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Clostridium difficile in Gnotobiotic Mice

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Germfree mice associated with *Clostridium difficile* developed intestinal disease characterized by polymorphonuclear cell infiltration of the lamina propria, diarrhea, and cecal cytotoxin concentrations positive at a 10^{-6} dilution. The numbers of viable bacteria never exceeded 10^{10} colony-forming units per g (dry weight). Despite the high toxin levels and chronic inflammation over a 30-day period, the mortality rate was low (<2%). Daily treatment of these animals with two oral doses of 2 mg of vancomycin resulted in stool levels of >200 μg/ml, well in excess of the minimum inhibitory concentration for *C. difficile*. This therapy decreased viable cell density by 2 to 3 logs and increased the spore counts from $10^{5.8}$ to $10^{7.8}$ colony-forming units per g (dry weight) by day 7, and animals were free of detectable toxin. However, once therapy was stopped, viable bacteria and spore counts and cytotoxin concentrations returned to previous levels. Treatment of mice with concentrations of clindamycin shown to be inhibitory in vitro had no effect on *C. difficile* toxin titers or bacterial counts, although the appearance of a clindamycin-resistant population was noted. These data indicate that vancomycin, given orally, decreases the concentration of toxin, but *C. difficile* survive as spores. By contrast, large populations of vegetative cells and high cytotoxin levels persist when clindamycin is used, even at an inhibitory concentration.

The role of *Clostridium difficile* in antibiotic-associated colitis (AAC) has been well established by recent studies. This work has shown that feces from humans or hamsters with AAC contain a cytopathic toxin which can be neutralized with monovalent antiserum (4). *C. difficile* are believed to be the source of this cytotoxin, since they are isolated at a high rate from stools containing the toxin (1), and *C. difficile* produce a similar or identical toxin in vitro. The role of this toxin in the pathogenesis of colitis was further clarified by reproducing the disease in experimental animals by intracecal inoculation of *C. difficile* cell-free supernatant (3) or the partially purified cytotoxin (10).

A curious feature of the pathogenesis of *C. difficile* is that these microbes appear to cause enteric disease almost exclusively in individuals given antimicrobial agents. This observation applies to both humans and experimental animals. Paradoxically, AAC is treated with another antibiotic, vancomycin.

The purpose of the present report was to study the in vivo interrelationship between antibiotics and *C. difficile*. Gnotobiotic mice were employed as a nonlethal experimental model in which *C. difficile* populations could be monitored over extended periods without interference from other microbial species. The agents tested were clindamycin, which is a major cause of this complication in humans, and vancomycin, which has become a favored therapeutic modality.

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MATERIALS AND METHODS

Animals. Male, germfree, CD-1 mice weighing 14 to 18 g were obtained from Charles River Breeding Laboratories, Wilmington, Mass. Animals were housed five per cage within flexible plastic isolators (Standard Safety Co., Palatine, Ill.). Sterilized food (Ralston Purina Co., St. Louis, Mo.) and sterile water were available ad libitum. Sterility of animals was confirmed by testing representative animals from each cage. Swabs of the fur, feet, mouth, anus, and ears were incubated at 37°C in chopped meat glucose broth (Scott Laboratories, Fiskeville, R.I.), examined for growth at 24 and 48 h, and plated onto Brucella base blood agar (BMB; Scott Laboratories) and Trypticase soy blood agar (BAP). The BMB was incubated at 37°C within an anaerobic chamber, and the BAP within a CO_2 jar for 48 h. The absence of growth on any of the plates or in the chopped meat glucose broth was interpreted as bacteriological sterility.

Bacterial strains. *C. difficile* strain HUC2-4 was used for all experiments. This strain had been isolated from an AAC patient and had been maintained as a stock culture in the Infectious Disease Research Laboratory, Boston Veterans Administration Hospital. *Bacteroides fragilis* (ATCC 23745) or *Escherichia coli* (NIH 1666) was also employed in some experiments. All cultures were grown in brain heart infusion broth for 24 h within an anaerobic chamber, and portions were frozen at −40°C. Before use, each strain was plated onto BMB, and a single colony was inocu-

lated into a fresh brain heart infusion tube. After incubation for 18 to 24 h, cultures were restreaked for purity.

