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Molecular, Immunological, and Biological Characterization of a Toxin A-Negative, Toxin B-Positive Strain of *Clostridium difficile*

S. P. BORRIELLO,[1]* B. W. WREN,[2] S. HYDE,[1] S. V. SEDDON,[1] P. SIBBONS,[3] M. M. KRISHNA,[1] S. TABAQCHALI,[2] S. MANEK,[4] AND A. B. PRICE[4]

Microbial Pathogenicity Research Group, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ,[1] Department of Medical Microbiology, St Bartholomew's Hospital Medical College, West Smithfield, London EC1A 7BE,[2] Department of Paediatric Surgery, Institute of Child Health, London WC1N 1EH,[3] and Department of Histopathology, Northwick Park Hospital, Harrow, Middlesex HA1 3UJ,[4] United Kingdom

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A cytotoxigenic *Clostridium difficile* strain that fails to produce toxin A but causes hemorrhage and bloody fluid accumulation in ligated ileal loops of rabbits and hemorrhage and diarrhea in hamsters is described. The lack of reaction of DNA from this strain in hybridization studies with a toxin A gene-specific 4.5-kb probe and polymerase chain reaction studies with six toxin A-specific primers indicate the absence of the toxin A gene. The cytotoxin produced by this strain was not responsible for the enterotoxic or hemorrhagic activity and shared characteristics with toxin B, i.e., its cytotoxicity was neutralized by antibodies to toxigenic strains of *C. difficile* and *Clostridium sordellii*. Polymerase chain reaction studies with toxin B-specific primers showed that the DNA from this strain produced a 690-bp product in addition to the expected 591-bp product.

The role of toxigenic strains of *Clostridium difficile* in antibiotic-associated diarrhea and colitis is now well established (3, 20). The relative roles of the two individual major protein toxins, toxins A and B, in the disease process have attracted a great deal of attention (6, 18, 19, 21, 24, 25, 33). The overall conclusion is that the gastrointestinal disease features can be reproduced by toxin A alone. However, a general limitation of these studies is that they have relied on the use of purified toxin administered as a single challenge either orally, directly into the cecum, or into ileal or colonic loops. It would be of interest to know the effects of either of these toxins, particularly toxin B, which generally appears to have little effect in an intact gut, when produced continually in the gut in the presence of other factors such as proteolytic enzymes (28, 29) or other putative toxins (1, 11, 31) produced by *C. difficile*. These sorts of studies require a cytotoxic strain of *C. difficile* that fails to produce toxin A. Further, the activity of any such additional enterotoxic factor(s) would be more apparent after infection with such a strain. In this report we characterize a natural strain of *C. difficile* that is cytotoxigenic but fails to produce toxin A (12, 32).

MATERIALS AND METHODS

Source of *C. difficile* and *Clostridium sordellii*. The following strains of *C. difficile* were used: a fully toxigenic virulent strain (B-1) isolated from a patient with antibiotic-associated pseudomembranous colitis and a nontoxigenic strain (M-1), both described in detail elsewhere (6); a toxigenic strain (VPI 10463) and a cytotoxigenic strain that fails to produce toxin A (strain 8864) (12, 32), provided by D. W. Burdon (The General Hospital, Birmingham, United Kingdom); and strains of *C. difficile* types B, D, E, W, X, and Z (all toxigenic) and types A, C, and Y (nontoxigenic), typed according to their [^{35}S]methionine-labelled sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profiles (30). *C. sordellii* 3703 (36) was provided by S. Nakamura (Kanazawa University, Japan).

Culture of *C. difficile* 8864 for toxin A determinations. To determine whether strain 8864 produced toxin A, it was cultured in brain heart infusion (BHI) broth (Oxoid) both in conventional batch culture and by the dialysis bag method as a means of increasing the yield of toxins (13, 17). The culture filtrates were examined for the presence of toxin A by using a commercial immunoassay (see below).

Preparation of inocula for hamster experiments. The total 48-h growth on a semiconfluent Columbia blood agar (CBA) plate (Difco Laboratories, Surrey, United Kingdom) was harvested and suspended in 10 ml of BHI broth containing 0.05% (wt/vol) L-cysteine HCl and 0.03% sodium formaldehyde sulfoxylate for reduction. For both of the *C. difficile* strains used in the animal experiments (B-1 and 8864), this consistently yielded ca. 10^6 CFU/ml.

Hamster experimental groups. Adult female Syrian hamsters (*Mesocricetus auratus*) supplied from the National Institute for Medical Research (Mill Hill, United Kingdom) were used in all experiments. Each received intraperitoneally 0.5 ml of a 10-mg/ml solution of clindamycin phosphate (Dalacin C; Upjohn, Crawley, West Sussex, United Kingdom). The hamsters were housed individually in filter-lidded isolater cages (Techniplast animal cages; Biotech Consultants Ltd., Clackmannanshire, Scotland, United Kingdom) and given food and water ad libitum. All but one animal received 0.5 ml of strain 8864 (toxin A negative) or strain B-1 (fully toxigenic) perorally. One animal received only clindamycin and was sacrificed 10 days later to provide negative control material. One group of 10 hamsters and two groups of 3 hamsters received strain 8864 5, 8, and 10 days after administration of clindamycin, respectively. All of the strain

* Corresponding author.

