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Role of Competition for Nutrients in Suppression of *Clostridium difficile* by the Colonic Microflora

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The cecal flora of mice is able to eliminate *Clostridium difficile* from the mouse cecum even when *C. difficile* is the first organism established. We used a continuous-flow (CF) culture model of the cecal flora to investigate the possibility that competition for nutrients is one mechanism for this antagonism. The medium for the CF cultures consisted of homogenates of fecal pellets from germfree mice. Carbohydrate analysis showed that mouse flora depleted 74 to 99.8% of the various carbohydrates from this environment-simulating medium. When inoculated into filtrates made from CF cultures of mouse flora, *C. difficile* multiplied slower than the dilution rate of the CF cultures unless glucose, *N*-acetylglucosamine, or *N*-acetylneuraminic acid was added. *C. difficile* did not synthesize hydrolytic enzymes able to cleave these monosaccharides from oligosaccharide side chains. As found previously, veal infusion broth did not support the growth of a microflora that could be transferred to gnotobiotic mice and fully suppress *C. difficile*. When mucin or monosaccharides found in mucin were added to veal infusion broth, the flora functioned normally in this regard. These data suggest that as yet unidentified organisms compete more efficiently than *C. difficile* for monomeric glucose, *N*-acetylglucosamine, and sialic acids found in colonic contents.

Clostridium difficile colitis occurs almost exclusively after the use of antibiotics. The most obvious explanation of this association is that antibiotics disrupt the colonic microflora, which normally suppresses *C. difficile*. This explanation is supported by extensive experimental evidence. In the hamster model of the disease, the pathogen cannot colonize the animal in the presence of an undisturbed microflora (24), yet it rapidly attains a large population size when introduced into the antibiotic-treated animal. When animals are inoculated with cecal flora from conventional hamsters after antibiotic treatment and before exposure to *C. difficile*, the population size of the pathogen is less than 1% of the level achieved in animals not inoculated with cecal flora and animals are protected from colitis (26). In gnotobiotic mice, although *C. difficile* is able to achieve a population of over 10^8 CFU per cecum when it colonizes alone, both hamster and mouse floras are able to suppress it to undetectable levels (25). The control that the colonic flora exerts over *C. difficile*, at least in these rodents, is absolute.

We have studied the mechanisms for this control in a continuous-flow (CF) culture model of the mouse cecum originally developed by Freter et al. (5). The model consists of an anaerobic CF culture inoculated with cecal contents from a mouse, and it reproduces very closely the ecosystem found in the live-mouse cecum. Previous work has shown that it specifically reproduces the complex interaction between *C. difficile* and the cecal flora (23). The CF culture model has allowed studies that would not be possible in vivo and has recently led to theoretical explanations of the competitive interactions of the colonic microflora (4). The present study concentrated on the possible role of single-nutrient competition in the control of *C. difficile* for two reasons. First, the very fact that a CF culture is able to reproduce the colonic ecosystem suggests that competition for growth-limiting substrates plays an important role in homeostasis (2, 4). Second, nutrient competition is a mechanism by which the colonic flora suppresses *Escherichia coli* (3), the organism that is probably the best studied with respect to control mechanisms.

MATERIALS AND METHODS

Animals. BALB/c mice were obtained from the colony of W. Murphy, Department of Microbiology and Immunology, University of Michigan. Gnotobiotic CD-1 mice were originally obtained from Charles River Breeding Laboratories and were maintained and bred for many generations in Trexler-type polyvinyl isolators. Gnotobiotic mice were associated (colonized) with *C. difficile* and CF culture contents as described previously (23).

CF cultures. The anaerobic CF culture system used was designed originally by Freter and has been described previously (5, 9). The flow rate was −0.157/h. The culture medium used in these experiments consisted of extracts of fecal pellets from germfree mice and was prepared as described previously (23). CF cultures were inoculated anaerobically with cecal contents from conventional mice and allowed 2 to 3 weeks to equilibrate before being used in experimental protocols.

Sterile filtrates were made from CF culture contents by removing 1 ml with a sterile pipette, centrifuging at 3,000 × *g* inside the anaerobic chamber for 15 min, and then passing the supernatant through a sterile membrane filter (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.). Because Freter has shown that growth in CF culture can improve the fitness of an organism to compete in the colonic ecosystem (4), the inoculum was prepared by growing *C. difficile* alone in CF culture for 24 to 48 h before inoculation. The inoculum was diluted in reduced balanced salts solution, and then 10^4 CFU in 10 μl was inoculated into 1 ml of filtrate made from contents of CF cultures harboring mouse flora. Growth curves were obtained by performing serial quantitative cultures.

