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Effects of the Two Toxins of *Clostridium difficile* in Antibiotic-Associated Cecitis in Hamsters

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Hamsters were vaccinated with toxoids containing toxin A, toxin B, both toxins, or a preparation containing neither toxin of *Clostridium difficile*, the causative agent of antibiotic-associated cecitis in hamsters and pseudomembranous colitis in humans. To determine whether these vaccines would reduce the severity of antibiotic-associated cecitis, the hamsters were injected subcutaneously with clindamycin. Nearly all of the hamsters protected against neither toxin or only one toxin died. These animals developed enlarged hemorrhagic ceca and diarrhea, although the ceca from the animals immunized against toxin B were less hemorrhagic. The hamsters immunized against both toxins survived clindamycin treatment and had ceca of normal size and appearance. Concentrations of both toxins were lower in the ceca of the latter animals than in the unprotected animals. To determine the effects of either toxin alone on the animals, nonimmunized hamsters were injected with either purified toxin A, which produced enlarged ceca with moderate hemorrhaging, or partially purified toxin B, which produced hemorrhagic ceca of normal size. All of the hamsters injected with either toxin at concentrations found in the ceca after clindamycin treatment died. These results suggest that toxin A causes the water influx, that both toxins cause hemorrhaging to different extents in the ceca of hamsters with antibiotic-associated cecitis and that either toxin alone can cause death. These studies may help explain the etiology of pseudomembranous colitis in humans.

Clostridium difficile has been reported to cause pseudomembranous colitis in humans (3, 10, 14) and antibiotic-associated cecitis (AAC) in hamsters (19). Both the hamster and human diseases are characterized by similar intestinal lesions with pseudomembrane formation (5, 11a, 16). There are at least two means of protecting against the effects of *C. difficile*. Vancomycin or other antibiotics will inhibit the growth of *C. difficile* and prevent or eliminate these symptoms in hamsters (4) and humans (12, 23). Passive immunization with the U.S. Bureau of Biologics *C. sordellii* antitoxin, a cross-reacting preparation which neutralizes *C. difficile* toxins, will protect hamsters from the lethal consequences of antibiotic treatment (1), but this has not been attempted in humans.

Bartlett et al. and Taylor have recently reported that two distinct toxins are produced by *C. difficile* (6, 22) instead of the single toxin previously described by several investigators (13, 18, 21). The evidence for this conclusion is the separation of two activities by DEAE Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) ion-exchange chromatography. The first toxin, toxin A, elutes at a lower salt concentration than does the second toxin, toxin B, and has been reported to be 17 times more lethal to mice but much less cytotoxic than toxin B (22). Antiserum to toxin A prepared by these investigators has failed to neutralize toxin B, demonstrating a significant difference between the toxins. Libby and Wilkins also have demonstrated that the two toxins of *C. difficile* are antigenically distinct (15).

We do not now know whether one or both of these toxins are responsible for the symptoms of pseudomembranous colitis in humans. However, we recently produced specific antitoxins for toxins A and B (15) and used this immunization procedure to study the role of these toxins in AAC of hamsters. This report describes the active immunization of hamsters against either or both toxins of *C. difficile* and the relative protective effect of these immunizations. We also studied the effects of toxin A or B preparations on hamsters after intracecal injection to determine the effects caused by these proteins and the roles they may play in AAC.

MATERIALS AND METHODS

Preparation of toxins A and B. *C. difficile* VPI 10463 was grown at 37°C for 48 h inside dialysis tubing containing 100 ml of saline suspended in 2 liters of

