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Pseudomembranous Colitis in *Clostridium difficile*-Monoassociated Rats

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Germfree rats were monoassociated with either a toxin-producing strain of *Clostridium difficile* (Tox^+) or a variant of this strain (Tox^R) which produced much less toxin (1/10,000) in vivo and in vitro. Monoassociation of germfree rats with *C. difficile* Tox^+ resulted in mortality (17%) and in pathology to the small and large intestines, livers, and lungs. Cecal filtrates from the Tox^+-monoassociated rats were cytotoxic for tissue culture cells. The cytotoxicity of cecal filtrates could be blocked by sera from Tox^+-monoassociated rats. Monoassociation of rats with *C. difficile* Tox^R resulted in no deaths or pathology, and much less toxin was detected in the cecal filtrates of these animals than in those of rats colonized with the Tox^+ strain. This gnotobiotic model may be useful for investigating the etiology, prophylaxis, therapy, and exacerbation of *C. difficile*-induced pseudomembranous colitis.

The association of pseudomembranous colitis (PC) with antibiotic therapy, particularly clindamycin therapy (9, 13, 21), has been frequently recognized since the advent of antibiotics. Numerous etiologies for antibiotic-associated colitis have been proposed, including direct cytotoxicity by the antibiotic (14), disruption of bile acid metabolism (12), and viral (23) or *Staphylococcus aureus* infection (9, 21). Current evidence on the etiology of antibiotic-associated colitis suggests that antibiotic therapy disrupts the ecological balance of the intestinal flora, resulting in growth and toxin production by *Clostridium difficile* (2, 21). Recently, Czuprynski and Balish induced PC in gnotobiotic rats by colonizing them with *Listeria monocytogenes* (5), demonstrating that factors other than *C. difficile* toxin can be involved in the etiology of PC. Some antibiotics are able to cause a pseudomembranous cecitis and mild inflammation of the terminal ilea and proximal colons in hamsters (16, 21, 27) and guinea pigs (20); however, severe damage usually is restricted to the cecum. Cecitis, but not PC, is also produced in hamsters by intracecal injection of *C. difficile* or its toxin (2, 15, 19).

Fecal filtrates from PC patients are cytotoxic for tissue culture cells (2, 19), lethal for hamsters or mice on intraperitoneal or intracecal injection (2, 19), and able to cause increased vascular permeability in rabbits (17, 19). Toxins produced by *C. difficile* are cytotoxic in vitro (1, 7, 8, 17, 24–26) and lethal in vivo (1, 15–17, 26). All these toxic manifestations can be prevented by pretreatment of the cecal filtrate or purified toxin with *C. difficile* or *Clostridium sordellii* antitoxin (1, 2, 15, 19, 26).

Gnotobiotic rodents are a unique animal model for studies on the interrelationship of bacterial species with other components of the microbial flora and with the host itself. Several investigators have studied the effect of *C. difficile* on the alimentary tract of germfree rodents (6, 10, 11, 18, 22, 26). Skelly et al. (22) have reported that *C. difficile* decreases the cecal size of gnotobiotic mice. Gustafsson et al. (10) have reported that *C. difficile* causes diarrhea and decreases the cecal size of gnotobiotic rats. Dabard et al. (6) have demonstrated that *C. difficile* causes diarrhea and is lethal for young gnotobiotic hares, but no pathology was observed when young germfree rabbits, mice, or rats were colonized with *C. difficile*. Dabard et al. (6) have also reported that gnotobiotic hares that are diassociated with *C. difficile* and *Clostridium perfringens* or triassociated with *C. difficile*, *C. perfringens*, and *Clostridium tertium* manifest acute inflammatory, hemorrhagic, and necrotic lesions in the small and large intestines. Wilkins et al. (26) have reported that *C. difficile* Tox^+ colonizes the gastrointestinal tracts of germfree rats and produces within the intestinal tracts a cyto-

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toxin that has a molecular weight similar to that of the cytotoxin produced by *C. difficile* in culture medium. Monoassociation with *C. difficile* decreased the cecal size of the rats and was lethal for 25% of the gnotobiotic rats. Wilkins et al. (26) also isolated from the Tox^+-monoassociated rats a low-toxin-producing strain of *C. difficile* (Tox^R) that was not lethal when it colonized the intestinal tracts of rats. Onderdonk et al. (18) have reported that germfree mice can be colonized with *C. difficile*. The latter monoassociated mice, which had cytotoxin in their intestinal tracts, experienced diarrhea, acute polymorphonuclear inflammation of the lamina propria, and decreased cecal weights; however, fewer than 2% of the mice died (18).

