INFECTION AND IMMUNITY, Feb. 1985, p. 349–352
0019-9567/85/020349-04$02.00/0

Vol. 47, No. 2

Effects of *Clostridium difficile* Toxins Given Intragastrically to Animals

DAVID M. LYERLY, KENNETH E. SAUM, DAVID K. MacDONALD, AND TRACY D. WILKINS*

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received 8 June 1984/Accepted 26 October 1984

We examined the activities of *Clostridium difficile* toxin preparations given intragastrically to hamsters, mice, and rats. The culture filtrate from a highly toxigenic strain of *C. difficile* caused hemorrhage and accumulation of fluid in the small intestine and cecum, diarrhea, and death in hamsters and mice. In rats, the culture filtrate caused only a small amount of fluid accumulation and slight hemorrhage along the small intestine. When toxin A was removed from the culture filtrate, the filtrate lost its activity. Preparations of homogeneous toxin A caused a response similar to that observed after the administration of culture filtrate. Hamsters were more sensitive to toxin A than mice or rats were. When hamsters were given multiple low doses of toxin A 1 week apart at a concentration which singly caused no response, they became ill and died, indicating that the toxin may have long-term effects. High amounts of toxin B did not cause any significant response when given intragastrically, unless initially mixed with low amounts of toxin A or given to hamsters with bruised ceca. These results suggest that toxins A and B act synergistically and that the action of toxin B may occur via the tissue damage caused by toxin A.

Clostridium difficile, the etiological agent of pseudomembranous colitis in humans, causes cecitis and colitis in a number of experimental laboratory animals, including hamsters, mice, rats, and rabbits (5, 6, 10, 22, 24, 27). The disease has commonly been associated with antibiotic therapy. More recently, the results of several studies have shown that the disease can occur in humans and experimental animals in the absence of antibiotics (9, 26).

At the present time, the animal model used most extensively to study *C. difficile* disease is the hamster. The disease progresses rapidly to cecitis, and hemorrhage, ulceration, and inflammation are evident in the intestinal mucosa. The animals become lethargic and develop severe diarrhea, and a high percentage of the animals die from the disease (5, 10, 24). Although *C. difficile* disease in other animals has not been studied in as much detail, it appears that animals such as mice, rats, and rabbits are not as susceptible to the disease as hamsters (10).

C. difficile produces at least two toxins (1, 30, 31). One of the toxins, toxin A, causes an accumulation of fluid in ligated intestinal loops of rabbits (20, 31) and has been referred to as the enterotoxin. The other toxin, toxin B, is a potent cytotoxin; subpicogram amounts of the toxin cause rounding of tissue culture cells (30). In the following study, we examined the effects of these toxins when they were given intragastrically to animals. Our findings showed that toxin A caused intestinal pathology, diarrhea, and death in animals and that toxin B, when given with low amounts of toxin A, caused death in hamsters. We also found that hamsters were more sensitive to toxin A than mice or rats were.

MATERIALS AND METHODS

Assays. (i) Protein determination. Protein was estimated by the method of Bradford (3) with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). Bovine gamma globulin was the standard.

(ii) Cytotoxicity assay. The cytotoxic titer was determined against Chinese hamster ovary K-1 cells as previously described (8).

(iii) Enzyme-linked immunosorbent assay. An indirect enzyme-linked immunosorbent assay specific for toxin A was performed as previously described (21).

Bacterial strains. *C. difficile* strains 10463 (tox^+), 11186 (tox^-), and 2037 (tox^-) were obtained from the collection of the Department of Anaerobic Microbiology of the Virginia Polytechnic Institute and State University (Blacksburg, Va.). Strain 10463 produces high amounts of toxins A and B, as determined by enzyme-linked immunosorbent assay and cytotoxicity assay, and strains 11186 and 2037 do not produce detectable amounts of either toxin (21, 30).

Preparation of toxin samples. *C. difficile* strains were grown in brain heart infusion dialysis flasks similar to those previously described (29). The culture filtrates and toxins A and B were purified as previously described (30).

Animals. The animals used in these experiments included BALB/c male mice (Dominion Laboratories, Dublin, Va.) weighing about 25 g, golden Syrian male hamsters (Engle Laboratory Animals, Inc., Farmersburg, Ind.) weighing about 160 g, and Sprague-Dawley male rats (Department of Animal Sciences, Virginia Polytechnic Institute and State University) weighing about 500 g.