brain heart infusion broth (7). The contents of the dialysis tubing were harvested by centrifugation at 8,000 × *g* for 10 min, and the culture fluid was filtered through a 0.45-micrometer membrane filter. This culture filtrate was concentrated to 5.0 ml with an XM-100 filter in a stirred-cell ultrafiltrator (Amicon Corp., Lexington, Mass.) and then diluted to 50.0 ml with 0.05 M Tris-hydrochloride, pH 7.5. The concentration and dilution steps were repeated twice and the final retentate (5 ml) was applied to a DEAE Sepharose CL-6B ion-exchange column (2.5 by 8.0 cm) equilibrated with 0.05 M Tris-hydrochloride, pH 7.5. With two gradients, toxin A was eluted at a concentration of 0.14 to 0.16 M NaCl, and toxin B was eluted at a concentration of 0.38 to 0.42 NaCl (20). This chromatographic procedure completely separated the two toxins; rabbit antitoxins specific for either toxin completely neutralized the cytotoxic activity of the homologous preparations, and neither toxin preparation given to rabbits elicited an antibody response to the heterologous toxin (15). The partially purified toxin A preparation contained 1.6 mg of protein per ml, and the partially purified toxin B preparation contained 0.6 mg of protein per ml. These preparations were used for all of the toxoids produced for hamsters.

Preparation of toxoids. Previous attempts to produce antitoxin to toxin B with Formalin-treated, partially purified toxin B have failed, although antibodies to toxin B are present in the antitoxin made in animals injected with a toxoid made from a crude culture filtrate containing toxin A, toxin B, and other *C. difficile* proteins (7). This latter observation suggested that a Formalin-treated culture filtrate of *C. difficile* containing toxin B but without toxin A might make a suitable toxoid. Therefore, toxoid B was prepared by the addition of 1.0 ml (0.6 mg of protein) of the partially purified toxin B preparation that had been concentrated 10 times with a Minicon B-125 (Amicon) to 9.0 ml of a dialysis culture filtrate of *C. difficile* VPI 2037, a nontoxigenic strain of *C. difficile*. Toxoid A was prepared in the same manner, with partially purified toxin A (0.35 mg of protein) concentrated 10-fold to 1.0 ml. Toxoid AB was prepared by the addition of 1.0 ml of 10-fold-concentrated toxin A preparation and 1.0 ml of the 10-fold-concentrated toxin B preparation to 8.0 ml of the *C. difficile* 2037 culture filtrate that had been concentrated from 9.0 ml with a Minicon B-15 concentrator. Formaldehyde-treated, nontoxigenic filtrate (NTF) was made by the addition of 1.0 ml of 0.05 M Tris-hydrochloride (pH 7.5) solution to 9.0 ml of *C. difficile* VPI 2037 culture filtrate. To each preparation 0.1 ml of 40% (vol/vol) formaldehyde (Fisher Scientific Co., Fair Lawn, N.J.) was added, and the preparations were then incubated overnight at 37°C. Each of the toxoids contained 6 mg of protein from the *C. difficile* VPI 2037 filtrate. The concentration of either toxin A or toxin B in the toxoids containing them were the same as the concentrations of both toxins in the toxigenic culture filtrate (*C. difficile* VPI 10463) as determined by the cytotoxicity assay.

Cytotoxicity assay. The toxicity for tissue culture cells was determined from the percentage of cells (Chinese hamster ovary, CHO-K1) that became round when exposed to toxin A or B. CHO-K1 cells were grown and the titers were determined as previously described (2, 7).

Immunization of hamsters. Male golden Syrian hamsters, 60 to 70 g (Charles River Breeding Laboratories, Wilmington, Mass.) were vaccinated once per week for 3 weeks by anguinal subcutaneous injections with 0.2 ml of a 1:1 mixture of Freund complete adjuvant (Miles Laboratories, Inc., Elkhart, Ind.) and toxoid. Each of the four toxoid mixtures was administered to 7 hamsters housed individually in one experiment and 10 hamsters housed in groups of 5 to a cage in a second experiment. After week 3 the animals were vaccinated with a 1:1 mixture of Freund incomplete adjuvant and toxoid on a weekly basis.

Testing of sera for antibodies to toxins A and B. In the first experiment, two hamsters from each group were bled from the orbital sinus with nonheparinized capillary tubes 3 days after the eighth injection. In the second experiment, the animals were bled, and their sera were tested after injections 8 and 10 since the sera titers were deemed insufficient after week 8. The sera from each animal were tested for the ability to neutralize both toxins A and B with the following neutralization assay. Sera were diluted in twofold series with 0.05 M Tris-hydrochloride (pH 7.5) and then mixed 1:1 with either toxin which had been diluted to a concentration 20-fold greater than the greatest dilution of toxin that would cause rounding of 100% of the CHO-K1 cells in a microtiter well. After incubation at room temperature for 1 h, each mixture was added to a final concentration of 10% to a microtiter well containing CHO-K1 cells. The titer was defined as the greatest dilution of serum which completely inhibited rounding of the cells.