8864-challenged hamsters were handled and housed in a room where no previous experiments with *C. difficile* had been performed. One group of three hamsters received strain B-1 5 days after the administration of clindamycin. Pellets were collected daily from animals directly into sterile plastic universal containers. Moribund animals were killed under CO_2. In some cases animals that had wet tail (diarrhea) but were not moribund were killed, since it was noted that progression from wet tail to death was rapid. The whole of the gut was removed from killed animals, the gross pathology was recorded, and the contents were removed after full-length incision and gentle washing by immersion in 10% Formol–saline. The gut tissue was then fixed in 10% Formol–saline in preparation for histological examination as described in detail by Price et al. (26).

Isolation of *C. difficile*. Pellets and cecal contents for culture were prepared as 10% emulsions in BHI broth, serial 10-fold dilutions were prepared in the same broth, and 10-μl aliquots were seeded onto a cycloserine-cefoxitin selective medium (6) by a micromethod detailed previously (4). In addition, 100 μl of the 10% emulsion was seeded onto the selective medium to facilitate detection of small numbers of *C. difficile* cells. After the seeded plates were incubated anaerobically for 48 h at 37°C in an atmosphere of 10% CO_2–10% H_2–80% N_2 in an anaerobe cabinet (Forma Scientific, Marietta, Ohio), colonies of *C. difficile* were detected by their characteristic colonial morphology and fluorescence under long-wave UV light (5, 10).

Toxin assays. Cecal contents or fecal pellets were diluted 10-fold in BHI broth and filtered (0.45-μm-pore-size filter), and 100-μl samples of serial 10-fold dilutions were applied to monolayers of African Green Monkey kidney (Vero) cells in the wells of microtitration trays for the detection of cytotoxicity. The level of cytotoxin was defined as the last dilution causing a cytopathic effect in 25 to 50% of the cell sheet and was recorded as the $\log_{10}$ value of the reciprocal of that dilution. For the specific detection of toxin A, a commercial enzyme immunoassay (Premier; Meridian diagnostics Inc.) was used. Filtrates of *C. difficile* 8864 cultured in BHI broth were similarly assayed. In addition, the ability of culture filtrates to hemagglutinate rabbit erythrocytes at 4°C as a measure of toxin A was tested as described by Krivan et al. (16) with the slight modification of Kamiya et al. (14).

Enterotoxicity was detected in the rabbit ligated intestinal loop assay. Female New Zealand White rabbits (1,500 to 1,700 g) purchased 1 week before the study were housed separately, fed normally, and allowed water ad libitum. Food, but not water, was withdrawn 24 h before surgery. A maximum of eight 5-cm-long ileal loops with 3-cm interloop spaces were formed. Material examined for enterotoxic activity included crude culture filtrates of *C. difficile* 8864, the cytotoxin of strain 8864 obtained after anion-exchange chromatography (see below), and the fractions in which toxin A would be expected to elute after dialysis against phosphate-buffered saline (PBSA). Crude culture filtrates of *C. difficile* VPI 10463 were used as positive controls, and saline and BHI broth were used as negative controls. For antibody neutralization tests, equal volumes of a 1:10 dilution of antiserum to strain 8864 (see below) and a crude undiluted culture filtrate of that strain were premixed and left at room temperature for 30 min. The control consisted of a 1:20 dilution of normal rabbit serum. In all cases, 1 ml (final volume) was injected into each loop. The rabbits were left for 18 h, the loops and control bowels were removed in toto, and the animals were killed by anesthetic overdose. The fluid contents were collected after incision of one end of the loop. The ratio of the volume accumulated to the length of the loop was calculated; a ratio of greater than 1 was considered positive.

Preparation of antisera and cross-neutralization and reactivity assays. Antisera to *C. difficile* 8864 and VPI 10463 were raised as follows. The strains of *C. difficile* were inoculated into BHI broth from CBA cultures and incubated for 7 h at 37°C anaerobically. The broth cultures were centrifuged at 10,178 × *g* at 4°C for 40 min; the resulting pellets were washed twice in PBSA, resuspended in 50 ml of 0.4% formol–saline, and stored at 4°C. Each of the Formol-treated antigens was injected subcutaneously into the scruff of the neck of a 3- to 3.5-kg New Zealand White rabbit at 3- to 4-day intervals with increasing doses of antigen (0.5, 0.75, and 1.0 ml and then 0.5-ml increases to 3.0 ml). Rabbits were bled via cardiac puncture 1 week after the final injection. Complement was removed from the separated sera by heating at 60°C for 30 min.

The antiserum to *C. difficile* 8864 was adsorbed with the nontoxigenic *C. difficile* M-1 to remove antibodies other than those directed to toxin B. Adsorption was achieved by mixing 5 ml of a 1:100 dilution of the antiserum with 1 g of pelleted *C. difficile* M-1 obtained after dialysis bag culture and rotation at 4°C overnight. The mixture was then centrifuged at 10,178 × *g* at 4°C for 20 min, and the resultant supernatant was decanted and retained. This supernatant was subjected to two identical adsorptions.

The antisera raised were used at a dilution of 1:200, and the *C. sordellii* antitoxin (Wellcome Research Laboratories) was used at a dilution of 1:50, in cytotoxicity neutralization assays with the following: crude culture filtrates of *C. difficile* 8864 and VPI 10463, partially purified toxin B obtained from *C. difficile* VPI 10463 after Mono Q anion-exchange chromatography (see below), and toxin A from strain VPI 10463 purified as described in detail elsewhere (14). The culture filtrates and toxin preparations were diluted 10-fold in PBSA. Then 50 μl of each dilution was added in triplicate to the Vero cells, and 50-μl aliquots of antiserum or normal rabbit or normal horse serum, as appropriate, were added immediately to one of the triplicate sets. Cells were examined for cytotoxicity after 24 h of incubation.