Development of oral animal model. Experiments were done to determine the optimal conditions for administering *C. difficile* toxin preparations intragastrically to mice, hamsters, and rats. The following conditions were tested: (i) pretreatment of animals with 0.1, 0.5, or 1.0 M $NaHCO_3$ 15 to 30 min before the administration of toxin (in $NaHCO_3$ buffer) to neutralize the gastric acidity (analysis of the stomach and gastrointestinal tract contents of animals treated in this manner showed that the pH in all three species was about 7.4); (ii) pretreatment of animals with cimetidine (Tagumet; Smith Kline & French Laboratories, Philadelphia, Pa.) at a dose of 50 mg/kg of body weight 2 h before the administration of toxin to decrease stomach acid production; and (iii) incorporation of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 10 mg/ml in the

* Corresponding author.

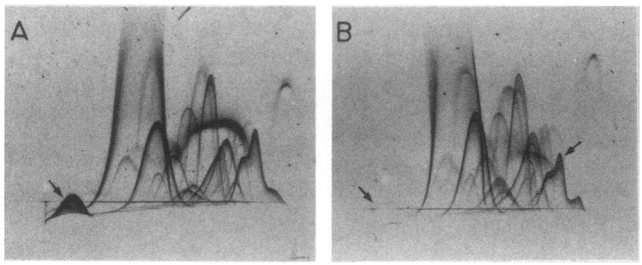

FIG. 1. Analysis of *C. difficile* strain 10463 culture filtrate adsorbed with toxin A antibody coupled to Affi-Gel 10. The upper portion of the gel in each plate contained 0.1 ml of goat antiserum against *C. difficile* strain 10463 culture filtrate (8). (A) The well contained 80 μg of strain 10463 culture filtrate. The arrow shows the location of the toxin A immunoprecipitin arc. (B) The well contained 80 μg of strain 10463 culture filtrate adsorbed with monospecific toxin A antiserum coupled to Affi-Gel 10. Note that the toxin A arc has disappeared (shown by arrow on left), demonstrating that the toxin has been removed by the gel. The toxin B arc in the adsorbed culture filtrate (shown by arrow on right) is still present.

toxin sample to minimize proteolytic degradation of the toxins. The cytotoxic activity of the toxin preparations was not affected by the concentrations of $NaHCO_3$ and soybean trypsin inhibitor used in these experiments. These conditions were tested individually and in conjunction with each other. Pretreatment of animals with 1 M $NaHCO_3$ and administration of toxin in 1 M $NaHCO_3$ gave optimal results. The administration of cimetidine or the incorporation of soybean trypsin inhibitor did not increase the toxicity of the *C. difficile* toxin preparations.

In subsequent experiments, mice, hamsters, and rats were given 0.5, 1, and 2 ml of 1 M $NaHCO_3$, respectively; 15 to 30 min later, they were challenged intragastrically with toxin samples (0.5-, 1-, and 2-ml volumes) in 1 M $NaHCO_3$. Food and water were withheld from the animals 24 and 4 h, respectively, before the administration of $NaHCO_3$ and were given ad libitum 4 h after challenge. The animals were observed for 4 days. Necropsies were performed on animals immediately after they died or after they were killed. Control animals received 1 M $NaHCO_3$.

Bruising of hamster ceca. Hamsters were anesthetized with methoxyflurane (Metofane) and the cecum was exposed through an incision in the abdominal wall. The cecum was bruised with forceps and injected with 0.5 ml of 1 M $NaHCO_3$. The incision was closed with sutures. Food and water were withheld from the animals 24 and 4 h, respectively, before the injection. Animals were observed for 4 days and necropsies were performed on animals immediately after they died or after they were killed.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed in low-electroendosmotic agarose as previously described (21).

Removal of toxin A from culture filtrate. A sample (1 ml) of *C. difficile* strain 10463 culture filtrate was passed through a column containing affinity-purified toxin A antibody (21) coupled to Affi-Gel 10 (Bio-Rad Laboratories). The gel (10 ml) contained about 0.5 mg of antibody per ml of gel. The gel was washed with 0.05 M Tris-hydrochloride buffer, pH 7.5, containing 0.5 M NaCl. The adsorbed culture filtrate was concentrated to 1 ml by ultrafiltration on a YM-10 membrane (Amicon Corp., Lexington, Mass.) and analyzed by crossed immunoelectrophoresis against crude goat *C. difficile* antiserum (8) to show that toxin A was removed. The adsorbed culture filtrate was mixed with 2 ml of 1 M $NaHCO_3$ and samples (1 ml) were given to three hamsters as described above.