Association of animals with bacterial strains. Animals were associated with bacterial strains by orogastric administration of 0.1 ml of a culture containing $\cong 10^9$ colony-forming units (CFU) per ml. The bacterial population density in mice was monitored by quantitative culture of either fresh fecal pellets or intestinal contents obtained from sacrificed animals. After collection, the samples were pooled, homogenized, weighed, and placed within an anaerobic isolator. A portion of each sample was then removed for dry-weight determinations. The samples were then diluted in phosphate-buffered saline and were plated onto BMB (for anaerobes) or MacConkey agar (for *E. coli*). After incubation, colonies were enumerated based on colonial morphology, and representative isolates were identified as a check on culture purity. Spore counts were obtained by heating a portion of homogenized specimen at 80°C for 10 min (7) before making serial decimal dilutions.

Histological evaluation. After association of animals with *C. difficile*, two mice were sacrificed at 0, 1, 2, 3, 5, 7, 9, 11, 13, 15, 21, and 28 days. Animals were sacrificed with CO_2, and tissue samples were immediately removed from the duodenum, jejunum, ileum, cecum, transverse colon, and rectum. Tissues were fixed in 10% Formalin on plastic grids. Longitudinal sections were cut and stained with hematoxylin and eosin before examination.

Cytotoxicity assay. *C. difficile* toxin titers were assayed by using a modification of the cytotoxicity assay described by Chang et al. (5). Intestinal contents were centrifuged at 10,000 × *g* for 20 min, and the supernatant was filtered through a 0.45-μm filter. Serial dilutions of the filtrates were made by using sterile phosphate-buffered saline, and portions of each dilution were added to WI-38 cells grown in medium 199 and incubated at 37°C for 24 h. The results were expressed as the greatest dilution showing cytopathic changes of at least 50% of the cells in the monolayer.

Experimental design. Six groups of 10 to 30 animals were associated with *C. difficile*. Mice in the first group were sacrificed at sequential intervals for bacteriological and histological evaluation. A second group received vancomycin at a dose of 2 mg twice daily by orogastric intubation, and a third group received clindamycin in the drinking water at a concentration of 10 μg/ml. An additional group of animals was associated with *E. coli* and received vancomycin as described above. Two groups of mice were first associated with *B. fragilis* and subsequently with *C. difficile*. These two groups received clindamycin at a concentration of 10 μg or 100 μg/ml, respectively, in the drinking water. All groups were monitored for total bacterial counts, *C. difficile* toxin levels, and spore counts. Randomly selected animals from each group were sacrificed for histological evaluation. Stool levels of clindamycin and vancomycin were assayed by using a hemolysis inhibition assay (8).

RESULTS

Monoassociation with *C. difficile*. Bacteriological analysis of pooled intestinal contents from two mice at each sampling time indicated that *C. difficile* could be detected along the entire length of the intestinal tract from the duodenum to the rectum (Table 1). Levels of viable *C. difficile* cells at 24 h after association ranged from $10^{4.6}$ CFU/g (dry weight) in duodenum contents to $10^{9.4}$ CFU/g (dry weight) in the contents from the rectum. Little change in the viable bacterial population density occurred over the next 2 days, although cecal contents consistently yielded the highest intestinal levels of *C. difficile* after day 3. The levels of *C. difficile* in the small intestinal contents were not thought to necessarily represent colonization per se; since mice are coprophagic, these counts may simply reflect ingestion of *C. difficile* in feces. No significant changes in viable cell density in intestinal segment contents were detected after day 3. The number of spores in cecal contents was also quite consistent, at $10^{5.6}$ CFU/g (dry weight).

Monoassociated mice appeared healthy during the course of the experiment, with the exception of persistent watery stools. Despite diarrhea, animals continued to thrive, gain weight, and consume water and food at a rate comparable to age-matched, unassociated, germfree animals from the same colony. At necropsy, all organs were comparable to those of unassociated animals with the exception of the large intestine and cecum. *C. difficile*-associated mice had ceca which were considerably smaller than those from germfree animals. A modest decrease in cecal size was first noticed on day 3 after association, and by day 7 the ceca were approximately 1/3 the size of those obtained from germfree animals. No hemorrhagic lesions were seen in the intestinal tract of *C. difficile*-associated mice, and blood cultures were uniformly negative.

Microscopic examination revealed minimal changes in the small intestine. Examinations of the large bowel revealed a persistent inflammation which was initially noted on day 2, became

TABLE 1. *Intestinal levels of C. difficile after monoassociation of mice*

Location	No. of *C. difficile* at:[a]		
	24 h	48 h	72 h
Duodenum	4.6	5.5	5.1
Jejunum	5.9	5.6	5.7
Ileum	7.1	6.5	7.7
Cecum	8.6	9.2	9.6
Colon	8.8	9.6	9.2
Rectum	9.4	9.6	9.4

[a] Log_{10} CFU/g (dry weight).

progressively more intense until day 7, and gradually subsided thereafter (Fig. 1). The inflammation was characterized by polymorphonuclear cell infiltration and edema of the lamina propria. Despite the intensity of the inflammation, only 1 of the 30 animals died during *C. difficile* association.