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Carbohydrate analysis. Total concentrations of amino sugars and neutral sugars were determined by fluorometric analysis of acid-hydrolyzed samples as described by Perini and Peters (18). Neutral sugars were derivatized to glycamines before samples were injected into the high-pressure liquid chromatography column. Concentrations of free amino and neutral sugars were determined by directly derivatizing and chromatographically analyzing unhydrolyzed samples. Sialic acid concentrations were determined by the method of Hammond and Papermaster (8).

Amino acid analysis. The amino acid compositions of both the CF culture medium and contents of CF cultures colonized with mouse flora were determined at the University of Michigan Protein Sequencing Facility, as described previously by Tarr (22).

Assays for carbohydrate hydrolases. *C. difficile* was assayed for intracellular and extracellular carbohydrate hydrolases. Cells were ruptured with glass beads as described by Moore et al. (16) to obtain intracellular enzymes. Hydrolase activity was determined by measuring release of ρ-nitrophenol from ρ-nitrophenyl glycosides as described previously (13, 14). Sialidase (*N*-acetylglucosaminidase) activity was determined by measuring the release of sialic acid residues from fetuin.

Quantitation of *C. difficile*. Samples were serially diluted and cultured quantitatively on prereduced cefoxitin-cycloserine fructose agar (6) containing 0.5% cholic acid, as described previously (26). Mice were placed in the anaerobic chamber before the peritoneal cavity was opened to culture cecal contents. *C. difficile* 49A was originally isolated from a hamster with antibiotic-associated colitis and has been used for previous studies (23–26; K. H. Wilson, M. Patel, P. Permoad, and L. Moore, Microb. Ecol. Health Dis., in press).

RESULTS

Carbohydrate utilization. Carbohydrate concentrations in CF culture medium were compared with those in contents of CF cultures colonized with mouse flora. Results were similar to those of earlier work comparing colonic contents of germfree versus conventional rats (12). The concentrations of the various free monosaccharides (i.e., sugars measured before hydrolysis) in samples of both the culture medium and culture contents were a small fraction of the total carbohydrate, indicating that the carbohydrates consisted mostly of oligosaccharides. Because the CF culture medium was made of homogenized fecal pellets from germfree mice, the overall composition of the sugar moieties was consistent with that of gastrointestinal mucin and the residues of undigested plant carbohydrates (Table 1). A comparison of the amounts of carbohydrates found in the culture medium (Table 1) with the amounts found in the growth chamber (Table 1) indicated that the colonic flora depleted from 74 to 99.5% of the various carbohydrate moieties present in the culture medium. Concentrations of free monosaccharides in the CF culture contents were in the micromolar range.

Amino acid utilization. Amino acid analysis of the same samples showed that, compared with carbohydrates, amino acids were not as extensively degraded (Table 2). The colonic microflora consumed less than half of most amino acids.

Nutrient limitation. When *C. difficile* was inoculated into culture filtrates, it either lost viability or failed to multiply as fast as the dilution rate of the CF culture (and thus would have been washed out). Similar experiments performed by inoculating *C. difficile* directly into CF cultures showed that it indeed was washed out and frequently disappeared faster than the dilution rate. The apparent loss of viability observed in some of these experiments was not a reproducible feature, and the mechanism for this effect was not determined.

Each of the carbohydrates detected in germfree mice and CF culture medium was then added to filtrates of CF cultures to a final concentration of 1 mM. The addition of galactose, *N*-acetylgalactosamine, mannose, xylose, arabinose, and fucose failed to increase the growth rate of the pathogen. However, glucose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid increased growth to a rate faster than the dilution rate. The results shown in Fig. 1A and B are representative of experiments in which *N*-acetylglucosamine ($N = 8$) or *N*-acetylneuraminic acid ($N = 5$) was added. Even at a concentration of 1 mM none of the amino acids

TABLE 1. Carbohydrates present in CF culture colonized with mouse flora

Carbohydrate	Concn (μmol/liter)[a]		
	Culture medium	Total in CF culture	Free monosaccharides in CF culture
Glucose	4,720	23.5	8.8
Galactose	4,010	21.6	7.8
Glucosamine	1,820	73.5	4.4
Galactosamine	820	21.0	2.0
Mannose	370	80.7	3.1
Fucose	300	65.0	6.0
Xylose	900	30.8	2.0
Arabinose	125	33.3	3.8
Sialic acids	1,090	45	<1.0

[a] The concentrations of neutral and amino sugars were determined by high-pressure liquid chromatography by using a fluorometric assay technique after acid hydrolysis of the specimen. Concentrations of free monosaccharides were determined by assaying unhydrolyzed specimens.