Anion-exchange chromatography. Since strain 8864 failed to produce toxin A but was cytotoxic, anion-exchange chromatography was performed to determine whether the cytotoxicity was due to toxin B and whether the strain 8864 toxin B was similar to the toxin B from strain VPI 10463 with regard to its point of elution.

Two kinds of anion-exchange gel, Q Sepharose FF (Pharmacia) and Mono Q (Pharmacia), incorporated into a fast protein liquid chromatography apparatus (Pharmacia) as described previously (14), were used. The sample was filtered through a membrane filter (0.2-μm pore size) and then applied to the column via a 10-ml Super loop (Pharmacia). The column was eluted with a 0 to 1 M NaCl gradient in 20 mM Tris-HCl (pH 7.5). Each fraction was examined for cytotoxicity and hemagglutination activity as described above and for A_{280}. After Q Sepharose FF anion-exchange chromatography, the cytotoxic but hemagglutination-negative fractions (containing toxin B) were dialyzed against 20 mM Tris-HCl (pH 7.5) overnight at 4°C and then subjected to Mono Q anion-exchange chromatography.

After strains 8864 and VPI 10463 were grown in dialysis bags, the cytotoxicity titers of crude culture filtrates were determined; cultures were adjusted by dilution in PBSA to be of equal cytotoxicity. These adjusted crude filtrates were then mixed in a ratio of 1:1 (vol/vol) and analyzed directly by

Mono Q anion-exchange chromatography to determine whether the peaks of cytotoxicity corresponding to toxin B were concordant.

Native gel electrophoresis. Cultures of strain 8864 grown in BHI broth were filtered, and the filtrates were analyzed by native polyacrylamide gel electrophoresis as described in detail previously (14).

Preparation of synthetic oligonucleotides. Synthetic oligonucleotides were synthesized by using phosphoramide chemistry in a DNA synthesizer (Applied Biosystems model 380). Two toxin A oligonucleotide primers (BW69, 5'-GAAGCAGCTACTGGATGGC; BW70, 5'-AGCAGTGT TAGTATTAAAG) were based on a 63-bp tandem repeat sequence at the 3' end of the gene (35). The sequence data for the other four toxin A gene oligonucleotides (I to IV) were taken from those reported by Dove et al. (7) and corresponded to regions 5' to the repeat units of the toxin A gene. The sequences are 5'-ATATCTAGACCTAGCTC TATTGGA (I), 5'-CAAACAATAGATTTTTCAGGCGAT (II), 5'-GTTTGTTATCTAAAAAGTCCGCTA (III), and 5'-TTTGAGTATTTTGCACCTGCTAAT (IV).

Toxin B oligonucleotide primers were deduced from a nonrepeating region of toxin B that showed no homology with toxin A (BW155, 5'-AAGGTTTATATGGATGAT; BW156, 5'-TACAACTTTATTAACACAACT), based on the sequence determined by Barroso et al. (2).

Toxin A gene-specific probe and hybridization studies. A 4.5-kb internal *Pst*I fragment from the toxin A gene was prepared as described previously (34) and used as a toxin A gene-specific probe. The toxin A probe was radiolabelled in vitro with [α-^{32}P]dCTP (Amersham International) by the random primer hexamer method of Feinberg and Vogelstein (8). Southern blot hybridizations were performed with dextran sulfate as an enhancer as described in detail previously (34), except that lower-stringency conditions (i.e., 55°C rather than 68°C) were used for the hybridization in the Southern blot. Briefly, filters were washed in 0.3 M sodium chloride–0.06 M Tris hydrochloride (pH 8.0)–0.002 M EDTA for 5 min and then in the same solution including 1.0% sodium dodecyl sulfate for 30 min at 55°C. All blots were dried and exposed to Fuji-RX X-ray film at −70°C for 16 h.

PCR methodology. Samples for the polymerase chain reaction (PCR) were prepared by harvesting a single colony of the strain to be examined into a 1.5-ml polypropylene tube containing 200 μl of sterile water. After the samples were boiled for 10 min, they were spun at 13,400 × *g* in a bench-top microfuge for 5 min. Two microliters of supernatant liquid was added to a 50-μl reaction volume with 1.5 mM magnesium chloride, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 200 μM each deoxyribonucleotide, 100 pM oligonucleotide primers, and 1.0 U of *Taq* polymerase (Perkin Elmer-Cetus). Reaction mixtures were overlaid with paraffin oil (50 μl) and placed in a thermal cycler (Hybaid Ltd., Twickenham, Middlesex, United Kingdom). Amplification for paired primers BW69 and BW70 and I and IV was for 30 cycles at 94°C (45 s), 46°C (45 s), and 72°C (15 s), and amplification for primers BW155 and BW156 was for 40 cycles of 94, 40, and 72°C, each for 1 min. For the mixed reaction with primers I through IV, the amplification conditions were like those for primers I and IV but with cycle temperatures of 94, 35, and 60°C, each for 1 min per cycle. Samples of 25 μl of the amplified products were electrophoresed in horizontal 1.5% agarose gels containing 0.5 μg of ethidium bromide per ml, and the bands were visualized by excitation under UV light.

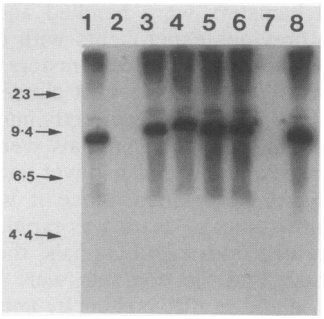

FIG. 1. Hybridization analysis after Southern transfer with the ^{32}P-radiolabelled toxin A-specific probe against 3 μg of *Hin*dIII-digested *C. difficile* genomic DNAs from the following (lanes): 1, standard type strain B (toxigenic); 2, strain 8864 (toxin A negative, toxin B positive); 3 through 6, standard toxigenic type strains D, E, W, and X, respectively; 7, standard type strain Y (nontoxigenic); 8, standard type strain Z (toxigenic). The major hybridization bands (9 to 13 kb) indicate the presence of the toxin A gene. Reference points (in kilobases) are indicated on the left.