Antibiotic-associated cecitis in hamsters. Two days after injection 9 of toxoid in the first experiment and 2 days after injection 10 of toxoid in the second experiment, all of the hamsters were administered clindamycin phosphate (The Upjohn Co., Kalamazoo, Mich.; 10 mg/kg of body weight) by intrascapular subcutaneous injection (16). The animals at this point weighed between 110 and 170 g each. The day of clindamycin treatment was designated as day 0, and all of the animals were observed for signs of distress, diarrhea, or death through day 14.

Enumeration of *C. difficile* in cecal contents. The cecal contents of six animals from the toxoid A, toxoid B, and NTF groups and three hamsters from the toxoid AB group were tested for the presence of *C. difficile* within 1 h of death. Cecal contents were diluted in anaerobic dilution medium (11) and then plated on cycloserine cefoxitin fructose agar (9). *C. difficile* colonies were identified by colony morphology and by the ability to produce a cytotoxin which could be neutralized by *C. difficile* antitoxin (7).

Testing for toxins in cecal contents. Cecal contents from seven animals of the toxoid A, B, and NTF groups and the three sacrificed animals from the toxoid AB group were centrifuged at 8,000 × *g* for 10 min, the supernatant fluids were filtered through 0.45-micrometer membrane filters, and 0.75 ml of each filtrate was applied to ion-exchange columns containing 4.0 ml of DEAE Sepharose CL-6B. The columns were then washed with 0.25 M NaCl and 0.45 M NaCl to elute any bound toxin A and B, respectively (N. Sullivan, personal communication). The fractions were tested for cytotoxicity, and the toxic activities were identified by neutralization with antitoxins specific for toxin A or B (15).

Testing for antibodies in cecal contents. Six hamsters from the toxoid AB group were given final injections of toxoid 4 weeks after clindamycin treatment; 4 days later the animals were bled from the orbital sinuses and each of the sera was tested for the ability to neutralize the cytotoxic effect of toxin A or B. On the fifth day after the last toxoid treatment, the animals were sacrificed and the cecal contents were removed and pooled. The mucus lining of each cecum was scraped and added to the pooled cecal contents. The pooled material (9.0 ml) was added to 4.5 ml of 0.05 M Tris-hydrochloride (pH 7.5), and the mixture was centrifuged at 8,000 × *g* for 10 min. The supernatant was filtered through a 0.45-micrometer membrane filter, and the filtrate was tested for the ability to neutralize toxin A or B in the same manner used to test the sera.

Intracecal injections. Hamsters were anesthetized with sodium pentobarbital, small abdominal incisions were made, and 1 ml of toxin preparation was injected into each cecum with a 26-gauge needle (5). Incisions were closed with wound clips, and the animals were kept at 37°C for 1 h before being returned to their cages. The toxin A preparation (0.35 mg of protein per ml) for intracecal injection was prepared with purified toxin A diluted 1:10 with 0.05 M Tris-hydrochloride (pH 7.5), to which had been added 0.01 ml of toxin B antitoxin (enough rabbit antitoxin to neutralize 10 mouse lethal doses of toxin B; 15) per 1-ml sample. Pure toxin A was obtained courtesy of N. Sullivan and S. Pellett and was prepared by DEAE chromatography and acetic acid precipitation (20). The purity of this preparation was determined by polyacrylamide gradient gel electrophoresis and crossed immunoelectrophoresis. The toxin B antiserum was added to ensure that any trace of toxin B present in the toxin A preparation would be neutralized and have no effect on the animals, even though we have not detected the presence of toxin B in this preparation. The toxin B preparation (0.6 mg of protein per ml) for intracecal injection was prepared with partially purified toxin B prepared in the same manner as that used for toxoid B production and diluted 1:10 with 0.05 M Tris-hydrochloride (pH 7.5) and 0.01 ml of toxin A antitoxin (enough rabbit antitoxin to neutralize 20 mouse lethal doses; 15) per 1-ml sample. Toxin A was not detected in the toxin B preparation. The preparations were incubated 1 h at room temperature before being used. The cytotoxic titers of the toxin A and B preparations were 10^3 and 10^6, respectively, at the time of injection. This experiment was performed three times with a total of 16 hamsters per group. Tenfold or greater dilutions of the toxin preparations were also administered intracecally to hamsters, six animals per dilution. A control group of 10 animals received 1 ml of 0.05 M Tris-hydrochloride (pH 7.5) and 0.01 ml of toxin A antitoxin. The ceca of animals that died within 48 h after injections were removed, weighed, and tested for the presence of toxin with the cytotoxicity assay. Control animals that received buffer were sacrificed at 8, 12, 24, 30, and 48 h after injection (two hamsters per time period), and their cecal contents were tested for cytotoxicity.