TABLE 2. Concentrations of amino acids in medium made from germfree mouse fecal pellet extract and in contents of CF cultures colonized with cecal flora from mice

Amino acid	Concn (μM) in[a]:		% Utilization
	Medium	Contents	
Ala	1,301	813	37
Cya[b]	213	152	29
Asp	2,381	1,347	43
Glu	2,054	968	53
Phe	484	243	50
Gly	2,105	1,087	48
His	237	124	48
Ile	526	283	46
Lys	711	562	21
Lev	788	425	46
Met	444	253	43
Asn	0	0	
Pro	1,534	922	40
Arg	0	61	
Ser	1,361	660	52
Thr	836	243	71
Val	829	456	45
Trp	0	0	
Tyr	346	202	42

[a] Assays were performed by the method of Tarr (22) and are reported as micromolar concentrations of total amino acids found in hydrolyzed samples of sterile filtrates.

[b] Cya, Cysteic acid.

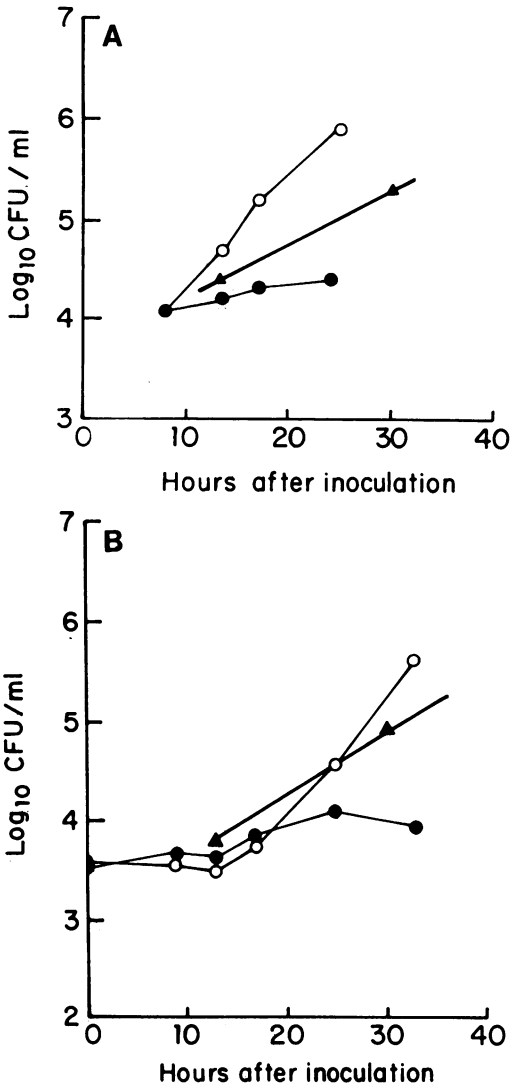

FIG. 1. Effect of adding *N*-acetylglucosamine (A) or *N*-acetylneuraminic acid (B) to sterile filtrates of CF culture contents. The growth of *C. difficile* inoculated into CF culture filtrates (●) was compared with that in CF culture filtrates to which 1 mM carbohydrate was added (○). *C. difficile* multiplied faster than the dilution rate (▲) only when a fermentable carbohydrate was added.

found in the colonic contents of germfree mice significantly increased the growth rate of *C. difficile* in CF culture filtrates.

Maximal growth rate. One way in which the indigenous flora could antagonize *C. difficile* would be to produce substances that decrease its maximal growth rate (2, 15). To determine whether such a mechanism existed, we compared the growth rate of *C. difficile* in tryptic soy broth (TSB) with that in filtrates of CF culture contents to which TSB powder had been added. The results presented in Fig. 2 (representative of seven experiments) show that after a slightly extended lag phase in the filtrate-containing medium, the pathogen multiplied with equal rapidity in both media.

