugation, the supernatants were filtered through 0.20-μm filters, dispensed into 1.0-ml screw-capped autosampler vials (Phase Separation, Inc., Norwalk, Conn.), and loaded into a Varian model 8000 autosampler. The following standard VFA mixture also was used for comparison and calculation of the unknowns: acetic acid (Mallinckrodt, Inc., St. Louis, Mo.; 91.0 mM) propionic acid (Aldrich Chemical Co., Inc., Milwaukee, Wis.; 35.1 mM); and butyric, isobutyric, valeric, and isovaleric acids (Eastman Chemical Products, Inc., Kingsport, Tenn.; 16.97, 2.18, 2.03, and 1.80 mM, respectively). A varian model 6000 gas chromatograph equipped with a flame ionization detector was used. The chromatograph and autosampler were controlled by a Varian 401 chromatography data system. Chro-

TABLE 2. Population levels of indigenous bacteria in the ceca of antibiotic-treated and control animals[a]

Animal treatment[b]	Log_{10} mean CFU/g (wet wt) of cecum ± SD	
	Facultative bacteria	Strictly anaerobic bacteria
Antibiotics	2.4 ± 0.8	<2.4
Antibiotics, followed by cecal filtrates	2.4 ± 0.7	<2.4
Antibiotics, followed by cecal homogenates	6.0 ± 0.8	8.8 ± 0.4
None	6.3 ± 0.7	8.9 ± 0.2

[a] Values expressed are from three animals per group.
[b] Antibiotic-treated animals were given VAG for 3 days as described in the text.

matographic conditions were as follows. The column was a silane-treated glass column (outer diameter, 1/4 in. [6 mm]; inner diameter, 1/12 in. [2 mm]; height, 8 ft [2.4 m]) packed with GP 10% SP-1200/H_3PO_4 on Chromosorb W AW. Phosphoric acid-treated glass wool was used to plug the inlet and outlet ends. The column was packed, leaving 80 and 60 mm on the inlet and outlet sides, respectively, to allow for on-column injection. When in place, the glass wool plugs could be seen. The initial conditioning of the column was as described by Supelco, Inc. (Supelco, Inc., Column conditioning, bulletin no. 739, supplement, 1976). Chromatograph temperatures were: oven, 130°C; inlet, 170°C; detector, 175°C. Gases were: air, 60 lb/in^2; hydrogen, 40 lb/in^2; nitrogen carrier, 77 lb/in^2 at a flow rate of 37.5 ml/min. The sample injection volume was 0.25 μl, and the septum was a Thermogreen LB-2 (Supelco, Inc., Bellefonte, Pa.). The chromatographic method used gave consistent results even after several hundred samples were analyzed, and very little deterioration of the liquid phase of the column was observed (R. C. Greening and J. A. Z. Leedle, personal communication). Periodic analysis of the standard VFA mixture by this method gave 100 ± 5% of the actual concentrations (data not shown). Triplicate injections for each standard and duplicate injections for each sample were made. Septa were changed after each series of eight samples to minimize carrier gas leakage after repeated punctures by the autosampler needle.

RESULTS

Indigenous bacterial populations and cecal characteristics. The VAG treatment of hamsters used in this study reduced the total population size of the indigenous bacterial flora more than a millionfold (Table 2; $P < 0.001$). Furthermore, aerobic and anaerobic cultures of IC from these animals revealed that the strictly anaerobic flora was effectively eliminated, whereas the facultative flora contained primarily enterococci. Gram stains also showed antibiotic-treated animals to contain few bacteria (mostly gram-positive cocci), with a complete disappearance of the predominant gram-negative and fusiform-shaped rods. In contrast, antibiotic-treated hamsters which had been recolonized with cecal bacteria by oral and rectal injections of cecal contents had numbers of facultative and anerobic bacteria colonizing their intestinal tracts that were similar to those found in conventional animals (Table 2). Gram stains of IC from recolonized hamsters showed a bacterial flora resembling that of untreated animals. *C. albicans*, or other *Candida* spp., were not found to be commensals of the GI tracts of the hamsters used in the present study.