RESULTS

Production of toxin A in vitro by strain 8864. Crude culture filtrates of strain 8864 failed to hemagglutinate rabbit erythrocytes at 4°C or produce a product that would react in an enzyme-linked immunosorbent assay for toxin A, regardless of whether cultures were grown in simple batches or in dialysis bags; this was despite production of cytotoxin to a titer of 1:3.2 × 10^4 or 1:10^7, respectively. The band characteristic of toxin A (ca. 540 kDa) was not evident after native polyacrylamide gel electrophoresis.

Detection of toxin A gene by DNA hybridization and the PCR. The 4.5-kb DNA probe failed to react with DNA from strain 8864 in hybridization studies (Fig. 1); it did react with the six toxigenic standard type strains but not with a standard nontoxigenic type strain (Fig. 1).

All of the toxigenic strains of *C. difficile* examined by the PCR with oligonucleotide primers BW69 and BW70 yielded characteristic profiles of at least five bands of 63 bp or multiples thereof, whereas a nontoxigenic strain and strain 8864 yielded nothing (Fig. 2). Oligonucleotide primers I and IV also failed to yield a product with strain 8864, as did a mixture of primers I through IV, whereas products were produced from the toxigenic strain VPI 10463 (data not shown).

Characterization of the toxin B gene by the PCR. The

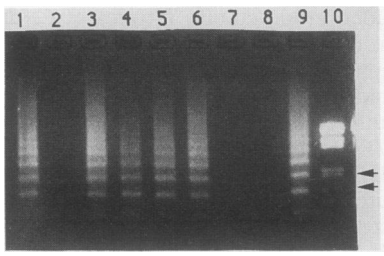

FIG. 2. PCR of strains of *C. difficile* with toxin A gene oligonucleotide primers BW69 and BW70. Lanes: 1, 3 through 6, and 9, toxigenic strains Z, X, W, E, D, and B, respectively; 2 and 7, Nontoxigenic strains Y and C, respectively; 8, strain 8864; 10, small size DNA marker (International Laboratories Services, London, United Kingdom). Arrows indicated on the right indicate 130 bp (lower arrow) and 190 bp (upper arrow).

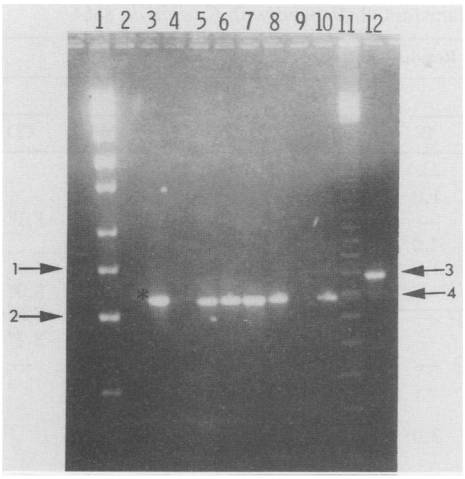

FIG. 3. PCR of strains of *C. difficile* with toxin B gene oligonucleotide primers BW155 and BW156. Lanes: 3, 5 through 8, and 10, toxigenic strains B, D, E, W, X, and Z, respectively; 2, 4, and 9, Nontoxigenic strains A, C, and Y, respectively; 12, strain 8864; 1, 1-kb ladder; 11, 100-bp ladder. Arrows: 1, 750 bp; 2, 500 bp; 3, 700 bp. 4, 600 bp. The asterisk (*) indicates the 591-bp product.

toxigenic strains all gave a single amplified product of the expected size (591 bp), whereas strain 8864 (toxin A negative, toxin B positive) gave a major amplification product of 690 bp and a minor product of 591 bp (Fig. 3). No amplification product was obtained from the nontoxigenic strains (Fig. 3).

Cross-neutralization of cytotoxic activity. In cytotoxicity neutralization assays (Table 1), the crude antiserum to *C. difficile* 8864 neutralized the cytotoxicity of a culture filtrate of *C. difficile* 8864 and partially neutralized purified toxin B from strain VPI 10463 but did not neutralize pure toxin A. The same neutralization pattern was obtained after adsorption of the antiserum with the nontoxigenic strain *C. difficile* M-1, although there was better neutralization of toxin B. These two antisera also partially neutralized the activity of crude culture filtrates of *C. difficile* VPI 10463. The antisera to VPI 10463 and *C. sordellii* neutralized all cytotoxic activities, although the antiserum to *C. difficile* VPI 10463 neutralized the cytotoxic activity of the crude culture filtrate of strain 8864 the least well.

Effect of strain 8864 in the hamster model. All three positive control animals were dead within 1 day of receiving the virulent fully toxigenic strain B-1 and had high levels of both cytotoxin and *C. difficile* in their ceca (Table 2). Post mortem examination showed distended and grossly hemorrhagic fluid-filled ceca and hemorrhagic fluid-filled small bowels.