This report describes the histopathology, microbiology, and immunology of rats that were monoassociated with either of two strains (Tox^+ or Tox^R) of *C. difficile* that differed in the capacity to produce both A and B toxins (Tox^R produced 10,000-fold less A and B toxin than the Tox^+ strain) in vivo and in vitro (26). The Tox^+ strain was lethal for 17% of the monoassociated rats, reduced their cecal size, caused diarrhea, and resulted in pathological damage to the intestinal tracts, livers, and lungs. The Tox^R strain has not been completely characterized for other virulence factors that could play a role in pathogenesis, but it colonized the gut in numbers comparable to the Tox^+ strain, reduced cecal size, and caused no obvious pathology for the gnotobiotic rats.

MATERIALS AND METHODS

Animals. Germfree Sprague-Dawley rats (males and females) 60 to 90 days of age were monoassociated with *C. difficile*. These rats were bred and reared in plastic isolators at the Gnotobiotic Laboratory, University of Wisconsin, Madison, Wis. The rats were given sterile lab chow and water ad libitum. Fecal samples were cultured weekly to detect any contaminating bacteria (aerobic or anaerobic) or fungi.

Rat monoassociation. *C. difficile* VPI 10463 Tox^+ or 10463 Tox^R was inoculated into 50 ml of prereduced brain heart infusion broth and was incubated for 48 h at 37°C in an anaerobic chamber. The *C. difficile* culture was aseptically introduced into a plastic isolator that contained germfree rats. The oral cavity of each rat was swabbed with the *C. difficile* broth culture; the remainder of the inoculum was then suspended in the drinking water of the rats. Similar procedures were followed to monoassociate germfree rats with a Tox^R strain of *C. difficile* which produces much less of both toxins (1/10,000) than the wild-type strain does (26). The Tox^R strain of *C. difficile* has not been completely characterized, but it had the same fermentation patterns and produced the same fatty acids as the Tox^+ strain. Since the Tox^+ and Tox^R strains of *C. difficile* are not isogenic, however, differences in traits other than toxin production may exist.

Organ distribution of *C. difficile*. Monoassociated rats were removed from the isolator, killed with ether, and put into an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.). The appropriate organs were removed aseptically, placed in glass tissue grinders which contained 9 ml of cold phosphate-buffered saline, and homogenized with a motor-driven Teflon pestle. The homogenates were serially diluted in phosphate-buffered saline and were plated on prereduced tryptic soy agar supplemented with 5% sheep blood. The plates were allowed to dry (inverted) and incubated at 37°C for 5 days in the anaerobic chamber. A portion of each tissue homogenate was dried in an oven to estimate its dry weight. The bacteriological data were expressed as the number of viable bacteria per gram of tissue (dry weight).

Histopathology. Organs removed from sacrificed rats or at necropsy were fixed in 10% buffered Formalin, sectioned, and stained with hematoxylin and eosin or with tissue Gram stain.

Cecal filtrate. Gnotobiotic rats were killed (cervical dislocation), and their cecal contents were put into sterile 15-ml centrifuge tubes. One ml of cecal contents (fluid) was diluted 1/10 in phosphate-buffered saline. The diluted cecal contents were agitated for 30 min and centrifuged (500 × *g*) to remove particulate matter, and the supernatant was passed through a 0.45-μm filter (Millipore Corp., Bedford, Mass.). The cecal filtrates were stored at −20°C until used in cell rounding or cytostasis assays.

Antitoxin assays. Serum samples from germfree, *C. difficile*-monoassociated, and conventional rats were assessed for their capacity to inhibit the cytotoxic activity of the *C. difficile* toxin (toxin B) that was present in cecal filtrates from monoassociated rats. Serial dilutions of serum samples were mixed with a 1/10 dilution of toxin-positive cecal filtrates. The titer of antitoxic activity was designated as the highest dilution of serum that completely prevented cell rounding by a 1/10 dilution of cecal filtrate from *C. difficile* (Tox^+)-monoassociated rats.