Addition of mucin to CF culture medium. Previous work has shown that when veal infusion broth (VIB) is used as a medium for CF cultures colonized with the colonic microflora, the contents of these cultures do not fully suppress *C. difficile* when transferred to germfree mice. This finding led

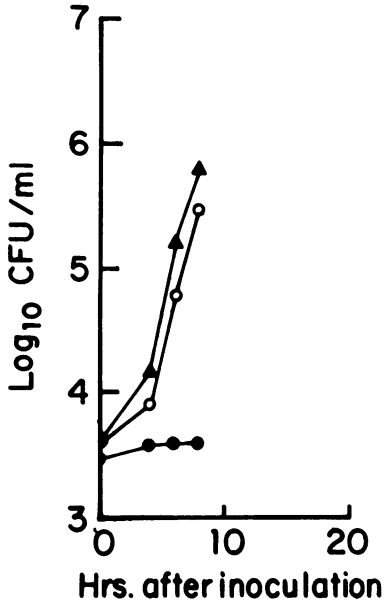

FIG. 2. Growth in nutritionally supplemented CF culture filtrate. The growth of *C. difficile* in CF culture filtrate (●) was compared with that in filtrate to which TSB was added (○) and with that in sterile distilled water to which TSB was added (▲).

to the use of homogenates of germfree fecal pellets as a medium for CF cultures to better simulate the interaction between *C. difficile* and the colonic flora (23). Because *N*-acetylneuraminic acid and *N*-acetylglucosamine are both components of mucin, we performed experiments to determine whether the absence of components found in mucin was a reason for the earlier failure of VIB. Four CF culture media were compared: (i) VIB, (ii) VIB containing 20% purified hog gastric mucin, (iii) VIB containing monosaccharides at the concentrations found in 20% hog gastric mucin, and (iv) unpurified hog gastric mucin alone at 12 g/liter. Hog gastric mucin (Sigma Chemical Co., St. Louis, Mo.) was purified by the method of Miller and Hoskins (14). The concentrations of the various monosaccharide components of mucin and VIB are shown in Table 3. CF cultures containing mouse flora growing on these media were used to conventionalize (i.e., colonize with conventional indigenous microflora) germfree mice as described previously (22). All mice had been monoassociated with *C. difficile* before the experiment started (i.e., *C. difficile* was the first organism present in these mice). After 3 weeks, the mice were sacrificed and the population sizes of *C. difficile* within the ceca were determined. In mice colonized by the contents of the CF culture grown on VIB alone ($n = 9$), *C. difficile* was suppressed from >8 to 4.1 ($\log_{10}$ CFU per cecum; standard

TABLE 3. Concentrations of carbohydrates found in VIB and purified hog gastric mucin

Carbohydrate	Concn (mM) in:	
	Mucin	VIB
Sialic acid	0.12	0.12
Glucosamine	5.8	0.65
Galactosamine	2.5	0.34
Galactose	3.0	0.22
Fucose	0.9	0

error of the mean = 1.6). No mouse receiving contents of CF cultures grown on VIB-mucin (n = 6), VIB-monosaccharides (n = 12), or mucin alone (n = 12) harbored *C. difficile* (limit of detection, 300 CFU).

Hydrolase activity. As noted above, *C. difficile* required a carbohydrate to multiply under the conditions found in CF cultures of the colonic flora. Because the carbohydrates found in CF culture contents occurred as oligosaccharides, we questioned whether *C. difficile* competed directly for these carbohydrates by cleaving them enzymatically from the oligosaccharide chains. Assays were performed to detect cell-associated or free α-fucosidase, β-galactosidase, β-*N*-acetylglucosaminidase, sialidase, and α-fucosidase, as described in Materials and Methods. None of these enzymes was found. Because two of the monosaccharides that *C. difficile* could utilize are specifically found in mucin side chains, the ability of *C. difficile* to degrade mucin was then tested more directly. Purified hog gastric mucin was added as the only source of carbohydrate to medium 75 (11), and the medium was inoculated with *C. difficile*. Carbohydrate analysis showed that the mucin carbohydrates were neither utilized nor hydrolyzed.