Histological examinations. Sections of the ceca of three hamsters of each of the immunized groups and six animals of each group injected intracecally with toxin A, B, both toxins, or buffer and the ceca of three hamsters that died 3 to 4 days after clindamycin treatment were processed to be embedded in paraffin. The ceca were fixed in 10% neutral buffered Formalin, cut in 6-μm cross sections, and stained with hematoxylin and eosin.

RESULTS

Prevention of cecitis in hamsters. Hamsters immunized with NTF which contained the extracellular proteins produced by a nontoxigenic strain of *C. difficile* did not produce antibody to either toxin A or B as determined by the neutralization assay (Table 1). The addition of either toxin A or B to this culture filtrate succeeded by the addition of Formalin resulted in toxoids that elicited specific antibody responses to the toxins. Hamsters receiving toxoid A produced antibody to toxin A but no detectable antibody to toxin B, whereas toxoid B elicited the opposite antibody response. Hamsters immunized with toxoid AB produced antibodies to both toxins.

The average time of death of the NTF-immunized hamsters occurred 3.4 days after the clindamycin treatment, and nearly all (16 of 17) of these animals died (Table 2). Most (13 of 17) of the hamsters immunized with either toxoid A or B died. The average time of death of both the toxoid A and B animals was 4.3 days after clindamycin treatment. Necropsies performed on the NTF and toxoid A groups revealed enlarged ceca with watery contents, intralumenal collections of bloody fluid, and hemorrhages into the bowel mucosa. The small and large intestines were also hemorrhagic. The toxoid B animals had enlarged ceca with watery contents, but less hemorrhaging of the ceca and intestines was observed with this group than with the toxoid A and NTF groups (Fig. 1). None of the animals in the toxoid AB group died, and most looked healthy and normal throughout the

TABLE 1. Titers[a] of sera from immunized hamsters

Titer against:	Titers of sera from hamsters immunized with:			
	Toxoid A	Toxoid B	Toxoid AB	NTF
Toxin A	10,240–20,480	<20[b]	10,240	<20[b]
Toxin B	<20[b]	20,480	10,240–20,480	<20[b]

[a] Titers are reported as the reciprocal of the highest dilution neutralizing toxin.
[b] Limit of assay: no neutralization detected.

TABLE 2. Deaths among immunized hamsters after clindamycin treatment[a]

Hamsters immunized with:	No. of deaths after clindamycin injection								
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Ratio of no. of deaths to no. of hamsters
Toxoid A	0	1	3	6	0	0	3	0	13/17
Toxoid B	0	2	5	1	2	0	2	1	13/17
Toxoid AB[b]	0	0	0	0	0	0	0	0	0/17
NTF	0	3	8	2	0	2	0	1	16/17

[a] Combined data from two experiments.

[b] One hamster from the toxoid AB group was sacrificed on day 4, one was sacrificed on day 6, and one was sacrificed on day 8 and examined for cecitis.

course of the experiment, although one had diarrhea on day 3 and another had it on day 4. This was in contrast to the animals of the other groups, almost all of which developed diarrhea. The necropsies performed on the toxoid AB animals revealed gastrointestinal tracts of normal appearance (Fig. 2).