Dissection of test and control hamsters revealed several different cecal characteristics according to the experimental group examined. Animals that possessed a complex bacterial flora had small ceca (approximately 1 to 2% of their total body weight), which contained thick, pasty contents. Hamsters given antibiotics to eliminate the indigenous microflora, in contrast, had enlarged ceca (5 to 10% of their total body weight) with very watery contents, a trait attributable to mice lacking a complex intestinal microflora (33, 55).

The concentrations of most of the individual and the total VFA in the cecal contents of antibiotic-treated hamsters were usually less than half the amounts found in control animals (Table 3). Isovalerate concentrations, however, were slightly higher in antibiotic-treated animals, and isobutyrate concentrations in the cecal contents of VAG-treated animals were ca. 3.5 times those of the control animals (Table 3).

***C. albicans* colonization and dissemination from the GI tract.** The ability of *C. albicans* to colonize and disseminate from the GI tracts of test and control hamsters is summarized in Table 4. Antibiotic-treated animals challenged with *C. albicans* had high cecal numbers of *C. albicans* colonizing the gut 24 h after oral inoculation, and viable *C. albicans* recovered from the visceral organs of 86% of the animals. Similarly, antibiotic-treated hamsters given cecal filtrates before yeast challenge had large numbers of *C. albicans* present in the GI tract, and viable *C. albicans* was recovered from the visceral organs of 85% of the animals. Control animals not receiving antibiotic therapy, and antibiotic-treated hamsters recolonized with the indigenous intestinal flora, in contrast, had significantly lower cecal populations of *C. albicans* ($P < 0.001$). *C. albicans* organisms spread systemically from the gut to visceral organs in 0 and 10% of these animals, respectively, and the incidence of systemic spread to these organs was significantly lower ($P < 0.001$) than that in hamsters lacking a complex intestinal microflora.

These data supported the hypothesis that threshold population levels of *C. albicans* are an important determinant of fungal dissemination (45). To test this hypothesis further, an untreated group of hamsters was inoculated intragastrically

TABLE 3. Concentration of VFA in the ceca of antibitoic-treated and control hamsters

Animal treatment[a]	VFA concn (mM) of cecal content[b]						
	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric	Total
None (control)	118.6 ± 19.9	20.4 ± 4.5	4.4 ± 0.8	20.7 ± 6.3	3.6 ± 0.4	1.3 ± 0.1	168.2 ± 31.3
Penicillin	29.1 ± 3.9 (24.5)	6.9 ± 1.0 (33.8)	2.7 ± 1.0 (61.4)	2.7 ± 0.7 (13.0)	3.9 ± 0.7 (108.3)	0.5 ± 0.1 (38.4)	45.6 ± 4.9 (27.1)
VAG	25.8 ± 2.9 (21.8)	3.3 ± 0.4 (16.2)	16.3 ± 2.7 (370.4)	3.3 ± 2.1 (15.9)	4.0 ± 0.5 (111.1)	0.7 ± 0.6 (53.8)	53.4 ± 5.0 (31.7)

[a] Animals were given nothing, penicillin, or VAG ad libitum in the drinking water as described in the text.
[b] Values are mean ± standard deviation of five animals per group. Values within parentheses are percents of control group.

TABLE 4. Incidence of dissemination and organ population levels of *C. albicans* in antibiotic-treated and control hamsters

Animal treatment[a]	Incidence of dissemination[b]	Log$_{10}$ mean CFU of *C. albicans*[c] in:			
		Cecum	Liver	Kidneys	Spleen
Antibiotics	13/15	7.7 ± 0.7[c]	2.5 ± 1.2	2.4 ± 1.4	2.1 ± 1.4
Antibiotics, followed by cecal filtrates	17/20	7.6 ± 0.6	2.6 ± 1.3	2.3 ± 1.4	2.0 ± 1.5
Antibiotics, followed by cecal homogenates	2/20	3.8 ± 0.7	0.8 ± 0.2	ND	ND
None	0/20	3.4 ± 0.8	ND	ND	ND
None (multiple *C. albicans* inoculations)	8/15	6.2 ± 0.6	1.4 ± 1.2	1.5 ± 1.4	1.5 ± 1.3