Animals receiving strain 8864 survived longer than did those receiving the virulent strain B-1 (Table 2). Hamsters challenged 5 days after the administration of clindamycin did not demonstrate any signs of diarrhea until day 3 (animals 2, 4, and 5), which corresponded to the appearance of cytotoxin in the fecal pellets. Deaths started to occur on day 4; all but one animal was dead or close to death by day 6. One animal (no. 9) survived for 9 days. In general, prolonged survival was associated with relatively low numbers of *C. difficile* in the fecal pellet; much higher levels were detected in the pellets of diarrheic animals and in the cecal contents of dead or moribund animals. Prolonging the period after administration of clindamycin to 8 or 10 days before challenge with strain 8864 reduced the time to death (Table 2). This was especially true for 10 days after the administration of clindamycin; diarrhea was seen in two of three animals on day 1, with relatively high levels of *C. difficile* present in all three animals, and all three hamsters were dead by day 2.

Diarrhea never occurred in animals receiving strain 8864 when there were fewer than $\log_{10}$ 5.9 CFU of *C. difficile* per g of fecal pellet (mean, $\log_{10}$ 6.9 CFU/g; range, 5.9 to 8.1 CFU/g), although in four cases (animals 1, 6, 11, and 15) more than $\log_{10}$ 5.9 CFU of *C. difficile* per g of fecal pellet were detected in the absence of diarrhoea. In all but one case (animal 4), toxin was only detected when levels of *C. difficile* were $\log_{10}$ 5.9 CFU or greater per g of examined material. Despite levels of *C. difficile* 8864 in the ceca of dead or moribund animals that were comparable to those in dead animals that received the fully toxigenic strain B-1, the levels of cytotoxic activity were 100-fold lower in all but one case (animal 2), with a mean $\log_{10}$ titer of 3.6 compared to 5.0. None of the cecal contents examined (animals 3, 7, 12, and 13) contained toxin A; the one positive control animal examined (animal 19) did contain toxin A.

Histological findings for animals infected with strain 8864. The gross pathology of sacrificed animals in all cases included hemorrhagic fluid-filled ceca, fluid-filled small bowels with milder hemorrhage, and normal colons. The histological picture, which was similar for all five sacrificed animals examined (animals 1, 3, 5, 7, and 10), was largely cecal disease. In animals given only clindamycin, there was simply a minor infiltration of polymorphonuclear cells (Fig. 4a), whereas in infected animals there was thickening of the mucosa with mild crypt hyperplasia (Fig. 4b) and increased mitotic activity, slight mucosal tufting in areas (Fig. 4c) (a feature more marked in animals 1 and 3), congestion, a mild to moderate increase in the inflammatory cell component of the mucosa (Fig. 4d), and submucosal edema. Similar but milder changes were present in the terminal ileum. The changes were much less severe in animals 7 and 10, which were also the animals with lowest levels of cecal cytotoxin (Table 2). There was no specific colonic disease in any animal.

Analysis of toxin B by anion-exchange chromatography and its effects in tissue culture. The cytopathic effects induced by the cytotoxins of the two different strains were slightly different. Toxin B of strain VPI 10463 caused cell rounding evenly distributed within the cell sheet, whereas that of strain 8864 also caused clumping (Fig. 5). Although the toxins basically coeluted, analysis of the characteristic cytopathic effects showed that the cytotoxin of strain 8864

TABLE 1. Neutralization of cytotoxic activity of *C. difficile* culture filtrates and toxin A and B preparations

Antibody	Cytotoxic activity ($\log_{10}$ reciprocal titer)			
	VPI 10463 toxin B	VPI 10463 toxin A	Crude culture filtrates of *C. difficile*:	
			VPI 10463	8864
No antibody	10^4	10^3	10^6	10^4
Anti-toxin B (raised from strain 8864)	10^1	10^3	10^4	10^1
Anti-8864	10^3	10^3	10^4	—[a]
Anti-VPI 10463	10^1	—	10^2	10^2
Anti-*C. sordellii*	10^1	10^2	10^3	—

[a] —, no cytotoxic activity detected.

TABLE 2. Culture and toxin findings in clindamycin-pretreated hamsters challenged with *C. difficile* 8864

Group (day of challenge)[a]	Animal no.	Results[b] on day: 1 CD	1 B	2 CD	2 B	3 CD	3 B	4 CD	4 B	5 CD	5 B	6 CD	6 B
1 (5)	1	3.4	—[c]	3.2	—	6.5	—	8.0[d]	NS[e]				
	2	3.6	—	3.0	—	6.2[f]	1.0	7.9[g]	5.0				
	3	3.8	—	3.2	—	3.1	—	2.5	—	4.9	—	8.1[d,f]	3.0
	4	3.2	—	4.2	—	7.7[f]	4.0	2.3[g]	4.0				
	5	3.6	—	3.4	—	6.0[f]	1.0	6.4[d]	3.0				
	6	3.3	—	2.7	—	—	—	3.3	—	7.0	1.0	8.0[g]	3.0
	7	NS	NS	3.0	—	2.0	—	2.3	—	—	—	7.5[d,f]	1.0
	8	3.2	—	—	—	2.6	—	2.0	—	4.3	2.0	8.2[g]	3.0
	9	3.5	—	2.5	—	2.9	—	2.3	—	2.0	—	—	—
	10	3.6	—	2.6	—	3.3	—	7.5[d,f]	1.0				
2 (8)	11	6.4	1.0	6.8	2.0	7.9[d,f]	3.0						
	12	NS	NS	NS	NS	NS		7.0[d]	4.0				
	13	NS	NS	7.3[g]	4.0								
3 (10)	14	5.9[f]	2.0	6.8[d]	4.0								
	15	6.5	1.0	7.3[g]	4.0								
	16	6.0[f]	3.0	7.0[g]	4.0								
Positive control group (5)	17	6.4[g]	5.0										
	18	7.3[g]	5.0										
	19	5.9[g]	5.0										