We assessed the cell rounding and cytostatic activity of the *C. difficile* toxin in the cecal filtrates by using C-26 colon carcinoma cells (derived from BALB/c mice that were treated with *N*-nitroso-*N*-methyl-urethane) (4) and cottontail rabbit kidney (CRK) cells by the following procedure. The C-26 colon tumor cells or the CRK cells (10^4 cells per well) in 0.2 ml of RPMI medium with 5% heat-inactivated fetal calf serum were added to microtiter wells and were incubated for 24 h in 5% CO_2 at 37°C. Dilutions of cecal filtrates were then added to wells that contained either the CRK cells or the C-26 tumor cells and were incubated at 37°C in 5% CO_2 for 24 h. The cultures were pulsed with 1.0 μCi of [^{3}H]thymidine and were incubated for 18 h at 37°C. The C-26 or CRK cells were next harvested onto fiber glass filters with an automatic cell harvester (Otto Hiller, Madison, Wis.). The filters were dried in an oven and were placed in scintillation vials. Scintillation cocktail was added, and the amount of [^{3}H]thymidine incorporated into the tumor cells was assessed.

RESULTS

Lethality. In two separate experiments (12 rats each), 17% (4 of 24) of the rats monoassociated with toxin-producing *C. difficile* Tox^+ died with-

in 1 to 5 days after monoassociation. There were no deaths in a group of 12 rats that were monoassociated with a strain of *C. difficile* (Tox^R) that was isolated during the initial monoassociation experiment and that produces much less of both *C. difficile* toxins than the wild type does (26).

Organ weight. Monoassociation with *C. difficile* Tox^+ decreased the cecal size of the rats in comparison with germfree controls (Table 1). Monoassociation with *C. difficile* (Tox^R) did not decrease cecal size of gnotobiotic rats to the same extent that the Tox^+ strain did.

Distribution of *C. difficile* in monoassociated rats. *C. difficile* Tox^+ and Tox^R colonized both the upper and lower gastrointestinal tracts. Low numbers of viable *C. difficile* were present in the stomachs (10^5 cells per g) and duodena (10^4 cells per g), greater colonization occurred in the ilea (10^8 cells per g), and maximal numbers of *C. difficile* were present in the ceca and colons (10^{10} to 10^{11} cells per g). *C. difficile* (Tox^+) was isolated from the spleens, livers, and kidneys of rats that were sacrificed at different times during the first 21 days after monoassociation. *C. difficile* Tox^R also colonized the gastrointestinal tracts of gnotobiotic rats in large numbers (similar to the Tox^+ strain), but the Tox^R strain was not isolated at any time from internal organs of the monoassociated rats that were sacrificed at various times. These data imply that the Tox^+ strain not only possessed the ability to colonize alimentary tracts and produce a cytotoxin but that it also entered bloodstreams and infected spleens, livers, and kidneys, possibly as a result of the colonic histopathology described below.

Histopathology. Rats monoassociated with Tox^+ *C. difficile* exhibited extensive histopathological changes in their guts and other organs. Rats which survived monoassociation and were sacrificed on the day after monoassociation demonstrated no stomach damage, but the ilea showed signs of mild to moderate inflammation, including mucosal edema, submucosal mononuclear cell infiltration, and hypercellular lacteals. Within the colons of these rats there was extensive submucosal edema and some mononuclear cell infiltration of the mucosae. Necropsy of moribund rats 5 days after monoassociation revealed severe damage to both small and large bowels. The small intestines were edematous with hypercellular glands and blunt degenerative villi which were sloughed off and trapped within fibrinopurulent pseudomembranes. Mucosal ulceration and pseudomembrane formation also occurred within the colons (Fig. 1). Numerous gram-positive bacilli were visible within the pseudomembranes; however, bacilli were not observed within the eroded mucosae (Fig. 2).

Monoassociation with the Tox^+ strain of *C. difficile* also caused extensive damage to livers and lungs, although in both cases there was very little infiltration of inflammatory cells into the damaged tissue. Hydropic degeneration of hepatocytes was evident at 24 h after monoassociation; by 48 h it had progressed into a severe hepatitis in which hepatocytes were visualized as thin rims of cytoplasm surrounding a centrally located nucleus (Fig. 3). Rats monoassociated with *C. difficile* Tox^+ also exhibited severe interstitial pneumonitis with hyperemia, pleuritis, and distended alveolar septa which contained erythrocytes, neutrophils, and mononuclear cells (Fig. 4). There were few cells and little exudate within the airways.

Rats monoassociated with the Tox^R strain of *C. difficile* did not manifest any abnormal pathology.