[a] Antibiotic-treated animals received 3 days of VAG treatment as described in Table 2, footnote *b*.
[b] Number of hamsters with *C. albicans* cultured from visceral organs/number of animals tested.
[c] Log$_{10}$ mean CFU of *C. albicans* per gram (wet weight) of organ ± standard deviation. ND, Not detectable.

with 10^7 CFU of *C. albicans* at 0, 8, and 16 h after the first challenge, in an attempt to maintain cecal numbers of *C. albicans* similar to those in antibiotic-treated hamsters. At 24 h after the first yeast challenge, animals were sacrificed and quantitatively cultured for *C. albicans* in ceca and visceral organs as described above (Table 4). The numbers of viable *C. albicans* present in the GI tracts of these animals were about 1,000-fold higher than those in either untreated or recolonized hamsters given a single *Candida* challenge, but approximately 100-fold less than those in animals treated with antibiotics. Of these animals, 53% had viable *C. albicans* cultured from their visceral organs.

Mucosal association studies. To determine the numbers of *C. albicans* that associated with intestinal slices after incubation in the various test solutions, slices and their surrounding solutions were quantitatively cultured and the association index was calculated (Table 1). Between 10^5 and 10^6 viable *C. albicans* were recovered per ml of the surrounding test solution. When *C. albicans* organisms were incubated in PBS with intestinal slices from antibiotic-treated animals (assay 3), large numbers of *C. albicans* were found to associate with intestinal slices. For instance, association indexes for small bowel and cecal slices were determined to be 13.41 and 13.04, respectively. Conversely, when *C. albicans* cells were exposed to intestinal tissues from untreated hamsters (i.e., slices which contained an indigenous mucosal-associated microflora), low numbers of *C. albicans* were found to associate with the intestinal slices (assay 6). Association index of 0.70 and 0.73 were determined for small bowel and cecal tissues, respectively, from this assay. Similarly, low numbers of *C. albicans* were observed to associate with intestinal slices when exposed to small bowel and cecal slices in the presence of IC, which contained the indigenous microflora (assays 1 and 4). Interestingly, the ability of *C. albicans* to associate with intestinal tissues from antibiotic-treated hamsters also was reduced when the association assay was performed in IC from untreated animals that were filtered to remove the indigenous microflora (assays 2 and 5). IF from antibiotic-treated animals (data not shown) also reduced *C. albicans*-mucosal association from that observed in PBS alone; however, this reduction was not as severe as that observed when association experiments were performed with IF from untreated animals. Thus, a certain unknown chemical substance(s) produced in the GI tract of untreated animals, probably by metabolic activities of those organisms which predominate in the gut, appeared to inhibit the ability of *C. albicans* to associate with intestinal mucosal surfaces. In an attempt to identify such factors, VFA and secondary bile acids were tested in the mucosal association assay. It was found that both of these types of substances reduced the ability of *C. albicans* to associate with intestinal mucosal tissues (assays 7 and 8).

The ability of *C. albicans* to associate with intestinal mucosal surfaces also was tested in vivo. Penicillin-treated and untreated animals were challenged orally with *C. albicans* and cultured for *C. albicans* 24 h after inoculation as described above. In penicillin-treated animals, large numbers of *C. albicans* were found in the IC and on intestinal mucosal surfaces (Table 5). Significantly lower numbers of *C. albicans*, in contrast, were found in the IC or on the mucosal surfaces of untreated animals ($P < 0.001$; Table 5). Viable *C. albicans* was recovered only from the visceral organs of penicillin-treated animals (Table 5). Facultative enteric bacilli were likewise found to increase significantly in number in the IC and on host mucosal surfaces after antibiotic treatment, concomitantly with a reduction in the numbers of strictly anaerobic bacteria colonizing these same habitats (Table 5).