Presence of *C. difficile* in cecal contents. All of the animals which were given clindamycin and which were tested had *C. difficile* in their ceca. No significant differences were observed between the four toxoid groups; all of the ceca tested had between 4×10^7 and 8×10^7 *C. difficile* cells per ml of cecal contents. Further examination of the cultures isolated from different animals revealed no detectable differences in colony morphologies or amounts of toxins produced.

Presence of toxins in cecal contents. Both toxins A and B were present in the cecal filtrates of the NTF group; cytotoxic activity eluted from the ion-exchange column in both steps of the salt gradient. The activity after elution with 0.25 M NaCl was due to toxin A since it was neutralized with antitoxin to toxin A but not with antitoxin to toxin B. The second activity, which appeared after elution from the column with 0.45 M NaCl, was neutralized by toxin B antitoxin but not by toxin A antitoxin. The animals in the toxoid A group were shown to have a high toxin B activity but no detectable toxin A activity in their cecal filtrates. Hamsters in the toxoid B group had the same large amount of toxin A present in their cecal filtrates did as the NTF group, but 100-fold less toxin B than either the NTF or toxoid A group. Animals receiving toxoid AB had reduced levels of both toxins in their cecal filtrates (Table 3).

Testing for antibodies in cecal contents. Although the animals at the time of this study had high serum titers against both toxins, the pooled and filtered cecal contents did not neutralize either toxin to a detectable extent at any dilution tested.

Intracecal injections. Hamsters were injected with either purified toxin A or partially purified toxin B to determine whether the toxins produced tissue damage similar to that observed in

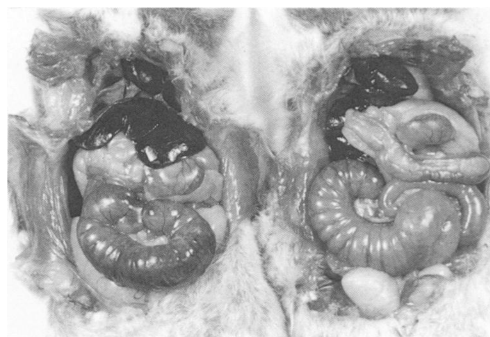

FIG. 1. Appearance of hamster ceca after clindamycin treatment and onset of cecitis. The animal on the left was immunized with toxoid A but died 4 days after administration of the antibiotic. The autopsy revealed an enlarged hemorrhagic cecum. Of 17 hamsters receiving NTF, 16 died and had ceca of identical appearances. The animal on the right was immunized with toxoid B but died 4 days after clindamycin treatment. Note that the cecum is enlarged but not very hemorrhagic.

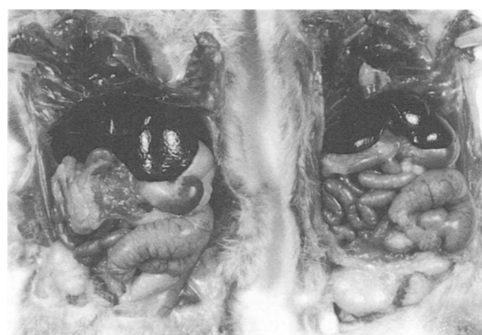

FIG. 2. Comparison of ceca from an untreated hamster and a hamster immunized with toxoid AB. The animal on the right demonstrated an antibody response to both toxins after an immunization program with toxoid AB. The cecum is of normal size and appearance, similar to that of the untreated animal seen on the left.

TABLE 3. Cytotoxic activity of hamster cecal contents after elution from DEAE Sepharose CL-6B[a]

Elution with:	Titer of cecal contents from hamsters receiving:			
	Toxoid A	Toxoid B	Toxoid AB	NTF
0.25 M NaCl	ND[b]	10^3	ND	10^3
0.45 M NaCl	10^6	10^4	10^4	10^6

[a] Data pooled from two experiments; no significant difference between experiments.
[b] ND, No cytotoxicity detected.