[a] Hamsters were challenged on the indicated day after administration of clindamycin. Control animals were challenged with strain B-1.
[b] CD, $\log_{10}$ CFU of *C. difficile* per g; B, $\log_{10}$ reciprocal titer. *C. difficile* and cytotoxin results respectively for days 7, 8, and 9 were as follows: 5.9 and negative, wet tail (no analysis), and 7.0 and 2.0 (animal sacrificed).
[c] —, not detected.
[d] Animal sacrificed.
[e] NS, no specimen.
[f] Diarrhea (wet tail).
[g] Animal died.

started to elute just before that of toxin B of *C. difficile* VPI 10463. To what extent the elutions of the two toxins overlapped could not be determined, since the cytopathic effect induced by toxin B of strain VPI 10463 would, as it increased in amount, mask that of strain 8864. No other cytotoxic activity was detected.

Rabbit ligated loop test. The results of the rabbit ligated loop test are presented in Table 3. The crude culture filtrates of strains 8864 and VPI 10463 were enterotoxic and caused hemorrhage, and the accumulated fluid contained mucus and blood. Culture filtrates of strain 8864 derived from two of the occasional cultures with low cytotoxin titers (titers of 10^5 and 10^3) were not enterotoxic, although the culture with a cytotoxin titer of 10^5 caused some accumulation (3.5 ml; volume/length ratio, 0.7) of a blood-red fluid that contained mucus. The toxin B of strain 8864 was negative for both activities. The fraction in which toxin A would be expected to elute if this strain produced toxin A was also negative. The enterotoxic activity of undiluted culture filtrate of strain 8864 was neutralized by a 1:10 dilution of homologous antiserum but not by normal rabbit serum. There was still measurable fluid in the loop (4.5 ml), but no mucus or blood was present.

DISCUSSION

In a study in 1986 on the effect of variation in nutrients on toxin production by *C. difficile*, a cytotoxigenic strain that failed to produce toxin A was described (12). The fact that in the present study crude culture filtrates of this strain were unable to hemagglutinate rabbit erythrocytes, failed to react in an enzyme immunoassay specific for toxin A, and failed to yield a product with the electrophoretic or anion-exchange chromatographic characteristics of toxin A confirms the absence of toxin A in this strain. These negative findings were obtained even after culture by the dialysis bag method, indicating that the negative findings obtained initially after routine batch culture were not the result of low levels of production under suboptimal conditions. The absence of toxin A could have been the result of the absence of the toxin A gene or a mutation that affects its expression. It has been reported that all strains of *C. difficile* examined produce both toxins A and B or are nontoxigenic (20). At the genetic level, it has been shown that all toxigenic strains contain the gene for toxin A but nontoxigenic strains do not (9, 15, 23, 27, 34, 35). Hybridization studies with a 2.1-kb *Pst*I fragment of the toxin A gene showed the gene to be present in six toxigenic strains and indicated its absence in four nontoxigenic strains (27), and a larger toxin A gene *Pst*I fragment of 4.5 kb hybridized to the DNA of 58 toxigenic strains but not to that of 17 nontoxigenic strains (34). McMillin et al. (23) used a 1.2-kb probe in hybridization studies showing hybridization with all 36 toxigenic strains examined and 1 of 9 nontoxigenic strains. The use of primers that amplify a 1,216-bp fragment of the toxin A gene showed this portion of the gene to be present in 28 toxigenic strains examined, absent in 5 nontoxigenic strains, but present in the nontoxigenic strain that was positive in hybridization studies (23). This strain also has the gene for toxin B (23). The PCR has also been used by others for such studies (9, 15, 35); their results and those of McMillin et al. (23) show the presence of toxin A

