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Intestinal Colonization of Infant Hamsters with *Clostridium difficile*

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Infant hamsters of different ages were examined for their susceptibility to enteric *Clostridium difficile* colonization. Intragastric administration of *C. difficile* to infant hamsters resulted in multiplication of the organism in the intestinal tracts of animals 4 to 12 days old; hamsters younger or older were resistant to *C. difficile* intestinal colonization. Toxicity to the colonized animals could not be demonstrated despite cytotoxin titers in some infant hamsters comparable to titers found in the intestinal tracts of adult hamsters with *C. difficile*-associated intestinal disease. When introduced into 4-day-old hamsters, *C. difficile* colonized the intestinal tract and remained at high levels until the animals were 13 days old, at which time the presence of intestinal *C. difficile* could no longer be demonstrated. The number of *C. difficile* required to colonize the intestinal tracts of 50% of 7-day-old hamsters was 18 viable cells. On the other hand, 10^8 viable cells of *C. difficile* failed to colonize the intestinal tracts of healthy, non-antibiotic-treated adult hamsters.

Toxigenic *Clostridium difficile* is the major etiological agent of antimicrobial agent-associated pseudomembranous colitis in humans and of ileocecitis in Syrian hamsters (3, 14). All major classes of antimicrobial agents, except vancomycin and parenterally administered aminoglycosides, can induce *C. difficile* intestinal disease in humans. *C. difficile* also is an etiological agent of pseudomembranous colitis or diarrhea without colitis in patients with no history of antimicrobial therapy (27), of diarrhea in patients receiving antineoplastic agents (10), and of inflammatory bowel disease exacerbations (5, 22). *C. difficile* is one of the most common bacterial enteropathogens in stool specimens submitted to hospital clinical laboratories (11, 28). Although the mechanism by which *C. difficile* causes diarrhea or mucosal injury is not known, at least three potential virulence factors of *C. difficile* have been described: an enterotoxin (toxin A), a cytotoxin (toxin B), and a motility-altering factor (21, 36, 38).

Several investigators have reported that a high percentage of neonates harbor both intestinal *C. difficile* and cytotoxin without apparent clinical consequences. *C. difficile* has been isolated from feces of up to 60% of healthy infants less than 1 year of age (7, 9, 19, 32, 34). Carrier rates for *C. difficile* fall sharply after 1 year of age, although in the second year they are still higher than the 4% carrier rate for adults (25, 34). Concentrations of *C. difficile* and its cytotoxin in the feces of healthy infants are frequently similar to those in the intestinal tracts of adults with pseudomembranous colitis (7, 32, 41), and *C. difficile* is found with equal frequency in children with and without gastroenteritis (19). The mechanism of this commensal intestinal colonization of newborns with toxigenic *C. difficile* is unknown.

In recent years, increasing numbers of infants with *C. difficile*-associated intestinal disease are being recognized (20, 24, 40). Thus, it appears that *C. difficile* colonization and toxin production can occur in both healthy and symptomatic infants. Nonetheless, most investigators agree that the incidence of antimicrobial agent-associated intestinal disease, pseudomembranous colitis in particular, is much lower in infants and older children than in adults.

The low incidence of pseudomembranous colitis in infants and the problems of controlling endogenous and exogenous parameters make it difficult to study *C. difficile* colonization of the intestinal tracts of newborns. In this report, we describe the nonlethal colonization by *C. difficile* of the intestinal tract of infant hamsters.

MATERIALS AND METHODS

Maintenance of hamsters. Syrian hamsters (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) were used throughout the course of this investigation. The adult (90- to 110-g body weight) and infant hamsters were housed in conventional animal rooms. Hamsters were randomly assigned to treatment or control groups and maintained ad libitum on Purina Laboratory Rodent Chow 5001 (Ralston Purina Co., Richmond, Ind.) and

water. Females were bred by housing one female and one male together for 7 days, after which the female was housed individually. All pups of a litter received the same experimental treatment. Infant hamsters were covered with fragrant baby powder after treatment, immediately before returning them to their mothers, to prevent maternal cannibalism.

Preparation of *C. difficile* challenge. *C. difficile* TTU614 was used throughout this investigation. This strain was isolated from an adult male hamster with clindamycin-induced ileocecitis, and it produced an in vitro cytotoxin titer of 10^3 after 48 h of incubation in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.). The strain was inoculated into reduced brain heart infusion broth and incubated for 24 h at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2). The cell suspension was then adjusted to approximately 10^8 viable bacteria per ml (absorbance at 600 nm = 0.65) with reduced modified tryptic soy broth (TSB) (30 g of TSB and 0.41 g of sodium carbonate per liter of distilled water).