SEM studies revealed that large numbers of *C. albicans* were present on the surface of the villi and mucus material in antibiotic-treated animals, whereas only very small numbers of *C. albicans* were observed associating with host mucosal surfaces of untreated animals (Fig. 1). Likewise, *C. albicans* cells were often observed to penetrate deep into intestinal tissues of antibiotic-treated animals, but not untreated animals. Furthermore, *C. albicans* was often seen attached to and embedded in mucus material adjacent to intestinal villi only in antibiotic-treated animals (Fig. 2). In animals not

TABLE 5. Populations of indigenous bacteria and *C. albicans* in the cecal contents, cecal walls, and visceral organs of untreated and penicillin-treated animals challenged with 10^7 *C. albicans*

Animal treatment	Log$_{10}$ mean no. (± SD) of bacteria or yeast in:						
	Cecal contents (per g [wet wt])			Cecal wall (per g [wet wt])			Visceral organs
	Enteric bacilli	Anaerobes	*C. albicans*	Enteric bacilli	Anaerobes	*C. albicans*	*C. albicans*
None	5.2 ± 0.5	9.3 ± 0.5	3.3 ± 0.3	3.2 ± 0.2	9.8 ± 0.3	2.1 ± 0.4	ND[a]
Penicillin	9.6 ± 0.3	7.4 ± 0.7	7.4 ± 0.2	8.2 ± 0.2	8.1 ± 0.3	5.5 ± 0.3	2.06 ± 1.71 (6/10)[b]

[a] ND, Not detectable.
[b] Number of animals with *C. albicans* cultured from visceral organs/number of animals tested; this was determined 24 h after intragastric *Candida* challenge.