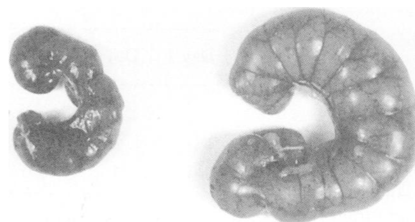

FIG. 3. Ceca of animals injected intracecally with either partially purified toxin B (left) or purified toxin A (right). Both hamsters died 8 h postinjection. The weight of the cecum from the animal receiving toxin A was twice that of the normal-sized cecum on the left from a hamster that received toxin B. The cecum on the left shows a hemorrhagic response typically seen after administration of toxin B. Toxin A also caused a hemorrhagic response, but to a lesser extent.

hamsters with AAC. The group challenged with the highest concentration of toxin A (0.035 mg of protein per hamster) and the group receiving the highest concentration of toxin B (0.06 mg of protein per hamster) all died within 8 to 14 and 8 to 36 h postinjection, respectively, whereas the animals injected with greater dilutions of either toxin did not die. The animals given toxoid AB had 100-fold less toxin B in their cecal contents after clindamycin treatment than did the animals vaccinated with NTF. This toxin B activity was determined to be 100-fold less than the minimum lethal dose by the intracecal injection experiment. In contrast to the immunization experiment, in which the animals with high intracecal concentrations of either toxin A or B had enlarged ceca, the only animals with enlarged ceca after direct intracecal injection of either toxin were the animals receiving toxin A. The average weight of the ceca from this group was 3.5 ± 0.6 g (mean weight $\pm$ standard deviation) whereas the ceca from the animals injected with toxin B averaged 1.9 ± 0.6 g; this was not significantly different from animals injected only with buffer whose ceca averaged 1.8 ± 0.5 g. The ceca from the toxin A group were moderately hemorrhagic, whereas those that had been injected with toxin B were very hemorrhagic (Fig. 3). The cecal contents of the animals injected with toxin A were liquid, whereas those of the animals receiving toxin B or buffer were not. After the death of animals injected with toxin A, the toxin was detected in the pooled cecal contents at a titer of 10^3. The titer injected was also determined to be 10^3 before a ca. threefold dilution with the cecal contents. This was due to the inability of the cytotoxicity assay to resolve less than a 10-fold difference in toxin concentration. However, the animals injected intracecally with toxin B had an average titer of 10^5 in the ceca after death (titer injected was 10^6). The cecal contents of the animals injected with the suspending buffer was 10^1, a titer often seen with the cecal contents of untreated hamsters, and as in this case, the activity was not due to a toxin of *C. difficile* since this activity was not neutralized by *C. difficile* antitoxins.

Histological examinations. The ceca from the animals immunized with toxoid A, toxoid B, or NTF demonstrated a variety of severe lesions. Among the different groups a number of common features were observed, including hemorrhage, focal necrosis of the surface epithelium with underlying congestion, and leukocytic infiltration of the lamina propria and focal aggregates of neutrophils. The severity of the lesions varied among all of the specimens examined, and in this experiment we could not associate specific histological changes observed with either toxin. The ceca from the animals immunized with toxoid AB, however, demonstrated none of these lesions and appeared normal (Fig. 4).

Injections of the minimum lethal dose of toxin

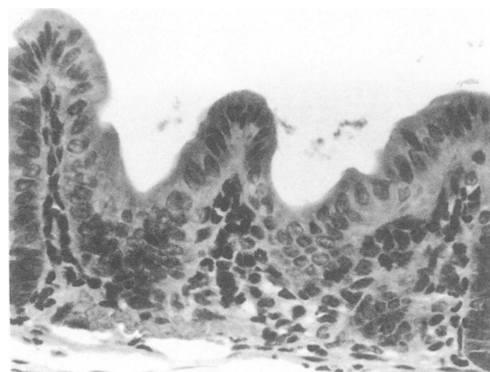

FIG. 4. Mucosa of cecum from a toxoid AB hamster 4 days after clindamycin treatment. The mucosa is of normal appearance. Hematoxylin and eosin; × 256.