Inoculation of hamsters with *C. difficile*. Each infant hamster in a litter received 0.1 ml of the *C. difficile* cell suspension administered orogastrically with a 23-gauge feeding needle. *C. difficile* was administered to four litters of newborn hamsters at each of the following ages: 1, 4, 7, 10, 13, 16, 19, and 22 days (total of 32 litters). In addition, four control litters of hamsters at each age received sterile modified TSB by orogastric inoculation. Two litters of both test and control infants of each age group were observed for evidence of toxicity, whereas the other two litters were sacrificed after 72 h by cervical dislocation under ether anesthesia. After being sacrificed, animals were placed in an anaerobic chamber and the intestinal tracts, from the stomach to the rectum, were removed and weighed. The entire intestinal tract, including the contents, was diluted fivefold (wt/vol) in reduced yeast extract diluent (0.05% yeast extract in distilled water) and thoroughly homogenized. All diluent and media were reduced inside the anaerobic chamber at least 24 h before use. In some cases, the excised gastrointestinal tract was aseptically divided into segments, with the small intestine divided into proximal and distal halves. Each intestinal segment was diluted fivefold in reduced sterile yeast extract diluent and thoroughly homogenized.

Isolation of *C. difficile*. Three serial 100-fold dilutions of the aseptically prepared intestinal homogenates were made in reduced yeast extract diluent. Samples (0.1 ml) of the homogenate and each serial dilution were plated onto a selective medium for the isolation of *C. difficile* (15). The inoculated agar plates were incubated anaerobically at 35°C for 48 h. Organisms with colonial morphology resembling *C. difficile* were enumerated. Representative colonies were restreaked, and isolated colonies were used to identify the organism by established procedures (18, 37). *C. difficile* isolates were subcultured in 10.0 ml of brain heart infusion broth and incubated anaerobically at 37°C for 48 h. The broth cultures were centrifuged (8,000 × *g* for 30 min), and the supernatants were sterilized by passage through 0.45-μm (pore size) membrane filters for cytotoxin assays.

Cytotoxin assay. After inoculation of the selective medium, the remaining intestinal homogenates were removed from the anaerobic chamber and centrifuged at 10,000 × *g* for 30 min, and the supernatant was filter sterilized (0.45-μm [pore size] membrane filter). Serial 10-fold dilutions of the intestinal homogenates and *C. difficile* broth filtrates were prepared in phosphate-buffered saline at pH 7.2 and assayed for cytotoxicity to HeLa tissue culture cells by previously described procedures (13, 31). The reciprocal of the highest dilution that produced actinomorphic changes in at least 50% of the cells in the monolayer was defined as the cytotoxin titer of the filtrate. The presence of *C. difficile* toxin in all cytotoxic intestinal and broth filtrates was confirmed by neutralization with *Clostridium sordellii* antitoxin (U.S. Food and Drug Administration, Bureau of Biologics, Rockville, Md.).

ID_{50}. A cell suspension of *C. difficile* was diluted in modified TSB to give viable bacterial concentrations of 10^9, 10^7, 10^5, 10^3, and 10^1 bacteria per ml. Bacterial concentrations in each challenge dose were determined by performing serial 10-fold dilutions of the cell suspensions and plating each serial dilution onto reduced brucella agar supplemented with 5% sheep blood. Two litters of 7-day-old hamsters were inoculated intragastrically with 0.1 ml of each dilution. At the same time, five adult male hamsters were inoculated intragastrically with 10^8 viable cells of *C. difficile*. Seventy-two h later, the hamsters were sacrificed, and their intestinal tracts were cultured for *C. difficile* and tested for cytotoxicity. The number of *C. difficile* cells required to colonize the intestinal tracts of 50% of the hamsters (ID_{50}) was calculated from the colonization data by the method of Reed and Muench (29).