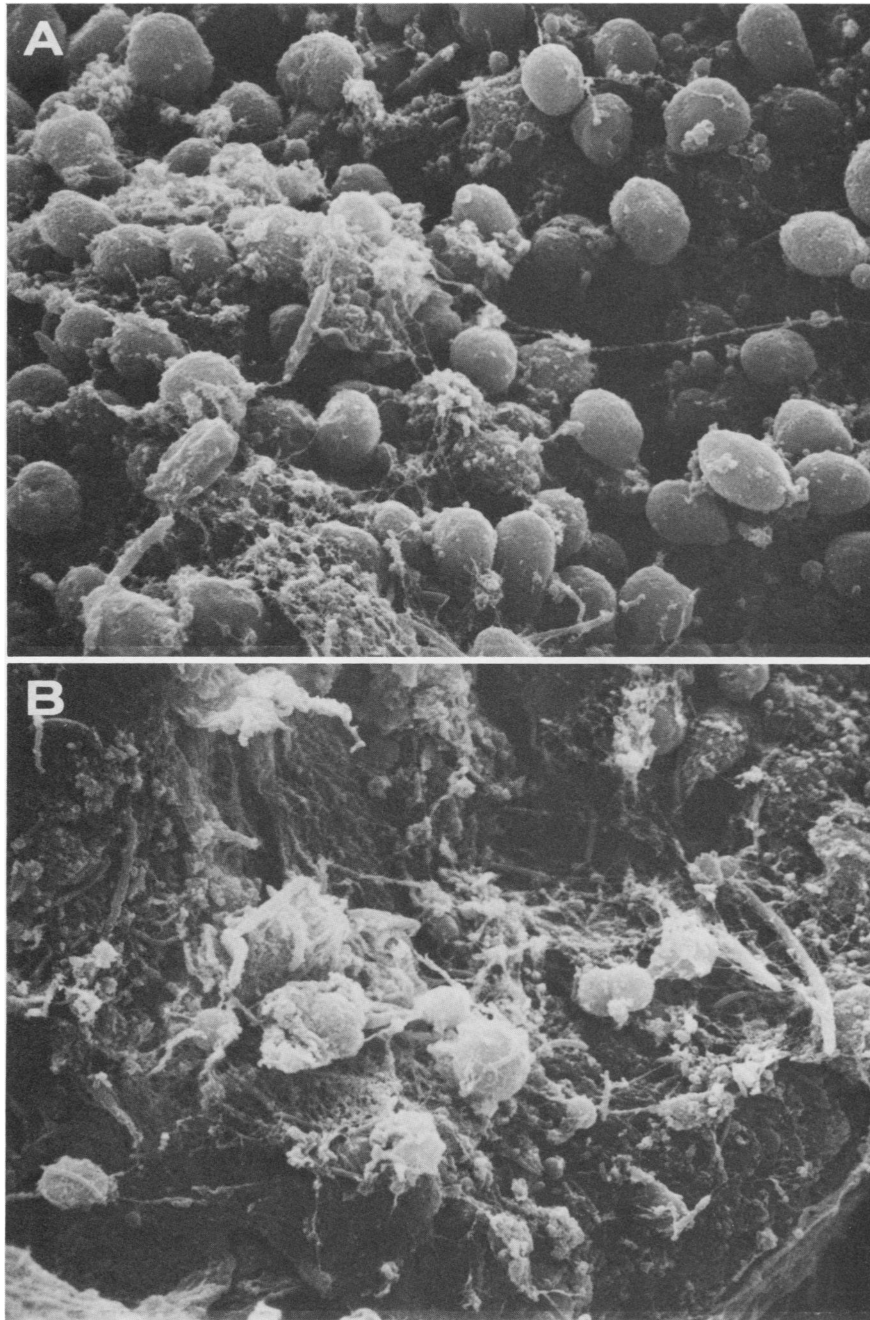

FIG. 1. SEM of ceca from antibiotic-treated (A) and control (B) animals challenged with *C. albicans*. Magnification, ×2,175 and ×1,400, respectively.

given antibiotics, yeast cells were probably associated with the loose top layer of mucus not preserved by the fixation procedure used for SEM studies. That is, most of the yeast cells found in untreated animals were probably associated with the thick layer of mucus gel covering the epithelium, whereas *Candida* cells were found throughout the cecal tissue of antibiotic-treated animals.

The rate of *C. albicans* disassociation from intestinal tissues from penicillin-treated and untreated animals (see above) also was tested. It was found that *C. albicans* cells were shed from intestinal mucosal surfaces at a faster rate from untreated animals than from antibiotic-treated animals. Nearly 74% (range, 58.7 to 76.2%) of the *Candida* cells that had associated with intestinal mucosal surfaces of untreated animals (n = 15) were removed by 1 h of incubation, whereas only a mean 31.3% (range, 1.8 to 60.9%) of the *Candida* cells were removed from animals treated with antibiotics (n = 15). It should be noted that it is not known whether the in vitro results of the present study adequately reflect the disassociation rates of *C. albicans* in vivo, although it is likely that *C. albicans* disassociation from intestinal tissues would be higher from animals that possessed an indigenous microflora since *C. albicans* cells would not be able to penetrate to the same degree into the mucus gel.

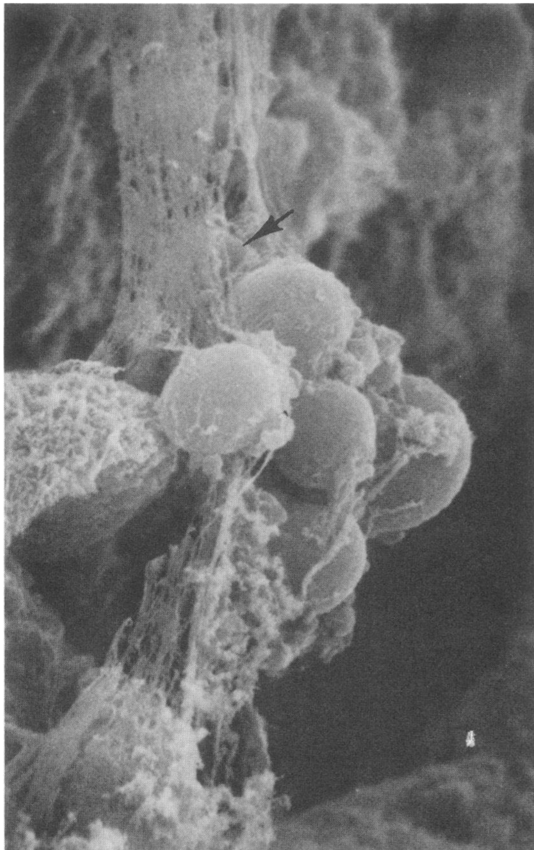

FIG. 2. SEM of the cecum from a penicillin-treated animal challenged with *C. albicans*. Yeast cells can be seen attached to and embedded (arrow) in mucus material. Magnification, ×7,000.