TABLE 1. Weight of ceca from germfree, conventional and *C. difficile*-monoassociated rats

Microbial status	Cecal wt (% of total body wt)[a]
Germfree	5–8
Conventional	1.8–2.2
C. difficile monoassociated	
Tox^R	3–5
Tox^+	1.8–4.1

[a] Range of cecal weight for at least 15 rats per group. The monoassociated rats were sacrificed at various times over a period of 40 days after monoassociation.

Cytotoxic activity of cecal contents from *C. difficile*-monoassociated rats. A previous report (26) has demonstrated that cecal filtrates from *C. difficile* (Tox^+)-monoassociated rats cause rounding of Chinese hamster ovary (CHO-K1) cells and are lethal for mice. The data in Table 2 demonstrate that the toxin in cecal filtrates of monoassociated rats caused rounding of C-26 colon tumor cells and CRK cells and also inhibited the capacity of the C-26 and CRK cells to incorporate [^{3}H]thymidine. The data in Table 2 also demonstrate that the Tox^+ strain produced much more toxin in vivo than the Tox^R strain did.

Antitoxin in sera of *C. difficile*-monoassociated rats. Sera from rats that were monoassociated with *C. difficile* Tox^+ for 21 days had antitoxic antibody to the *C. difficile* cytotoxin (Table 3). This antitoxin abrogated both cell rounding and inhibition of thymidine uptake. No antitoxin was demonstrable in sera from age-matched germfree, Tox^R-monoassociated, or conventional rats. *C. difficile* and *C. sordellii* antitoxin also neutralizes the cytotoxin in cecal filtrates from *C. difficile*-monoassociated rats (26).