RESULTS

Colonization of infant hamsters. Infant hamsters of different ages received *C. difficile* or TSB orogastrically and 72 h later were sacrificed and their intestinal tracts examined for the presence of *C. difficile* and cytotoxicity to HeLa tissue culture cells. The results show that hamsters have an age-dependent susceptibility to *C. difficile* enteric colonization (Fig. 1). Colonization was arbitrarily defined as the presence of viable cells of *C. difficile* in the intestinal tracts. The intestinal tracts of colonized hamsters had from 1.3×10^3 to 4.5×10^7 *C. difficile* cells per g of intestinal homogenate. Toxin titers in the intestinal filtrates prepared from infant hamsters colonized with *C. difficile* ranged from undetectable to 10^4. Intestinal filtrates prepared from hamsters not colonized with *C. difficile* were negative for cytotoxicity. Control animals receiving modified TSB were not colonized with *C. difficile*. Hamsters receiving *C. difficile* or TSB remained healthy and gained weight at a rate comparable to that of untreated controls. *C. difficile* isolates from the intestinal tracts produced toxin titers of 10^3, which was comparable to the titers produced by the stock culture.

To better characterize the age-dependent susceptibility to *C. difficile* enteric colonization, 10^7 cells of *C. difficile* were administered orogastrically to two litters of hamsters between 1 and 17 days old. Hamsters in one of the paired litters

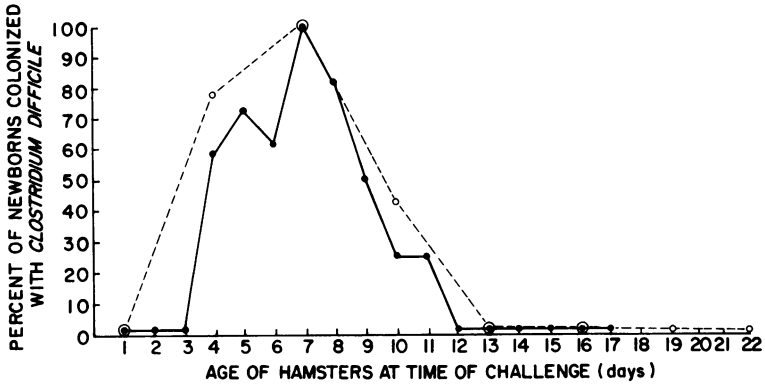

FIG. 1. Colonization of newborn hamsters with *C. difficile*. Hamsters between the ages of 1 to 22 days were challenged with *C. difficile*, and 72 h later their intestinal tracts were examined for the presence of *C. difficile*. ○, Experiment 1; ●, experiment 2.

were observed for evidence of toxicity, whereas those in the other litter were sacrificed after 72 h, and their intestinal tracts were examined for *C. difficile* and cytotoxin. Figure 1 depicts the percentage of infant hamsters of different ages that were colonized with *C. difficile*. Each point plotted represents the results of tests on 8 to 13 infant hamsters. *C. difficile* only colonized the intestinal tracts of hamsters between 4 and 11 days old. The viable cell count of *C. difficile* per gram (wet weight) of the intestinal tracts and the cytotoxin titer in hamsters colonized with *C. difficile* are shown in Table 1. All hamsters not colonized with *C. difficile* were negative for cytotoxin. None of the infant hamsters receiving *C. difficile* displayed evidence of toxicity.

TABLE 1. Concentrations of *C. difficile* and titers of cytotoxin in newborn hamsters

Age of hamsters at time of challenge (days)[a]	*C. difficile*		Cytotoxin	
	No. of hamsters colonized	Mean concn[b]	No. of hamsters positive	Mean titer[c]
4 (n = 12)	7	6.7 ± 2.4	3	1.9
5 (n = 11)	8	6.8 ± 2.3	5	2.2
6 (n = 13)	8	7.1 ± 1.5	8	3.2
7 (n = 11)	11	7.6 ± 1.5	11	4.4
8 (n = 11)	9	7.5 ± 2.1	7	3.8
9 (n = 10)	5	6.6 ± 1.9	4	2.3
10 (n = 8)	2	5.4 ± 1.8	1	0.3
11 (n = 12)	3	5.6 ± 3.2	1	0.4

[a] Hamsters were challenged orogastrically with 10^7 viable cells of *C. difficile*. n, Number of hamsters tested.

[b] Mean ($\log_{10}$) ± standard deviation per gram (wet weight) of intestine. Only hamsters colonized with *C. difficile* are included.

[c] Reciprocal of the highest dilution causing actinomorphic changes of at least 50% of the cells in the monolayer ($\log_{10}$). Only hamsters with detectable levels of cytotoxin are included.