Cytotoxin assays of cecal contents and feces obtained before *C. difficile* association were negative. After association, these were positive at a 10^{-6} dilution.

Treatment of *C. difficile*-associated mice with vancomycin. Vancomycin administration produced a decrease in the viable cell density of *C. difficile* from $10^{9.5}$ CFU/g (dry weight) to $10^{7.1}$ CFU/g (dry weight) of the cecal contents (Fig. 2). Pooled cecal contents had a vancomycin concentration of >200 μg/ml, which is well in excess of the minimum inhibitory concentration against this strain of *C. difficile* (minimum inhibitory concentration = 0.5 μg/ml). The concentration of spores increased from $10^{5.8}$ CFU/g (dry weight) before treatment to $10^{7.8}$ CFU/g (dry weight) during vancomycin therapy. These relatively high spore counts were almost equal to the viable cell counts and suggested that *C. difficile* persisted during vancomycin treatment primarily as spores. The gradual decrease in both viable cell density and spore counts (Fig. 2) suggests a washout effect for a static bacterial culture. Interestingly, cecal toxin titers decreased from a positive response at a 10^{-6} dilution to an undetectable level within 24 h after initiation of vancomycin therapy. These data indicate that, despite the absence of detectable toxin, *C. difficile* can persist in large numbers during vancomycin therapy.

Additional animals were associated with *C. difficile* for 5 days and then given vancomycin for 7 days (Fig. 3). Viable counts, spore counts, and toxin levels for *C. difficile* duplicated the earlier results. When the antimicrobial agent was discontinued, toxin levels and viable bacterial counts returned to pretreatment levels, and the animals redeveloped the diarrheal symptoms seen before treatment. These results indicate that *C. difficile* not only survives during vanco-

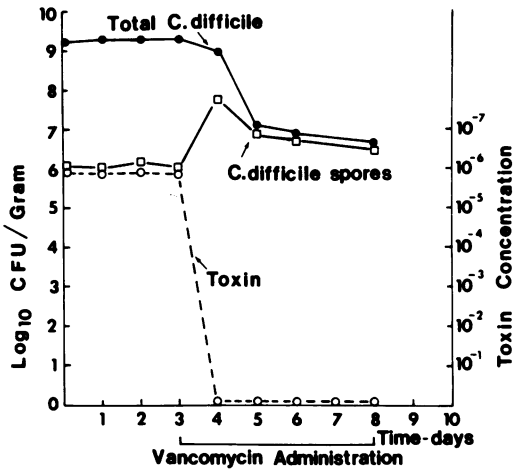

FIG. 2. *Effect of vancomycin on C. difficile in monoassociated mice.*

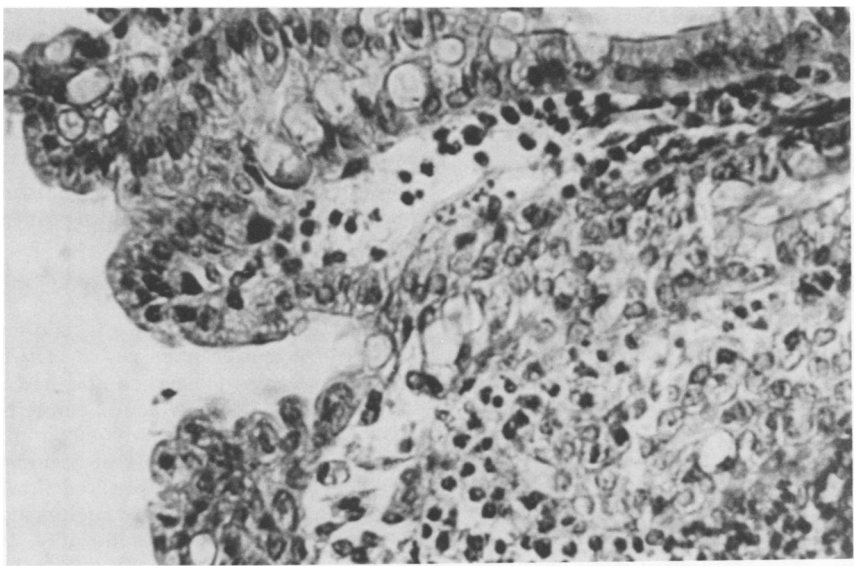

FIG. 1. *Cecal tissue obtained from a mouse 8 days after C. difficile association. Note the polymorphonuclear infiltration of the lamina propria (×250).*

mycin therapy, but is capable of repopulating the animals and reproducing symptoms once therapy is discontinued.