Preparation of lavage fluids from mouse intestine. The small intestine and cecum were removed from mice, sutured with nylon at each end, and lavaged three times with 3 ml of phosphate-buffered saline. The lavage specimens were pooled and insoluble material was removed by centrifugation. The supernatant was filtered through a 0.45-μm membrane.

RESULTS

Effects of culture filtrates given intragastrically. We compared the effects of culture filtrate from toxigenic and nontoxigenic strains of *C. difficile* given to hamsters, mice, and rats. All four of the hamsters given undiluted samples of culture filtrate from toxigenic strain 10463 developed hemorrhagic fluid in the stomach, small intestine, and cecum within several hours. Diarrhea was evident in all of the animals by 5 h and two of the animals died within 15 h. In mice, the small intestine and cecum were distended with fluid by 12 h, but only 4 of 16 mice had diarrhea. Some hemorrhage was apparent along the intestinal mucosa. By 24 h, six of the mice were dead and five of the surviving mice had diarrhea. Rats accumulated some fluid and slight hemorrhage in the stomach, small intestine, and cecum within 24 h. None of the rats developed diarrhea or died. Hamsters, mice, and rats that were given undiluted culture filtrate from nontoxigenic strains 11186 and 2037 did not become ill or develop intestinal pathology.

To determine which toxin caused the effects, toxin A in culture filtrate was removed by immunoadsorption (Fig. 1) and the filtrate was given to hamsters. Culture filtrate treated in this manner did not cause diarrhea or intestinal pathology in any of the three hamsters given the sample. Hamsters which received culture filtrate diluted to the same extent, but which had not been adsorbed, developed diarrhea and intestinal pathology, and five of the six animals died.

Effects of toxins A and B given intragastrically. When toxin A was given to hamsters, the animals accumulated hemorrhagic fluid in the cecum and small intestine, and the mucosa was inflamed. Mice which were given the toxin also accumulated fluid in the small intestine, but there was considerably less hemorrhage than observed in hamsters. Rats did not accumulate fluid at the doses we tested. The amount of toxin A necessary to cause intestinal pathology, diarrhea, and death in the three species is given in Table 1. Hamsters are more sensitive to the toxin than mice or rats are.

To further investigate the difference in sensitivity, we gave three mice a large dose of toxin A (1 mg/kg). At this concentration, the mice did not become ill or develop intestinal pathology. After 1 h, the mice were killed and the gastrointestinal tracts were lavaged. Samples (1 ml) of the lavage fluid were given intragastrically to three hamsters, and within 16 h the hamsters became lethargic and developed diarrhea. Three hamsters which were given lavage fluid from untreated mice did not become ill. Toxin A was incubated with intestinal lavage fluid from mice to detect any factors in the mouse gastrointestinal tract which might inactivate or neutralize the toxin. When we gave toxin A treated with the lavage fluid to hamsters, there was no decrease in toxicity.

Multiple doses of low amounts of toxin A (0.02 mg/kg) had cumulative effects when given to hamsters at weekly intervals. A single dose of toxin A at this concentration did not cause diarrhea or intestinal pathology. However, five of the

six hamsters developed diarrhea after the second dose and four of the animals died after the third dose.

High amounts of toxin B (0.5 mg/kg) did not cause intestinal pathology, diarrhea, or death when given intragastrically to animals. We were able to recover a cytotoxic titer of 10^5 from the ceca and small intestine of hamsters which received toxin B preparations; therefore, all of the toxin was not inactivated when given by this method. When we mixed a low amount of toxin A (0.01 mg/kg) with toxin B (0.5 mg/kg) and gave this mixture to hamsters, all six of the animals became lethargic and four died within 24 h. Necropsies revealed some mucus and edema in the small intestine and hemorrhage in the lungs.

We investigated the effects of toxin B given intragastrically to hamsters with bruised ceca. When toxin B (0.5 mg/kg) was given intragastrically to hamsters treated in this manner, all four of the animals became lethargic and two died within 24 h. None of the four control hamsters which received buffer instead of toxin B became ill.

TABLE 1. Effects of intragastric administration of toxin A on hamsters, mice, and rats[a]

Animal species[b]	mg of toxin A per kg of body wt necessary to cause:		
	Intestinal pathology	Diarrhea	Death
Hamster	0.08[c]	0.08	0.16
Mouse	2[d]	2	16
Rat	>2[e]	>2	>2

[a] The minimum amount of toxin A necessary to elicit the observed responses is given.