A into the ceca of untreated hamsters produced rapidly progressing mucosal necrosis, primarily affecting epithelium. The lesions appeared to begin as multifocal necrosis of superficial epithelium, which rapidly (within 4 to 8 h) progressed to diffuse involvement of these cells with extension into the glands of Lieberkuhn (Fig. 5). Although there was associated laminal proprial and submucosal acute inflammation, congestion, and hemorrhage, the epithelial necrosis was the predominant lesion. Bacterial masses, polymorphonuclear leukocytes, and necrotic cells were noted on the mucosal surface.

Eight hours after intracecal administration of a minimum lethal dose of toxin B, multiple regions of epithelial cell necrosis with associated prominant polymorphonuclear leukocytic infiltration, congestion, and hemorrhage were noted. The inflammatory and vascular reactions often extended to the submucosa. Although these necrotic, inflamed lesions were multifocal, intervening mucosa sometimes had milder, diffuse, inflammatory and degenerative changes. Thirty hours after toxin B administration the necrotic, inflamed lesions were more extensive but still maintained a multifocal pattern (Fig. 6). The predominant features of this (toxin B) model were the multifocality and intensity of associated inflammation of the necrosis.

DISCUSSION

A recent report has described the passive immunization of hamsters with U.S. Bureau of Biologics *C. sordellii* antitoxin and the protective effect of this preparation against *C. difficile*-mediated AAC (1). This suggested to us that

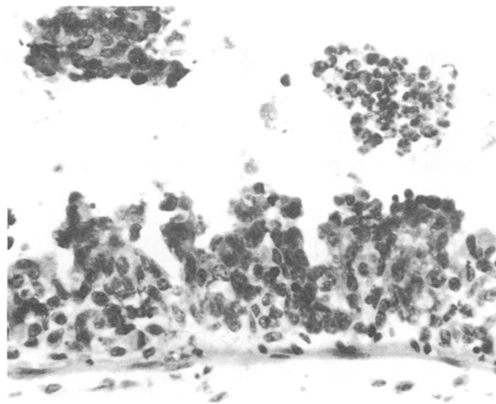

FIG. 5. Mucosa of cecum with extensive necrosis of epithelium 8 h after administration of toxin A. Small clumps of necrotic cells and polymorphonuclear leukocytes are present in the lumen. Hematoxylin and eosin; × 256.

FIG. 6. Mucosa and submucosa of cecum 30 h after administration of toxin B. There is epithelial necrosis with an extensive inflammatory reaction in the mucosa. Hematoxylin and eosin; × 102.

actively immunizing hamsters by injection of toxoids made with either or both toxins of *C. difficile* might result in protection from AAC and thus allow us to ascertain which toxin causes which symptoms of the disease. We produced *C. difficile* antitoxin containing antibodies to both toxins (7) but failed to produce antitoxin specific for toxin B until we added a crude culture filtrate of a nontoxigenic strain (*C. difficile* VPI 2037; 15). More recent evidence suggests that antibody production to toxin B may be affected in a nonspecific fashion by an increase of the total protein content of this preparation by the addition of *C. difficile* VPI 2037 culture filtrate, which thus minimizes the effect of formaldehyde on toxin B. By incorporating *C. difficile* VPI 2037 culture filtrate in all of the hamster toxoids, we minimized differences between these preparations in the concentration of nontoxin proteins and thus essentially limited the differences among the groups of immunized hamsters to the presence or absence of antibodies to toxin A or B.

An analysis of the sera of hamsters chosen from the four groups of immunized animals indicated that we had successfully immunized these animals in a specific fashion against one or both of the toxins of *C. difficile*, except in the case of the NTF group, which did not receive toxins and thus did not produce antibodies to them. We next presented the hamsters with a challenge likely to be lethal to untreated animals: clindamycin therapy. The fact that hamsters vaccinated with either toxoid A or B produced high titers of antibodies to one of the toxins but were not protected against the lethal consequences of antibiotic treatment indicates

that both toxins are involved in the pathology of AAC. The cecal contents of these animals contained concentrations of the toxin not used for immunization that were greater than the minimum lethal level determined from the intracecal injection experiment. The two toxins together seem to cause all of the disease symptoms since hamsters that were injected with a toxoid vaccine made from both toxins (toxoid AB) were completely protected because of the reduction of toxin A and B activity in the ceca.