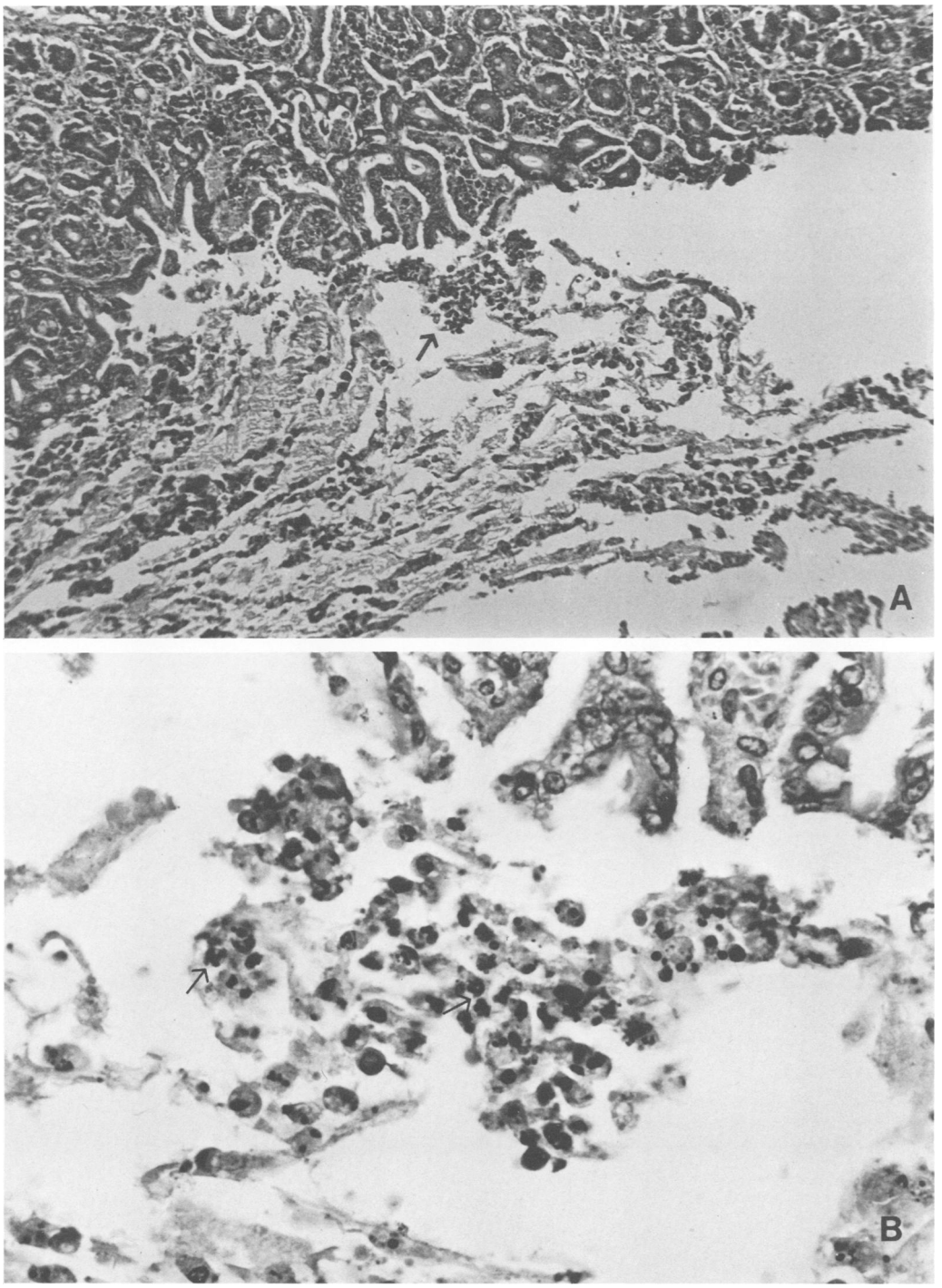

FIG. 1. (A) Colonic ulceration and pseudomembrane formation (arrow) in moribund rat 5 days after *C. difficile* (Tox^+) monoassociation. Hematoxylin and eosin stain, ×82. (B) Neutrophils (arrows) in pseudomembrane shown in (A). Hematoxylin and eosin stain, ×328.

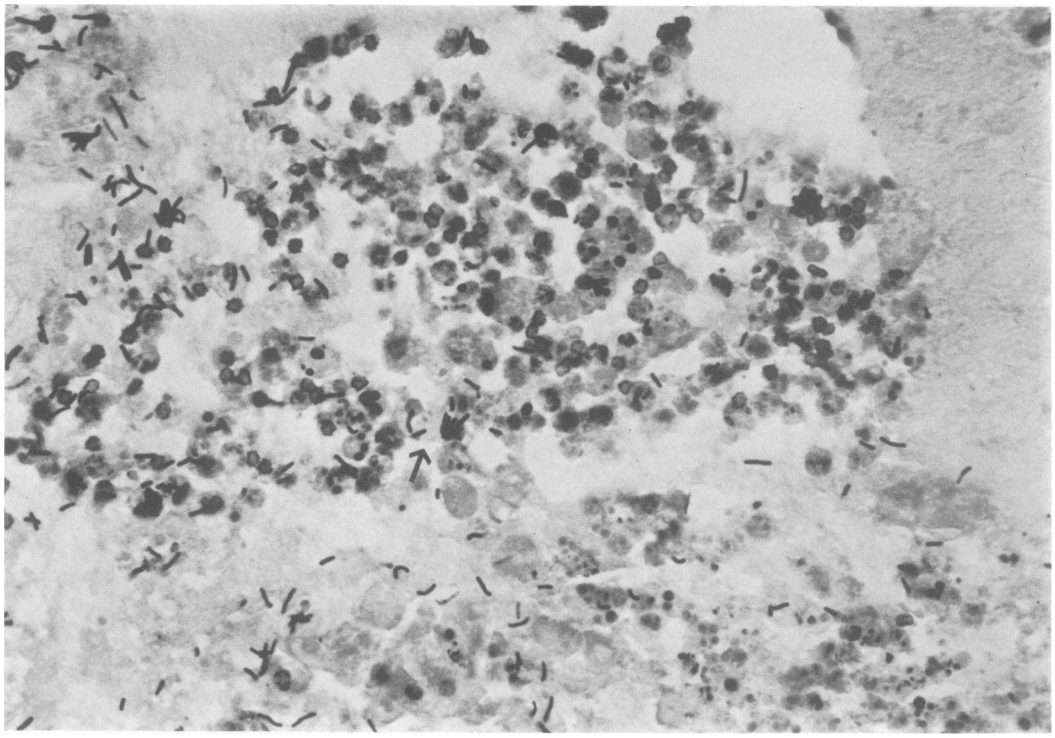

FIG. 2. Gram-positive bacilli (arrow) within a pseudomembrane. Gram stain, ×336.