[b] Twofold dilutions of toxin A were administered intragastrically to each group of animals (three animals per group), and the animals were observed over a 72-h period. Necropsies were performed on animals immediately after they died or were sacrificed.

[c] This dose caused hemorrhage and fluid in the small intestine and cecum.

[d] This dose caused some fluid in the small intestine and hemorrhage along the mucosa of the intestine.

[e] The highest dose of toxin A given to rats was 2 mg per kg of body weight, which did not cause any significant intestinal pathology, diarrhea, or death.

DISCUSSION

C. difficile disease has been described in a number of experimental laboratory animals (5, 6, 10, 22, 24, 27). Hamsters are highly susceptible to the disease and develop severe cecitis, usually resulting in death. Other experimental animals do not appear to be as susceptible as hamsters. For example, germfree mice containing high numbers of toxigenic *C. difficile* in their feces develop only chronic inflammation along the intestine (22). In the present study, we examined the effects of *C. difficile* toxin preparations given intragastrically to hamsters, mice, and rats. Our results showed that culture filtrate from a toxigenic strain of *C. difficile* caused intestinal pathology, diarrhea, and death in animals. Further analyses revealed the following: (i) culture filtrate in which toxin A was removed was not active in this model; (ii) homogeneous toxin A caused intestinal pathology, diarrhea, and death in animals; and (iii) preparations of toxin B did not cause any significant pathology or death of the animals. Based on these observations, we suspected that toxin A caused most of the responses elicited by the culture filtrate from the toxigenic strain. However, when we gave a mixture of toxin B containing low amounts of toxin A intragastrically to hamsters, they died. These findings suggest that toxins A and B act synergistically. These findings suggest also that both toxins are involved in the pathogenesis of *C. difficile* disease since no toxin A^-/toxin B^+ clinical isolates have been reported (21).

We previously showed that toxin B, at a cytotoxic titer of 10^5, was lethal when injected into the ceca of hamsters (18). In the present study, we found that hamsters which had a cytotoxic titer of 10^5 in their ceca after the intragastric administration of toxin B did not become ill. During an intracecal injection, there is significant trauma to the tissue around the injection site. It is possible that toxin B is active via the damaged tissue. To investigate this possibility, we gave toxin B intragastrically to hamsters in which the ceca had been bruised and injected with buffer. Our findings showed that toxin B was active when given intragastrically to hamsters treated in this manner, suggesting that the integrity of the mucosa plays a role in the action of toxin B. Based on these observations, it is feasible that the tissue damage caused by toxin A during *C. difficile* disease may increase the action of toxin B. Also, any manipulations which result in damage to the integrity of the gastrointestinal tract (e.g., surgical wounds, trauma due to handling of the intestine) in persons infected with *C. difficile* may enhance the action of toxin B. Our results may explain why some persons with high levels of cytotoxicity due to toxin B do not become ill. It is conceivable that some persons do not have receptors for toxin A and that the action of the toxin is not expressed; as a result, toxin B is not active.

When we exposed hamsters repeatedly to low doses of toxin A, the effects were cumulative and the hamsters developed diarrhea and died. Bullen and Batty (4) previously described a similar situation involving the epsilon toxin of *C. perfringens* type D. They found that if mice were repeatedly given low doses of the toxin, the animals died. These investigators suggested that the initial dose of toxin increased the permeability of the intestine, resulting in absorption of lethal amounts of toxin in subsequent doses. In the experiments of Bullen and Batty (4), the challenge doses of toxin were given several hours apart. We obtained a similar effect when the doses of toxin A were given a week apart; therefore, toxin A may exert a long-term effect on the mucosa. If the same phenomenon occurs in humans, this effect could be one cause of the relapses that occur with this disease (2, 16, 25). We have initiated experiments to examine the effects of these toxins on the permeability of the intestine and to detect the toxins, after intragastric administration, in the blood and various organs.

When we determined the dose of toxin A necessary to elicit a response in animals, our findings showed that hamsters were more sensitive to the toxin than mice or rats were. The greater sensitivity of hamsters to toxin A probably explains why hamsters are more susceptible to *C. difficile* disease than mice or rats are. We also found that toxin A caused considerably less hemorrhage in mice than in hamsters and that, in the mouse, the intestinal pathology (i.e., inflammation, mild hemorrhage, fluid response) occurred primarily in the small intestine. These findings are consistent with those of Onderdonk et al. (22), who noted that mice monoassociated with toxigenic *C. difficile* did not develop the rapidly fatal, hemorrhagic cecitis which occurs in hamsters, and with those of Lonnroth and Lange (19), who reported that toxin A injected into ligated intestinal loops in mice caused a watery hypersecretion with little hemorrhage.