Presumably, both toxins were produced by the bacteria in all of these hamsters in sufficient concentrations to cause death since *C. difficile* was detected in the same numbers in the cecal contents of all four groups of immunized hamsters after clindamycin treatment. However, the toxins were neutralized by the immune response of the animals, depending on the toxoid treatment given. The sites of neutralization within the animals have not been determined. The cecal contents from the toxoid AB group did not possess the ability to neutralize either of the toxins. The sites of neutralization in vivo may not be the cecal lumen; other studies (8) have demonstrated that the neutralization of cholera toxin may occur near the lamina propria, which produces immunoglobulin A capable of binding this toxin. Intestinal antibodies of rats immunized against cholera toxin are known to interfere with toxin attachment to the intestinal microvillus surface of the rats (24). A similar mechanism may have protected the hamsters immunized against one of both of the toxins of *C. difficile*.

The toxoid A hamsters had more hemorrhaging than did the toxoid B hamsters, suggesting that the effects of the two toxins on hamster ceca may be different and that both toxins may act together to produce the symptoms associated with AAC. The separate effects of these toxins were verified with the intracecal injection experiments, in which it was demonstrated that toxin A caused enlargement of the ceca and moderate hemorrhaging, whereas toxin B did not cause cecal enlargement but caused extensive hemorrhaging. We cannot say with certainty that toxin B, instead of another of the *C. difficile* proteins present in this preparation, caused the hemorrhaging, but this seems likely; culture filtrates of nontoxigenic strains do not cause death or hemorrhaging in animals (unpublished data). Both the intracecal injections and the immunization experiments suggest that toxin A causes a water influx response. However, the results of these experiments regarding the effect of toxin B on water influx appear at odds: the toxoid A animals, which had toxin B but no detectable toxin A present in the ceca, had enlarged ceca, but the animals injected intracecally with toxin B did not develop enlarged ceca. It is possible that a small undetected amount of toxin A was present in the ceca of the animals immunized against this toxin and caused cecal enlargement. This idea is supported by the fact that of the two toxins, only toxin A produces a positive response in the rabbit ileal loop assay and hence is alone responsible for water influx in this model (6, 17).

Although a variety of lesions were found in the ceca of animals immunized with toxoid A, toxoid B, or NTF, we could not associate these lesions with the effects of a particular toxin since specimens from the different groups occasionally possessed common features. However, the ceca from the animals immunized against both toxins appeared normal. This suggests that a synergistic effect may be taking place; the effect of either toxin on ceca may be enhanced by the presence of the other toxin at high (lethal) levels.

The predominant lesion induced by intracecal injection of a minimum lethal dose of toxin A (the amount of toxin A found in hamster ceca after clindamycin treatment) was diffuse epithelial necrosis of rapid onset and progression. In contrast, a minimum lethal dose of toxin B (the amount of toxin B found in hamster ceca after clindamycin treatment) elicited multifocal necrotic, inflammatory lesions. These appeared to progress at a slower rate, which may have allowed for the more intense inflammatory reaction.

Some of the effects of the toxins in hamsters are similar to those seen in humans with *C. difficile*-mediated colitis (5, 11a, 16), and these similarities may extend to the causes of these diseases; the lethal concentration of toxin B present in hamster ceca after clindamycin treatment is similar to the concentration of toxin B found in the feces of some patients with pseudomembranous colitis (unpublished data). The concentration of toxin A in human feces has not been determined.

The findings presented here suggest that both toxins are involved in the pathogenesis of *C. difficile*-mediated cecitis in hamsters and that the presence of either toxin in hamster ceca can cause death. Only by protection against both toxins are the animals certain to be spared the lethal consequences of clindamycin treatment. These data suggest the possibility of a similar relationship between the two toxins of *C. difficile* and the symptoms observed in humans with pseudomembranous colitis.

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