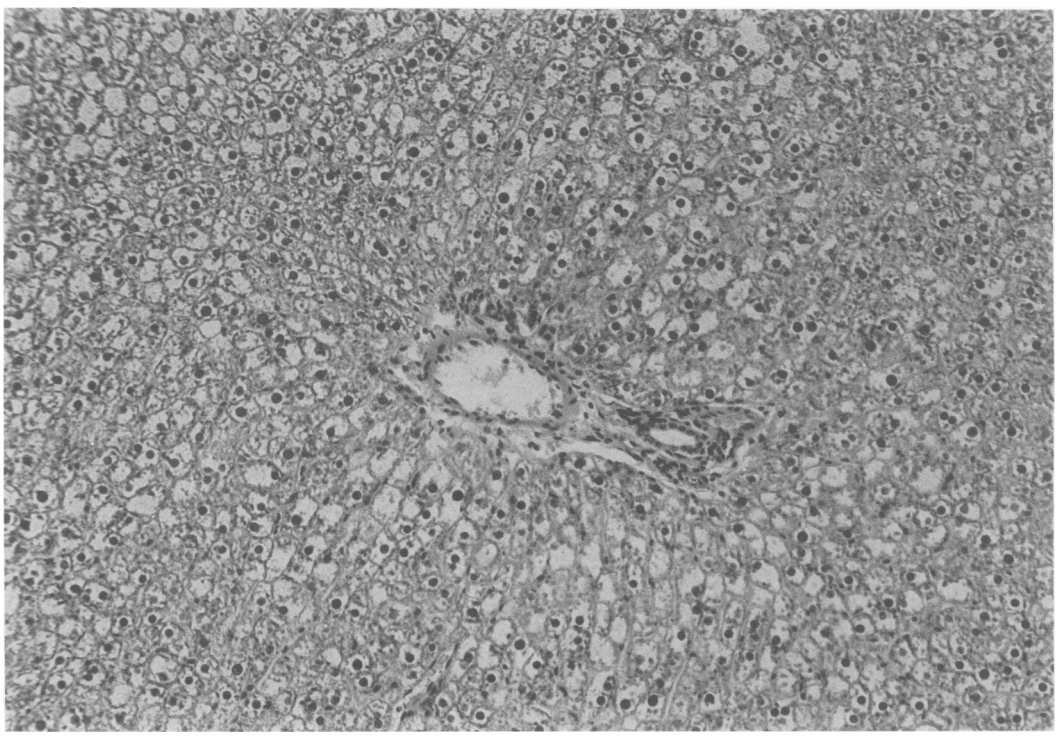

FIG. 3. Hydropic degeneration of hepatocytes in a rat 48 h after *C. difficile* (Tox^+) monoassociation. Hematoxylin and eosin stain, ×84.