To determine whether a competing microflora would alter the establishment of *C. difficile* after vancomycin treatment, a group of mice were associated with *E. coli* before *C. difficile* was added to the microflora. Cultures of pools of fecal material from two to three animals revealed that both species established in the large intestine in high numbers, with *C. difficile* being present at $10^{9.0}$ CFU/g (dry weight) and *E. coli* at 10^{10} CFU/g (dry weight). As in previous experiments, treatment of these mice with vancomycin resulted in a decline in both the *C. difficile* population and cytotoxin levels. No change in the numbers of *E. coli* was seen during therapy. The *C. difficile* population and toxin levels returned to pretreatment values within 24 h after vancomycin was discontinued.

Effect of clindamycin on *C. difficile* in vivo. *C. difficile*-associated mice were given clindamycin in their drinking water to evaluate the effects of an antimicrobial known to induce AAC. Preliminary experiments showed that no change in viable cell density, spore counts, toxin titer or looseness or frequency of stools occurred during clindamycin administration. Two additional groups of mice were then associated with a combination of *C. difficile* and *B. fragilis* to determine whether the presence of an additional microbe would alter the effect of clindamycin on *C. difficile*. Clindamycin was administered to associated mice at a concentration of either 10 or 100 μg/ml in the drinking water. In both groups, the *B. fragilis* population measured in fecal samples decreased from $10^{9.5}$ CFU/g (dry weight) to 10^{5} CFU/g (dry weight) within 48 h after initiating clindamycin (Fig. 4). *C. difficile* populations remained unchanged at $10^{9.0}$ CFU/g (dry weight) in both groups, despite the sensitivity of this strain to clindamycin (minimum inhibitory concentration = 1.0 μg/ml) and the >50 μg/ml level of clindamycin in the stool. *C. difficile* isolates from these animals before and during therapy showed that 19 of 20 pretreatment isolates were sensitive to 1 μg of clindamycin per ml, whereas 11 of 20 isolates obtained during treatment were resistant to 64 μg/ml. Culture of pooled feces from these animals onto media incorporating 10 μg of clindamycin per ml (Fig. 5) showed that the number of clindamycin-resistant organisms increased from undetectable levels before clindamycin treatment to $10^{7.8}$ CFU/g (dry weight) by day 5 of antimicrobial exposure.

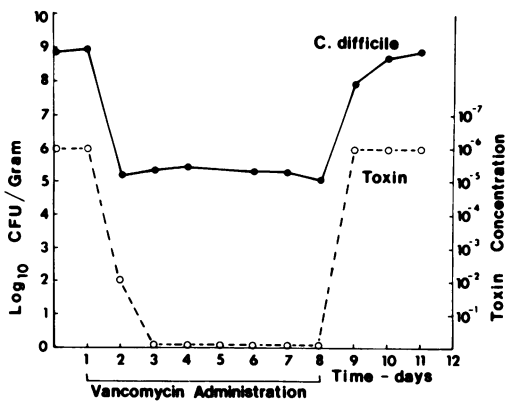

FIG. 3. *Effect of discontinuation of vancomycin therapy on C. difficile toxin levels in monoassociated mice.*

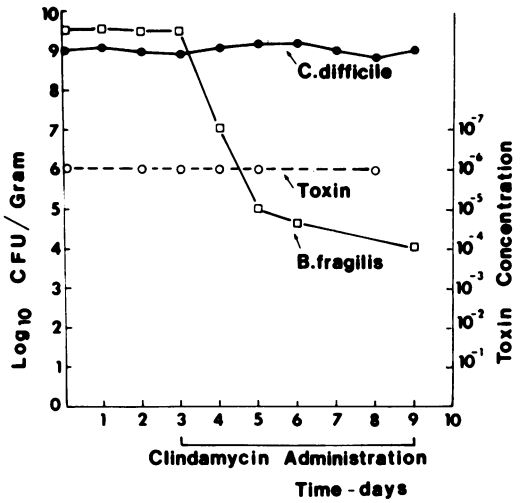

FIG. 4. *Effect of clindamycin on C. difficile in diassociated mice.*

These data suggest either that *C. difficile* rapidly develop resistance to clindamycin or that a low percentage of resistant organisms in these mice become dominant during antimicrobial therapy.