It is unclear why hamsters are more sensitive to toxin A than mice or rats are. We did not detect any inactivation or neutralization of the toxin by soluble extracts prepared from mouse intestine. We were able to recover toxin A from the small intestine of mice and show that the toxin still caused diarrhea in hamsters; thus, the toxin was not inactivated as it passed through the stomach of the mice. It is possible that

receptors for toxin A are present in higher numbers in the hamster, particularly in the cecum.

In addition to the variation in sensitivity among animal species, there appears to be an age-dependent variation. Human infants can have titers of toxins A and B equivalent to those found in adults with pseudomembranous colitis, yet show no clinical symptoms of the disease (7, 12, 17, 28). As many as half of the normal neonate population may carry *C. difficile* as part of their colonic flora so any effects of toxin A on gut permeability could influence the susceptibility to other intestinal diseases and toxins. For example, an increase in susceptibility to botulinum toxins could result in more severe forms of infant botulism.

Various procedures have been described for orally administering bacterial toxins to animals. The toxins of *Vibrio cholerae* and *Escherichia coli* have been given to animals pretreated with bicarbonate solutions or cimetidine to reduce the gastric acidity of the stomach. Amino acids have been added to samples to protect toxins against proteolytic degradation in the gastrointestinal tract. In addition, pH-dependent microspheres have been used to transport the heat-labile toxin of *E. coli* through the stomach of rats (13–15, 23). In our study, we found that toxins A and B were sufficiently protected by pretreating the animals with a high-molarity $NaHCO_3$ solution and incorporating the toxin samples in the $NaHCO_3$ solution.

Gurian et al. (11) recently described pseudomembranous colitis in a patient with hypochlorhydria and noted that the patient had not been receiving antibiotic therapy. They suggested that the disease resulted from the ingestion of contaminated food. These investigators also showed that the cytotoxic activity of the strain isolated from the patient was inactivated by gastrointestinal secretions. Our finding that toxin A is protected against gastric acidity by $NaHCO_3$ corroborates these observations; however, additional studies are needed to determine whether *C. difficile* toxin can be a cause of food poisoning in humans.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 15749 from the National Institutes of Health and state support grant 2124520 from the Commonwealth of Virginia.

We thank R. Van Tassell for help in preparing this manuscript.