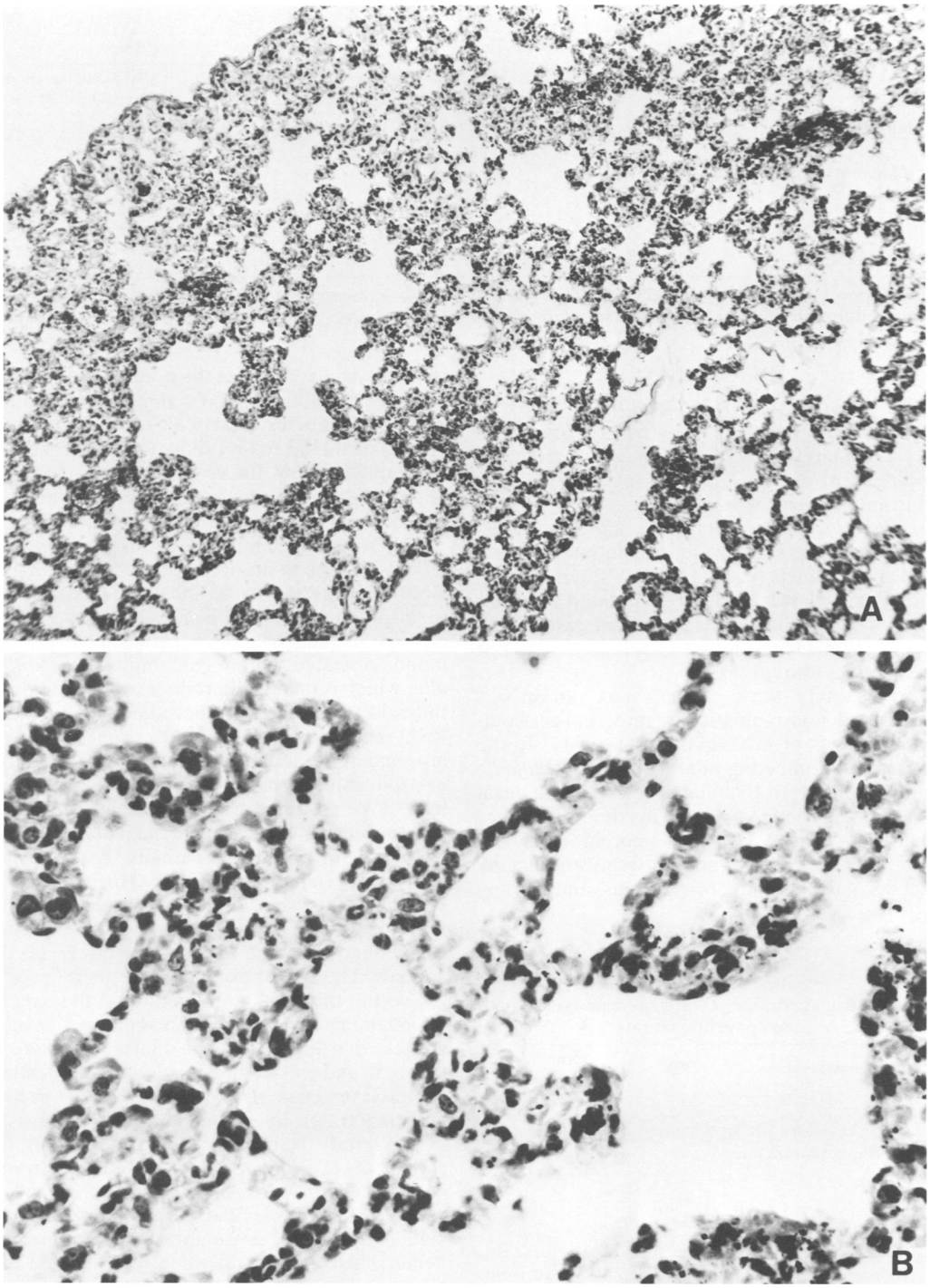

FIG. 4. (A) Interstitial pneumonitis in a rat 4 days after monoassociation with *C. difficile* (Tox^+). Hematoxylin and eosin stain, ×82. (B) Detail of (A); Magnification, ×328. Hematoxylin and eosin stain.

TABLE 2. Cytotoxic activity of cecal filtrates from germfree, *C. difficile*-monoassociated, and conventional rats

Microbial status	Cytotoxic activity in cells[a]:			
	C-26 Tumor		CRK	
	Rounding	[^{3}H]thymidine uptake inhibition	Rounding	[^{3}H]thymidine uptake inhibition
Germfree	0	10^{-1}	0	10^{-1}
C. difficile monoassociated				
Tox$^+$	10^{-11}	10^{-11}	10^{-9}	10^{-9}
Tox^R	10^{-2}	10^{-2}	10^{-2}	10^{-2}
Conventional	0	10^{-1}	0	0

[a] Dilution of filter-sterilized cecal contents from *C. difficile*-monoassociated rats that caused >90% of the tissue culture cells to round up, or that interfered (>90%) with the capacity of cells to incorporate [^{3}H]thymidine.

DISCUSSION

We believe that the *C. difficile* (Tox$^+$)-monoassociated rats in this study represent the first animal model of PC caused by a cytotoxin-producing strain of *C. difficile* (originally isolated from a patient with antibiotic-associated PC; 26). *Bacillus pumilus* (3) and *C. difficile*, or their toxins, cause primarily a cecitis in hamster and guinea pig models (2, 15, 16, 20, 27). Czuprynski and Balish have also demonstrated that *L. monocytogenes* can produce PC in gnotobiotic rats by a mechanism that apparently does not involve a cytotoxin (5).

In this study, the *C. difficile* Tox$^+$ strain was introduced nontraumatically into the gastrointestinal tracts of rats via the oral cavity. In the absence of competing microbes, the *C. difficile* multiplied (10^{10} to 10^{11} viable cells per g of cecal contents), produced toxin in the alimentary tract, and caused PC. This gnotobiotic model eliminates the need for antibiotics to study many aspects of the etiology, prophylaxis, and therapy of colitis, and it also has the potential for clarifying the interactions of *C. difficile* with other microbial species that can either suppress growth or inhibit toxin production. Such knowledge might allow future manipulations of the microbial flora to prevent or decrease the incidence of antibiotic-associated PC in patients.