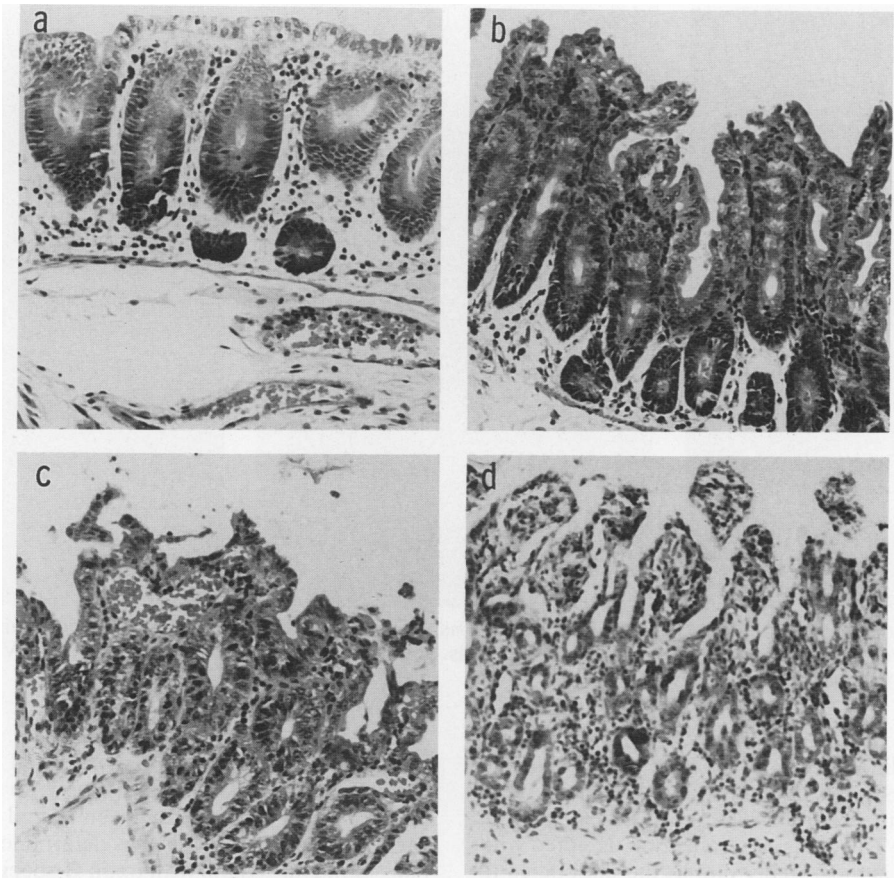

FIG. 4. Sections of hamster ceca (fixed with hematoxylin and eosin; Magnification, ×157) from sacrificed animals after infection with strain 8864. (a) Control 5 days after administration of clindamycin; minor polymorphonuclear cell infiltration is visible. (b) Crypt hyperplasia, loss of crypt architecture, and mucosal thickening in hamster 3 of Table 2. (c) Tufting in hamster 1 of Table 2. (d) Frayed mucosa and infiltration in hamster 5 of Table 2.

genetic determinants in all of 135 toxigenic strains and in 1 of 64 nontoxigenic strains.

Although less work has been done on the gene for toxin B, its simultaneous presence with the toxin A gene in toxigenic strains and absence in nontoxigenic strains has been shown by use of the PCR and in hybridization studies in 75 toxigenic strains and 7 nontoxigenic strains (9, 23). We have shown in this study that the lack of toxin A production by strain 8864 is not due to the presence of a silent copy of the gene but rather to the definite absence of a large section of the 3′ end of the gene containing repeat units and parts of the 5′ end nonrepeat components of the gene. These findings indicate that the whole gene may be absent.

It is of interest, therefore, that despite this the strain produced diarrhea and death in clindamycin-pretreated hamsters. In initial experiments it appeared that this strain was less virulent than a fully toxigenic strain, with symptoms and death occurring at least 2 days and up to 8 days later than in hamsters receiving the fully toxigenic strain 5 days after the administration of clindamycin. It is possible that these results reflect differences in the intrinsic virulence of the two strains or that strain 8864 was more sensitive than the fully toxigenic strain to clindamycin and its in vivo-produced active metabolites. Therefore, the period between administration of antibiotic and exposure to this toxin A negative strain of *C. difficile* was extended in a number of experiments. The periods of 8 and 10 days were chosen because these represented the days after administration of clindamycin when symptoms first started to appear and when most animals had succumbed to disease, respectively. Extending the period between antibiotic and challenge reduced the time to disease after challenge. It therefore appears that there is a minimal difference between these two strains with respect to their intrinsic virulence capabilities in this model of disease.

It was a surprise that this toxin A-negative strain caused diarrhea in hamsters and that cecal damage characteristic of infection with fully toxigenic strains occurred. Great care was taken to minimize the risk of inadvertent infection; all animals were housed individually in sterile isolators, and experiments with the toxin A-negative strain were conducted in a room not previously used for *C. difficile* studies.

That the symptoms were not due to inadvertent infection with a fully toxigenic strain is evident from the negative finding for toxin A in the cecal contents of the animals and the fact that the control animal receiving clindamycin only did not succumb to infection.

Because of the above observations, we had to consider the possibility that the cytotoxin was the cause of the pathology seen in infected animals, especially since the cytopathic effect in Vero cells caused by the toxin was slightly different from that caused by crude culture filtrates of fully toxigenic strains (data not shown) or purified toxin B from one of these