LITERATURE CITED

1. **Banno, Y., T. Kobayashi, K. Watanabe, K. Ueno, and Y. Nozawa.** 1981. Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic-associated colitis: purification and some characterization. Biochem. Int. **2**:629–635.
2. **Bartlett, J. G., F. J. Tedesco, S. Shull, B. Lowe, and T. Chang.** 1980. Symptomatic relapse after oral vancomycin therapy of antibiotic-associated pseudomembranous colitis. Gastroenterology **78**:431–434.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Bullen, J. J., and I. Batty.** 1956. The effect of *Clostridium welchii* type D culture filtrates on the permeability of the mouse intestine. J. Pathol. Bacteriol. **71**:311–323.
5. **Chang, T., J. G. Bartlett, S. L. Gorbach, and A. B. Onderdonk.** 1978. Clindamycin-induced enterocolitis in hamsters as a model of pseudomembranous colitis in patients. Infect. Immun. **20**:526–529.
6. **Czuprynski, C. J., W. J. Johnson, E. Balish, and T. Wilkins.** 1983. Pseudomembranous colitis in *Clostridium difficile*-monoassociated rats. Infect. Immun. **39**:1368–1376.
7. **Donta, S. T., and M. G. Myers.** 1982. *Clostridium difficile* toxin in asymptomatic neonates. J. Pediatr. **100**:431–434.
8. **Ehrich, M., R. L. Van Tassell, J. M. Libby, and T. D. Wilkins.** 1980. Production of *Clostridium difficile* antitoxin. Infect. Immun. **28**:1041–1043.
9. **Ellis, M. E., B. M. Watson, P. J. Milewski, and G. Jones.** 1983. *Clostridium difficile* colitis unassociated with antibiotic therapy. Br. J. Surg. **70**:242–243.
10. **Fekety, R.** 1984. Animal models of *Clostridium difficile* infection, p. 119–132. *In* S. P. Borriello (ed.), Antibiotic associated diarrhoea and colitis. Martinus Nijhoff Publishers, Boston.
11. **Gurian, L., T. T. Ward, and R. M. Katon.** 1982. Possible foodborne transmission in a case of pseudomembranous colitis due to *Clostridium difficile*. Gastroenterology **83**:465–469.
12. **Gurwith, M. J., C. Langston, and D. M. Citron.** 1981. Toxin-producing bacteria in infants. Am. J. Dis. Child. **135**:1104–1106.
13. **Klipstein, F. A., R. F. Engert, and J. D. Clements.** 1982. Arousal of mucosal secretory immunoglobulin A antitoxin in rats immunized with *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **37**:1086–1092.
14. **Klipstein, F. A., R. F. Engert, and R. A. Houghten.** 1983. Protection in rabbits immunized with a vaccine of *Escherichia coli* heat-stable toxin cross-linked to the heat-labile toxin B subunit. Infect. Immun. **40**:888–893.
15. **Klipstein, F. A., R. F. Engert, and W. T. Sherman.** 1983. Peroral immunization of rats with *Escherichia coli* heat-labile enterotoxin delivered by microspheres. Infect. Immun. **39**:1000–1003.
16. **LaMont, J. T., and Y. M. Trnka.** 1980. Therapeutic implications of *Clostridium difficile* toxin during relapse of chronic inflammatory bowel disease. Lancet **i**:381–383.
17. **Libby, J. M., S. Donta, and T. D. Wilkins.** 1983. *Clostridium difficile* toxin A in infants. J. Infect. Dis. **148**:606.
18. **Libby, J. M., B. S. Jortner, and T. D. Wilkins.** 1982. Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. Infect. Immun. **36**:822–829.
19. **Lonnroth, I., and S. Lange.** 1983. Toxin A of *Clostridium difficile*: production, purification and effect in mouse intestine. Acta Pathol. Microbiol. Immunol. Scand. **91**:395–400.
20. **Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins.** 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1147–1150.
21. **Lyerly, D. M., N. M. Sullivan, and T. D. Wilkins.** 1983. Enzyme-linked immunosorbent assay for *Clostridium difficile* toxin A. J. Clin. Microbiol. **17**:72–78.
22. **Onderdonk, A. B., R. L. Cisneros, and J. G. Bartlett.** 1980. *Clostridium difficile* in gnotobiotic mice. Infect. Immun. **28**:277–282.
23. **Pierce, N. F., W. C. Cray, Jr., and J. B. Sacci, Jr.** 1982. Oral immunization of dogs with cholera toxin, crude cholera toxin, or B subunit: evidence for synergistic protection by antitoxic and antibacterial mechanisms. Infect. Immun. **37**:687–694.
24. **Price, A. B., H. E. Larson, and J. Crow.** 1979. Morphology of experimental antibiotic-associated enterocolitis in the hamster: a model for human pseudomembranous colitis and antibiotic-associated diarrhoea. Gut **20**:467–475.
25. **Rampling, A., R. E. Warren, and H. V. Sykes.** 1980. Relapse of *Clostridium* colitis after vancomycin therapy. J. Antimicrob. Chemother. **6**:551–552.
26. **Rehg, J. E., and Y. Lu.** 1982. *Clostridium difficile* typhlitis in hamsters not associated with antibiotic therapy. J. Am. Vet. Med. Assoc. **181**:1422.
27. **Rehg, J. E., and S. P. Pakes.** 1982. Implication of *Clostridium difficile* and *Clostridium perfringens* iota toxins in experimental lincomycin-associated colitis of rabbits. Lab. Anim. Sci. **32**:253–257.
28. **Rietra, P. J. G. M., K. W. Slaterus, H. C. Zanen, and S. G. M. Meuwissen.** 1978. Clostridial toxin in faeces of healthy infants. Lancet **ii**:319.
29. **Sterne, M., and L. M. Wentzel.** 1950. A new method for the large scale production of high-titre botulinum Formol-toxoid types C and D. J. Immunol. **65**:175–183.
30. **Sullivan, N. M., S. Pellet, and T. D. Wilkins.** 1982. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun. **35**:1032–1040.
31. **Taylor, N. S., G. M. Thorne, and J. G. Bartlett.** 1981. Comparison of two toxins produced by *Clostridium difficile*. Infect. Immun. **34**:1036–1043.