DISCUSSION

Chemostat theory holds that if two or more populations of bacteria are growth limited by the same substrate in a chemostat, they will compete for that substrate. The population that is able to deplete the substrate to the lowest concentration while dividing at the dilution rate of the chemostat will then displace the other populations (2, 4, 15). The finding that CF cultures can serve as a model of the colonic ecosystem therefore suggests that single-nutrient competition plays a role in homeostasis. Freter et al. (3) have shown that *E. coli* is suppressed by the colonic flora by several mechanisms, including nutrient competition, and that it is growth limited in the colonic ecosystem by lack of a carbon source. This control mechanism for *E. coli* has been confirmed by Guiot (7). It is therefore not surprising that *C. difficile* was unable to compete with mouse flora for carbon sources. Under the conditions found in CF culture, *C. difficile* required a fermentable carbohydrate and could not utilize an amino acid for a carbon source. Although the presence of branched-chain volatile fatty acids in colonic contents suggests that fermentation of amino acids does occur in the gut, our data indicate that the colonic ecosystem as a whole did not degrade amino acids as extensively as carbohydrates for use in energy metabolism.

In the milieu of the colonic microflora, *C. difficile* was able to utilize the same carbohydrates that it characteristically ferments under less stringent conditions, i.e, glucose but not mannose, xylose, or arabinose (10). It also utilized *N*-acetylglucosamine and *N*-acetylneuraminic acid, neither of which occurs in nature as a monosaccharide and both of which are found in relatively large amounts in the side chains of gastrointestinal mucin. However, it did not have the enzymatic machinery to cleave these monosaccharides from oligosaccharide side chains. Lack of a neuraminidase has previously been shown to be characteristic of this pathogen (19). Further evidence that *C. difficile* may compete directly for free monosaccharides was provided by an experiment in which the monosaccharides were added to a suboptimal CF culture medium. VIB alone supports growth of a microflora that closely resembles mouse cecal flora (5), but when introduced into mice, this flora does not totally suppress *C. difficile* (23). In the present work, when monosacccharides found in mucin were added to VIB, the medium supported growth of a microflora that could eliminate *C. difficile* from gnotobiotic mice. Since the carbohydrates in question were already present in VIB, the most likely explanation is that the addition of the carbohydrates increased the population sizes of the required organisms to a level high enough for them to become established when inoculated into mice. Indeed, it is known that the population size of an organism in a chemostat culture is directly related to the quantity of growth-limiting substrate in the culture medium (15). Other explanations of these results are, of course, possible. For instance, the sialic acid in VIB may not have been *N*-acetylneuraminic acid.

It is possible that the indigenous flora may produce substances that decrease the growth rate of a suppressed organism even when that organism is provided with a nutritionally rich medium. The effect would be to decrease the maximal growth rate achievable by that organism. A decrease in the maximal growth rate would directly hinder the competitive ability of an organism in a chemostatlike environment (2, 15), could be directly quantified, and therefore could have predictive value when used in a mathematical model. Although we found no evidence that such a phenomenon occurred with *C. difficile*, it cannot totally be ruled out because some inhibitory substances have been found to be highly volatile (3).

It appears likely that *C. difficile* is controlled by other mechanisms as well. The finding that it loses viability when introduced into the cecal flora of the conventional hamster (24) or into human fecal filtrates (1) cannot be explained on the basis of nutrient depletion. Rolfe (20) has found that *C. difficile* also loses viability when inoculated into brain heart infusion broth containing volatile fatty acids at the concentrations found in hamster cecal contents. Borriello and Barclay (1) using another in vitro model found no such effect of volatile fatty acids. While the work of Su et al. (21) suggests that volatile fatty acids may not be important for the suppression of *C. difficile* in vivo, their work does not specifically address the situation in which an animal is suddenly challenged with large numbers of *C. difficile*. We could not study the loss of viability in CF culture because it was not reproducible in our system.

It has been possible to isolate a collection of organisms that suppress *C. difficile* from over 10^8 CFU per cecum in monoassociated mice to about 10^6 CFU per cecum in the presence of the synthetic microflora (Wilson et al., in press). Although this level of suppression is adequate to protect mice from cecitis caused by a hypertoxigenic strain, it does not compare with the total suppression caused by a complete microflora. Because the collection of isolates able to decrease the population size of *C. difficile* by a factor of only 2 logs already consists of over 100 isolates, the addition of more random isolates to this collection is likely to produce diminishing returns. Further work to develop a synthetic microflora to suppress *C. difficile* should be based on a knowledge of the control mechanisms normally active against this pathogen.

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