Persistence of *C. difficile*. Seventeen litters of 4-day-old hamsters were inoculated intragastrically with 10^7 viable cells of *C. difficile*, and every 24 h the infants in one litter were sacrificed and their intestinal tracts cultured for *C. difficile* and tested for cytotoxin. *C. difficile* could be recovered from infant hamsters up to 8 days after the intragastric challenge (Fig. 2). The concentrations of *C. difficile* in the intestinal homogenates of these colonized hamsters closely paralleled the concentrations presented in Table 1. In addition, the titers of cytotoxin present in the intestinal homogenates of colonized hamsters were directly related to the concentrations of *C. difficile*. The highest cytotoxin titers (10^2 to 10^4) were found in those hamsters with the highest concentrations of *C. difficile* (10^6 to 10^7 CFU/g [wet weight] of intestinal homogenate).

ID_{50}. The ID_{50} for 7-day-old hamsters was 18 viable cells. The intestinal tracts of all infant hamsters receiving $\geq 10^4$ viable cells of *C. difficile* were colonized. Of 21 infant hamsters receiving 10^2 viable cells of *C. difficile*, 17 were colonized, and 10 of 22 infant hamsters receiving 10 viable cells of *C. difficile* were colonized. The concentration of *C. difficile* in the ceca of colonized hamsters ranged from 6.4×10^6 to 8.2×10^8 viable cells per g (wet weight) of intestinal homogenate. On the other hand, 10^8 viable cells of *C. difficile* failed to colonize the intestinal tracts of normal adult males.

Intestinal localization of *C. difficile* and cytotoxin. The site of *C. difficile* colonization in the intestinal tracts of infant hamsters was determined by orogastrically inoculating 10^7 cells of *C. difficile* per animal into three litters of 7-day-old hamsters. Seventy-two hours later the hamsters were sacrificed, and their gastrointestinal tracts were divided into segments. In addition, adult male hamsters receiving only clindamycin

(3 mg of clindamycin per 100 g of body weight) were sacrificed when moribund, and their intestinal tracts were removed and divided into segments. The concentrations of *C. difficile* and cytotoxin titers in the intestinal segments were determined (Table 2 and Fig. 3). Intestinal segments in which *C. difficile* was not isolated were negative for cytotoxin. In addition, the proximal and distal small intestinal segments of infant hamsters were consistently negative for cytotoxin despite the presence of low concentrations of *C. difficile*.

DISCUSSION

The results of this investigation demonstrate that hamsters have an age-dependent susceptibility to enteric *C. difficile* colonization similar to the restricted-age distribution of human newborn colonization. Intragastric administration of *C. difficile* to infant hamsters resulted in recovery of the organism in the intestinal tracts of 4- to 12-day-old animals. Colonized animals did not become ill, despite cytotoxin titers in some hamsters approximating those in the intestinal tracts of adult humans and hamsters with *C. difficile*-associated intestinal disease. The evidence that *C. difficile* colonized the intestinal tracts of infant hamsters is twofold. First, greater numbers of *C. difficile* than were introduced were recovered from many of the infant hamsters. Second, *C. difficile* could be recovered from infant hamsters up to 8 days after intragastric challenge.

Why *C. difficile* readily colonizes the intestinal tracts of infants and is relatively rare in healthy adults remains an enigma. A majority of the theories proposed to explain the mechanisms by which *C. difficile* overgrows in the intestinal tract consider the interactions which undoubtedly exist between *C. difficile* and the normal intestinal bacterial flora (33). Several investigators have presented experimental evidence to show that the normal bacterial flora of the gastrointestinal tract constitute an extremely important defense mechanism which effectively interferes with the establishment of many enteric pathogens (4, 12, 16, 35). The dramatic quantitative and qualitative fluctuations in the bacterial populations of the normal intestinal flora which occur immediately after birth up until the time the animal begins to sample solid food indicate that the normal flora are not well balanced (17, 26). It has been suggested that this may contribute to some of the intestinal diseases seen in young children since the protective mechanisms of the normal flora are probably diminished or absent. This may contribute to the overgrowth of *C. difficile* in the intestinal tracts of many infants. An intriguing experiment of nature appears to confirm the importance of resistance to gastrointestinal colonization by *C. difficile*. Approximately 50% of newly born hares develop a spontaneous and lethal diarrheal disease involving *C. difficile*, whereas adult hares do not develop this illness (8). Presumably, the incompletely developed intestinal flora of the newborn hare do not possess adequate resistance to *C. difficile* colonization.