DISCUSSION

This study evaluates the effects of monoassociation of germfree mice with *C. difficile*. The animals developed diarrhea and had cytotoxin in their stools and an acute, nonlethal colitis characterized by intense polymorphonuclear cell infiltration and edema of the lamina propria. Previous studies have implicated *C. difficile* as a cause of antibiotic-induced cecitus in hamsters with a conventional microflora (4, 9). Major differences between the disease in monoassociated gnotobiotic mice and in conventional hamsters given antibiotics are the rapidly lethal

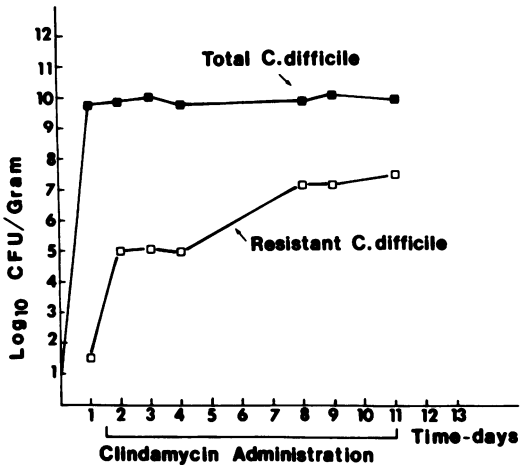

FIG. 5. *Development of clindamycin resistance in gnotobiotic mice associated with C. difficile.* ■, *total counts;* □, *counts obtained using media containing 10 μg of clindamycin per ml.*

course and the intense mucosal hemorrhage seen in conventional hamsters. With human patients, the disease course and histological changes in colonic mucosa appear more comparable to those produced in gnotobiotic mice. A prior report indicated that *C. difficile* caused colonic lesions in germfree hares but did not cause pathological change in monoassociated, germfree mice, rats, or rabbits (6). The discrepancy in observations with germfree mice in the two studies is unexplained except for vagaries in the mouse strains or the *C. difficile* isolate used.

C. difficile persisted in both monoassociated and diassociated gnotobiotic animals despite stool levels of the antibiotic which were well in excess of the in vitro minimum inhibitory concentration. However, during vancomycin therapy the concentrations of *C. difficile* decreased, the cytotoxin could not be detected, the diarrhea subsided, and the intensity of inflammation decreased. The number of spores increased during vancomycin treatment and appeared to account for virtually all the *C. difficile* enumerated.

The failure of vancomycin to eradicate *C. difficile* is of interest in view of clinical relapses. In most instances, vancomycin gives a prompt clinical improvement of pseudomembranous colitis due to *C. difficile* (11). Nevertheless, some patients have a persistence of the organisms despite vancomycin levels in the stool which are several hundred-fold higher than the minimum inhibitory concentration (unpublished data). Nearly all patients respond to vancomycin, but some of these patients experience a recurrence of symptoms in association with a reappearance of the cytotoxin. Our studies with gnotobiotic mice suggest that spores survive during vancomycin administration and recolonize the gut when the drug is discontinued. The presence of *E. coli* did not prevent reestablishment of *C. difficile* as a dominant component of the flora. However, it is known that vancomycin administration to hamsters with a conventional flora results in the lethal cecitis when the antimicrobial agent is discontinued. These animals have no detectable cytotoxin, no diarrhea, and no colonic lesions while vancomycin is administered. When vancomycin is discontinued, these animals develop large populations of vancomycin-sensitive *C. difficile*, have cytotoxin concentrations which are positive at 10^{-5} to 10^{-7} dilutions, and expire after 5 to 7 days (2). These observations in conventional hamsters appear comparable to those in gnotobiotic mice given vancomycin except for the rapidly lethal outcome.

Administration of clindamycin to monoassociated gnotobiotic mice did not result in any change in the *C. difficile* population or in the concentrations of the cytotoxin. The addition of a clindamycin-sensitive strain of *B. fragilis* to the flora had no important effect on either the concentrations of *C. difficile* or of cytotoxin. Counts of *B. fragilis* in these diassociated animals decreased with clindamycin administration, as would be anticipated. However, this decrease also had no impact on the concentrations of *C. difficile* or the levels of cytotoxin.

The persistence of *C. difficile* during clindamycin therapy is curious in view of the fact that the strain employed was sensitive to clindamycin, and antimicrobial levels in the stools were well in excess of the minimum inhibitory concentrations. A partial explanation was seen in the increased numbers of antibiotic-resistant cells that were present. These data suggest that *C. difficile* may rapidly develop resistance to clindamycin or that the inoculum contains a low percentage of resistant organisms which emerge as a greater portion of the flora in the face of antimicrobial pressure. These data are consistent with observations that clindamycin may cause antibiotic-induced colitis in both human patients and hamsters during the course of treatment (2, 3).

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