DISCUSSION

The results presented in this paper strongly support the hypothesis that the indigenous intestinal microflora suppresses *C. albicans* numbers in the GI tract and reduces the incidence of dissemination from the intestinal lumen to visceral organs (37, 37a, 43, 61, 62). The administration of normal cecal contents to antibiotic-treated animals before oral *C. albicans* challenge, for instance, led to a significant decrease in the cecal population of *C. albicans* with a concomitant reduction in dissemination. Furthermore, filtration of cecal contents through 0.20-μm Millipore filters eliminated their protective effect, indicating that viable bacteria, and not some unknown biochemical factor, were important in *Candida* suppression. Any biochemical substance present in hamster cecal contents that might suppress *Candida* growth probably would have been washed out of the gut before yeast inoculation, since intestinal tracers were rapidly removed from the GI tract by peristalisis (24, 25). Viable bacteria injected with cecal contents, in contrast, continue to multiply and colonize the intestinal lumen and mucosal surfaces (63).

Our findings, then, clearly demonstrate the importance of the intestinal microflora in the suppression of gut colonization and dissemination by *C. albicans*. Our data also clear up previous contradictory results reported in the literature regarding the effects of the normal intestinal microflora on GI colonization by this fungus. For instance, Balish and Phillips reported that *C. albicans* was established in "large numbers" in the gut of both germfree and conventional animals (5, 49), whereas De Maria et al. (13) and Helstrom and Balish (30) reported that conventional animals were significantly more resistant to GI colonization than were antibiotic-treated and germfree animals, respectively. Such discrepancies, however, may be due to the animals selected for study, since many strains of mice are unsuitable for research on intestinal flora interactions because they lack a true indigenous microflora (24, 27).

The data presented here indicate further that the indigenous microflora inhibited *C. albicans* colonization and dissemination from the intestinal tract by at least two possible mechanisms: (i) decreasing the size of the *Candida* population in the gut and (ii) inhibiting the mucosal association of *Candida* organisms by forming thick layers of bacteria in the mucus gel (14) covering the epithelium. Hamsters treated with antibiotics, for instance, had high numbers of *Candida* organisms opportunistically colonizing their GI tracts, with a high incidence of dissemination to visceral organs. Animals that possessed an indigenous intestinal microflora, in contrast, had low numbers of *C. albicans* residing in their GI tracts, with only 2 of 40 animals showing signs of *Candida* dissemination. Of untreated hamsters that received multiple *Candida* challenges to maintain high cecal populations, however, 53% had viable *C. albicans* recovered from their visceral organs. Thus, the suppression of *Candida* population levels appears to be an important factor controlling fungal dissemination from the GI tract (15, 37, 37a). Similarly, it has been shown that high intestinal populations of bacteria were required to promote *E. coli* dissemination (translocation) from the GI tract (8). When intestinal population levels dropped below the threshold level due to antagonism by the indigenous intestinal microflora, *E. coli* cells could no longer disseminate from the gut (6, 9). Likewise, work in our laboratory previously has shown that certain fungi unable to maintain high population levels in the intestinal tracts of antibiotic-treated mice could not disseminate to visceral organs, although viable fungi remained in the gut for several days (36, 37). Other investigators also have suggested a direct relationship between GI populations of other microbes and systemic dissemination (60, 64).

Nevertheless, it also appears that the dense bacterial layers lining the mucosal epithelium may have provided an important defense mechanism that inhibited both *Candida* colonization and dissemination from the GI tract. It is apparent that the first step in mucosal association must be the penetration of the mucus gel (18, 20). In infant mice, which lack a complete bacterial flora including the dense microbial populations in the mucus gel (12, 56), *C. albicans* can readily associate with and pass through the gut wall to initiate systemic infection (15, 50). In the present study, in contrast, it was shown that intestinal tissues that possessed an indigenous wall-associated microflora strongly inhibited mucosal association and dissemination of *C. albicans* from the GI tract. Mucosal association of *C. albicans* appeared to be blocked by competing for adhesion sites and physically blocking the larger yeast cells from penetrating into the mucus gel. For example, when intestinal slices from antibiotic-treated hamsters, yeast cells, and intestinal bacteria were mixed at the same time (assay 1), mucosal association by *C. albicans* was as strongly inhibited as when the mucosal association assay was performed with IC and slices from untreated hamsters (assay 4). Although this may seem surprising at first, it should be noted that most indigenous mucosal bacteria are very motile (58, 59) and may have been chemotactically attracted to the mucosal surface very rap-