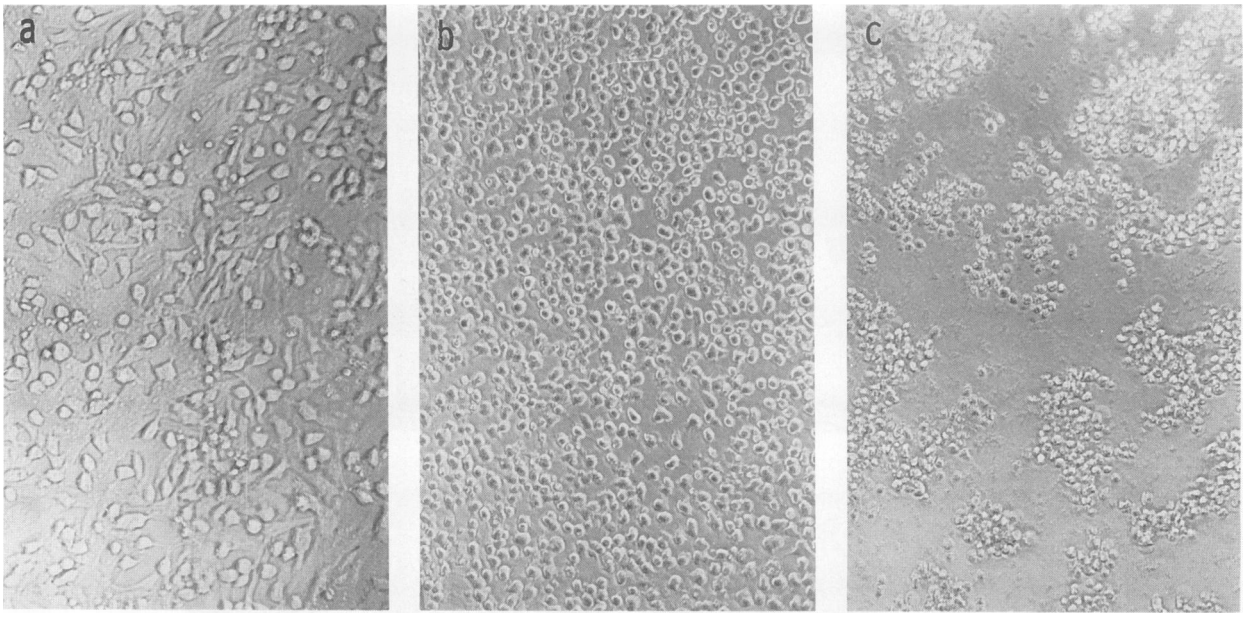

FIG. 5. Comparison of the cytopathic effects of toxin B preparations. Purified toxin B (Mono Q derived) from strains 8864 and VPI 10643 was adjusted to a final titer of $1:10^3$, and 100 μl was applied to Vero cells. (a) Normal cell sheet; (b) toxin B from strain VPI 10643; (c) cytotoxin from strain 8864. Magnification, ×262.5.

strains. Despite the difference in cytopathic effect, neutralization studies demonstrated immunological cross-reactivity between the cytotoxin of strain 8864 and toxin B but not toxin A of *C. difficile*. The cross-neutralization detected with *C. sordellii* antitoxin is most likely due to the antibodies to the lethal *C. sordellii* toxin, which is similar to toxin B (22). However, neutralization by antibodies to the hemorrhagic toxin, which is similar to toxin A (23), or other factors produced by *C. sordellii* cannot be excluded, although this is unlikely. Analysis of the cytotoxin of strain 8864 by anion-exchange chromatography showed that it started to elute fractionally earlier than did toxin B of strain VPI 10463. While this work was in progress, a publication reporting that the cytotoxin of strain 8864, which caused a slightly different effect in human lung fibroblasts, could by neutralized with antiserum to toxin B but not with antiserum to toxin A and that gel immunodiffusion demonstrated partial identity with toxin B appeared (32). Interestingly, in that study the cytotoxin of strain 8864 was between 10 and 30 kDa smaller than toxin B of strain VPI 10463 as determined by gel electrophoresis (32). Our results with toxin B-specific primers based on a nonrepeating region of the toxin B gene showed that there was a difference between strain 8864 and the six fully toxigenic strains examined; in the PCR, strain 8864 produced a product of ca. 690 bp in addition to the expected 591-bp product.

TABLE 3. Cytotoxicity and enterotoxicity of *C. difficile* 8864

Strain and test material	Cytotoxicity (log_{10} reciprocal titer)	Enterotoxicity	
		Fluid accumulation	Hemorrhage
8864			
Culture filtrate (undiluted)	6.0	+ (2.2)[a]	+
Culture filtrate			
With homologous antiserum	–	– (0.9)	–
With normal rabbit serum	6.0	+ (2.4)	+
Toxin A[b]	–	–	–
Toxin B (200 μg)	6.0	–	–
VPI 10463 culture filtrate (1:5 dilution)	7.0	+ (1.00)	+

[a] Numbers within parentheses indicate the ratios of volume (milliliters) accumulated to length (centimeters) of the loop. Where no value is given, there was insufficient loop content to make any measurement.

[b] Strain 8864 anion-exchange chromatography fractions in which toxin A would be expected.

Although the cytotoxin of strain 8864 is slightly different from toxin B, it appears that it is not the cause of the diarrhea, since 200 μg of purified cytotoxin with a cytopathic titer of $1:10^6$ failed to cause fluid accumulation or hemorrhage in the rabbit ligated ileal loop assay, whereas crude culture filtrate of this toxin A-negative strain (which had the same cytopathic titer of $1:10^6$) did cause extensive hemorrhage.

The evidence indicates that *C. difficile* 8864 produces a toxic factor(s) that causes fluid accumulation and hemorrhage. Although the features are similar to those induced by toxin A, the factor(s) in strain 8864 differs in that it is not cytotoxic to Vero cells. This can be deduced from the observation that among the fractions derived by anion-exchange chromatography only those corresponding to toxin B were cytopathic.

How this non-toxin A-mediated enterotoxic activity relates to the other enterotoxins of *C. difficile* is difficult to determine. The first description of such a factor was by Banno and colleagues (1), who detected a relatively unstable noncytotoxic factor of ca. 200 kDa that elicited a clear fluid response in ligated ileal loops of rabbits. A factor with similar properties was later described by Giuliano et al. (11). More recently, three enterotoxic factors, termed C1, C2, and C3, produced by *C. difficile* were described (31). These also induced clear fluid accumulation (but in these experiments ligated small intestinal loops of rats were used) and were

cytotoxic to Chinese hamster ovary cells. In all cases these purified enterotoxic factors cause clear fluid accumulation (1, 11, 31), which contrasts with the hemorrhagic activity and blood-stained fluid induced by crude culture filtrates of strain 8864. It is possible that the enterotoxic activity seen in this strain is due to one of the non-toxin A enterotoxic factors described by others and that the tissue damage and presence of blood and mucus in the fluid that accumulates is due to another factor(s). It is unlikely to be due to the toxin B-like cytotoxin that is produced, since this alone did not cause hemorrhage in rabbit ligated loops.

From the above observations it can be concluded that a noncytotoxic factor(s) that causes fluid accumulation and tissue damage in rabbit ligated intestinal loops and clindamycin-treated hamsters is produced by a cytotoxigenic strain of *C. difficile* that lacks the gene for toxin A. The extent to which this factor(s) is produced by other strains of *C. difficile*, including noncytotoxigenic strains, remains to be determined.

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