C. difficile is not unique in its selective colonization of the intestinal tracts of newborns. Infant botulism is a recently recognized form of botulism that results when the intestinal tracts of newborns become colonized with *Clostridium*

TABLE 2. Intestinal localization of *C. difficile* in infant and antibiotic-treated adult hamsters[a]

Intestinal segment	*C. difficile*			
	No. of hamsters colonized[b]		Mean concn[c]	
	Newborn	Adult	Newborn	Adult
Stomach	0/27	13/15		5.7
Proximal small intestine	16/27	15/15	3.5	4.9
Distal small intestine	27/27	15/15	3.8	7.2
Cecum	27/27	15/15	7.6	7.6
Colon	27/27	15/15	6.8	6.2

[a] Infant hamsters were challenged orogastrically with 10^7 viable cells of *C. difficile*, and adult hamsters were given clindamycin (3 mg of clindamycin per 100 g of body weight).

[b] Number of hamsters colonized with *C. difficile* at particular intestinal segment/total number of hamsters examined.

[c] Only hamsters colonized with *C. difficile* are included. Mean ($\log_{10}$) per gram (wet weight) of intestine.

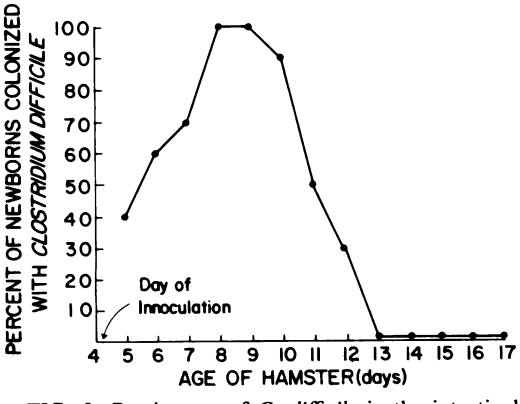

FIG. 2. Persistence of *C. difficile* in the intestinal tracts of 4-day-old hamsters. Seventeen litters of 4-day-old hamsters were challenged with *C. difficile*, and every 24 h the newborns in one litter were sacrificed and their intestinal tracts examined for the presence of *C. difficile*.

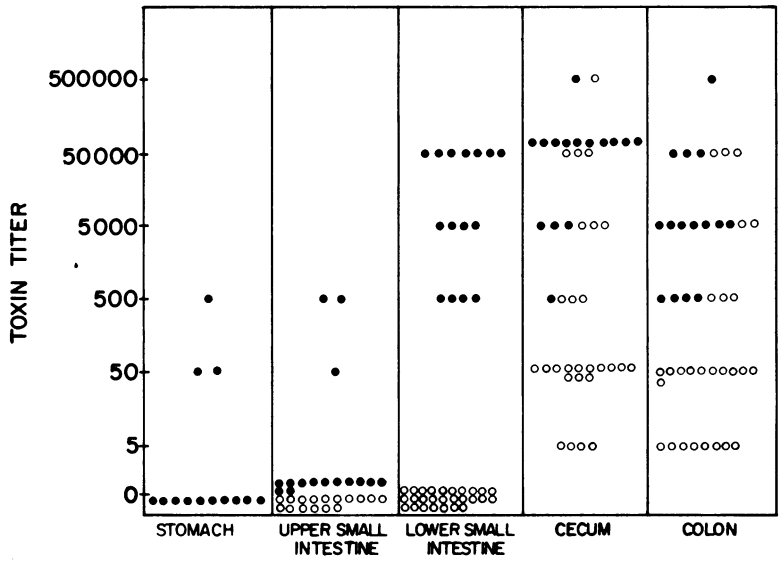

FIG. 3. Titer of cytopathic toxin present in different locations of the intestinal tract of hamsters colonized with *C. difficile*. Toxin titer is expressed as the reciprocal of the highest dilution causing actinomorphic changes of at least 50% of the cells in the monolayer. Only hamsters colonized with *C. difficile* are included. Symbols: ○, 10-day-old hamsters; ●, clindamycin-treated adult hamsters. All circles showing a toxin titer of <5 represent undetectable toxin titers in each particular intestinal segment.