TABLE 3. Neutralization of *C. difficile* cytotoxin with sera from germfree, *C. difficile*-monoassociated, and conventional rats

Serum source	Neutralization titer[a]
Germfree	0
C. difficile monassociated	
Tox$^+$	1,024
Tox^R	0
Conventional	0

[a] Reciprocal of serum titer that completely prevented (>90%) rounding of C-26 tumor cells or CRK cells by cecal filtrates from *C. difficile* (Tox$^+$)-monoassociated rats. Toxin produced by *C. difficile* (Tox$^+$) in culture was also inhibited by sera from *C. difficile*-monoassociated rats.

This study further substantiates the role of *C. difficile* and its toxin in the pathogenesis of PC. Germfree rats that were inoculated orally with toxin-producing *C. difficile* (Tox$^+$) sustained damage to their intestinal tracts; however, rats monoassociated with a Tox^R mutant of *C. difficile*, which is unable to produce as much toxin as the wild type (26), were not affected adversely. Rats monoassociated with *C. difficile* (Tox$^+$) also manifested damage to organs other than the gastrointestinal tract, primarily the lungs and livers. We are unaware of reports of such damage to internal organs in the hamster or guinea pig model, or even in PC patients. We were able to isolate viable *C. difficile* Tox$^+$ from the spleens, livers, and kidneys of monoassociated rats, suggesting that *C. difficile* Tox$^+$ had disseminated from the alimentary tracts of the animals. The absence of visible bacteria (histology) within the lungs and livers, and the paucity of inflammatory cells within damaged tissues, suggest that lung and liver damage was due to the toxin and was not the result of overwhelming bacterial invasion. The pathology to the gastrointestinal tracts, livers, and lungs in the rats used in this study is not surprising in light of the ability of *C. difficile* toxin to damage tissue culture cells in vitro (1, 7, 8, 15, 17, 24–26), to cause increased vascular permeability in rabbit skin (17, 19), to cause intestinal and hepatic hemorrhages and death when it is injected into mice (1, 26) or hamsters (15, 16), and to cause cecal enlargement in hamsters and guinea pigs (15, 16, 20).

Rats monoassociated with Tox$^+$ *C. difficile* exhibited a marked reduction in cecal size, a

phenomenon which has been reported for gnotobiotic rodents by several investigators (6, 10, 11, 18, 22, 26). Hammerstrom et al. (11) also found autoantibodies to colonic antigen in *C. difficile*-monoassociated rats. Current information regarding the pathogenesis of *C. difficile*-induced PC suggests that autoantibody production might be secondary to colonic damage caused by *C. difficile* toxin rather than a result of immunological stimulation by *C. difficile*, as was proposed originally by Hammerstrom et al. (11).

As indicated previously (26), the Tox^R strain, a spontaneous mutant of *C. difficile* that produces much less of both toxins (1/10,000), was recovered from rats that had been monoassociated with Tox^+ *C. difficile*. Rats monoassociated with this Tox^R mutant have smaller ceca than germfree rats but did not exhibit any pathology in the gastrointestinal tract or internal organs.

Our study also demonstrates that rats monoassociated with the Tox^+ strain of *C. difficile* were able to produce antitoxin, indicating that the toxin does interact with the immune system. Antitoxin to toxins A and B of *C. difficile* have been reported in persons over 6 months of age, and increased titers to *C. difficile* toxins have been demonstrated in the sera of patients with *C. difficile*-induced colitis (R. Viscidi, R. Yolken, B. Laughon, P. B.-Linn, T. Moench, and J. G. Bartlett, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 595, 1982). We were unable to detect antitoxin activity in serum from germfree, Tox^R-monoassociated, or conventional rats. We have no good explanation why germfree rats can survive their initial colonization with *C. difficile*. Since the colitis and other pathological changes were not observed in rats sacrificed 28 days after monoassociation, the antitoxin could explain the lack of a chronic colitis in these monoassociated rats, despite the continued presence of very high levels of *C. difficile* toxin in their alimentary tracts. An alternative explanation for the lack of a chronic colitis in the monoassociated rats could be the absence of other intestinal bacteria. The microbe that initiates the colitis may be very different from the factors or other intestinal microbes that could be responsible for sustaining or exacerbating the colitis. The *C. difficile*-monoassociated rat again will be a very useful animal model for studies on the interesting and clinically relevant aspects of etiology, prophylaxis, and exacerbation of bacterium-induced bowel diseases.

Immunization with *C. difficile* toxoid can prevent cecitis in hamsters (15). This gnotobiotic rat model would be an excellent way to assess whether immunization with *C. difficile* toxoid can also protect monoassociated rats against PC.

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