idly (1, 19, 20, 59). That is, indigenous bacteria may have reached attachment sites first by guided motility before yeast cells randomly bumped into intestinal tissues. In addition, SEM and disassociation studies suggest that in conventional animals, *C. albicans* was associated with only the top layer of mucus gel lining the lumen. Approximately 74% of the yeast cells associated with the cecal walls of these animals were shed in the surrounding PBS solution, whereas only about 31% of the associated *Candida* cells were shed from the cecal walls of antibiotic-treated animals. SEM analysis of intestinal mucosal surfaces also showed large numbers of yeast cells attached to intestinal villi only in antibiotic-treated animals. Presumably then, the dense layer of bacteria colonizing the mucus gel in conventional animals acts as a resistance barrier to mucosal association and dissemination by *C. albicans*.

Finally, it also was shown that certain chemical factors present in the normal gut environment inhibited the association of *C. albicans* with intestinal mucosal surfaces. Since environmental parameters are known to influence *Candida* adhesion to vaginal and buccal epithelial cells (39, 57), it follows that bacterial and host substances produced in the intestinal tract (probably as a result of the metabolic activity of those organisms which control the indigenous microflora [23]) also reduced the ability of *Candida* cells to attach to certain mucosal structures. Secondary bile acids and VFA, for example, may have reduced the mucosal association of *C. albicans* by modifying *Candida* adhesin(s) or mucosal receptor(s), or both (26), thus rendering *Candida* cells unable to attach to intestinal tissues. This is in agreement with the finding that intestinal levels of VFA dropped significantly in animals given antibiotics to eliminate certain components of their microflora (Table 3). Likewise, antibiotic treatment of hamsters has previously been shown to cause an increase in intestinal levels of primary bile acids and a decrease in the levels of secondary bile acids (R. Fekety, R. Browne, J. Silva, and A. F. Hofmann, Program Abstr. 18th Intersci. Conf. Antimicrob Agents Chemother., abstr. no. 129, 1978).

Indirect evidence suggests that the anaerobic organisms, which dominate the intestinal flora, normally suppress *C. albicans*. Treatment of animals with penicillin decreased the total populations of strictly anaerobic bacteria in the cecum, allowed an increase in facultative bacteria, and promoted *C. albicans* adhesion, colonization, and dissemination from the GI tract. Wingard et al. (67), in contrast, could not produce *Candida* dissemination in mice treated with gentamicin, an antibiotic active against most intestinal aerobes but sparing of the anaerobes. Pope et al. (50), moreover, reported *C. albicans* adhesion, colonization, and dissemination from the GI tracts of 5- to 6-day-old infant mice. They also have shown *C. albicans* to maintain a stable population of 10^3 to 10^4 in the cecum and large intestine of infant mice for the first 2 weeks of life, after which *Candida* counts decline to undetectable levels by 4 weeks (15). The timing of elimination of *Candida* organisms in infant mice, then, coincides with the acquisition of anaerobic bacteria, previously shown to occur during weeks 2 and 3 after birth (12, 56). The role of intestinal anaerobes in suppression of *C. albicans* within the GI tract should be investigated further.

ACKNOWLEDGMENTS

We thank Rolf Freter, Garth Jones, Ken Wilson, and Robert Yancey, Jr., for valuable suggestions and critical review of the manuscript. We also thank Ken Wilson for providing some of the hamsters used in this study, Rolf Freter and Jane Leedle for use of equipment, and Chris Edwards for assistance with the SEM. We also thank Richard Greening for kindly performing the VFA analyses.

This study was supported in part by the Upjohn Co., Kalamazoo, Mich.

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