botulinum, with subsequent production of botulinal toxin (1). There are many striking similarities between *C. botulinum* and *C. difficile* colonization of newborns. *C. botulinum* colonization of the intestinal tracts of infants can result in a wide range of clinical symptoms. A few infants will be transient asymptomatic carriers of *C. botulinum*, whereas other infants may die suddenly and unexpectedly in a way that is indistinguishable by history and autopsy from typical cases of sudden infant death syndrome (1, 2). Although the majority of infants colonized with *C. difficile* remain asymptomatic, a few infants have developed fulminating pseudomembranous colitis (24, 39, 40). One of the most characteristic features of infant botulism is its restricted age range; more than 75% of all recognized cases have occurred in patients between 1 and 6 months of age (1, 2). It has also been shown that the intestinal tracts of infant mice and rats between 7 and 13 days of age are readily colonized with *C. botulinum* after orogastric challenge; animals older and younger are resistant to *C. botulinum* colonization (35). Preliminary experiments demonstrate that infant mice and rats have a similar susceptibility to *C. difficile* intestinal colonization (R. D. Rolfe, unpublished observations).

In this investigation, we found that the ID_{50} for 7-day-old animals was 18 viable *C. difficile* cells. Larson et al. were able to induce fatal ileocecitis in hamsters previously treated with vanomycin by administering 1 CFU of *C. difficile*, which suggests that infant hamsters are more resistant to *C. difficile* intestinal colonization than vancomycin-treated adult hamsters (23). This greater resistance to *C. difficile* intestinal colonization in infant hamsters may explain our inability to detect intestinal *C. difficile* in uninoculated control animals.

Some investigators have suggested that the transient nature of *C. difficile* carriage in infants may account for the lack of clinical manifestations (6, 23). Other investigators, however, have been able to repeatedly isolate *C. difficile* from stool specimens of asymptomatic neonates over periods of several months (9, 34). In infant hamsters, *C. difficile* could be recovered up to 8 days after orogastric challenge.

Why there are no deleterious conseqences resulting from *C. difficile* colonization of neonates is unknown. The asymptomatic colonization may be related to the site of *C. difficile* colonization in the intestinal tract. In this investigation, the concentrations of *C. difficile* in the ceca and large bowels of 10-day-old hamsters were comparable to the concentration of *C. difficile* at these same locations in antibiotic-treated hamsters. On the other hand, antibiotic-treated adult hamsters possessed higher concentrations of *C. difficile* in their stomachs and small intestines than infant hamsters. In addition, cytotoxin was not present in the upper and lower small intestines of 10-day-old hamsters, whereas cytotoxin was present in many of these intestinal segments of antibiotic-treated adult

hamsters. This difference may be related to ingestion of *C. difficile* toxin(s) by the adult hamsters through coprophagy and may explain why *C. difficile* colonization is lethal to adult hamsters and innocuous to newborn hamsters. We have not observed coprophagy by 10-day-old hamsters.

C. difficile produces two immunologically and biochemically distinct toxins: toxin A (enterotoxin) and toxin B (cytotoxin) (36, 38). It is not known which of these toxins is responsible for the pathological changes seen in *C. difficile*-associated intestinal disease. The lack of toxic symptoms in infant hamsters colonized with *C. difficile* may be due to the low levels of one or both toxins in their intestinal tracts. In this investigation it was found that the majority of infant hamsters colonized with *C. difficile* possessed intestinal toxin titers 10-fold to 1,000-fold less than those present in the intestinal tracts of adult hamsters with *C. difficile*-induced ileocecitis. However, some of the infant hamsters colonized with *C. difficile* possessed toxin titers comparable to those present in the intestinal tracts of adult hamsters with *C. difficile*-associated intestinal disease. Investigators have shown that the titer of fecal toxin present in human newborns colonized with *C. difficile* also varies considerably (7, 30, 32, 39–41). Since tissue culture neutralization assays primarily detect toxin B, it is unknown at what levels toxin A was present in the intestinal tracts of colonized infant animals.

More studies are needed to adequately understand the mechanisms which permit the proliferation of *C. difficile* in the intestinal tracts of newborns and to understand the exact importance of this colonization in the health and development of neonates and young infants. The infant hamster model of *C. difficile* nonlethal intestinal colonization may help delineate the mechanism of *C. difficile* colonization of human newborns in the absence of intestinal disease. In addition, this animal model may help explain the pathogenesis of *C. difficile*-associated disease in adults, as well as possible means of prevention and treatment.

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