Binary Toxin–Producing, Large Clostridial Toxin–Negative *Clostridium difficile* Strains Are Enterotoxic but Do Not Cause Disease in Hamsters

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Binary toxin CDT or its genes have been identified in some strains of *Clostridium difficile* that also produce the large clostridial toxins, toxins A and B ($A^+B^+CDT^+$), including a newly recognized epidemic strain in the United States and Canada. To study the effects of binary toxin alone, we characterized 4 binary toxin CDT–positive only ($A^-B^-CDT^+$) *C. difficile* strains. Unlike other clostridial binary toxins, binary toxin CDT required exogenous trypsin for activation. Supernatants from all $A^-B^-CDT^+$ strains caused marked fluid accumulation in the rabbit ileal loop assay after concentration and trypsinization. In addition, the ileal loop response was neutralized by antisera raised against other binary toxin–producing clostridia. Challenge of clindamycin-treated hamsters with these strains resulted in colonization but not diarrhea or death. Binary toxin CDT may play an adjunctive role to toxins A and B in the pathogenesis of *C. difficile*–associated disease but by itself may not be sufficient to cause disease.

The pathogenesis of *Clostridium difficile*–associated disease (CDAD) has been attributed to 2 large clostridial toxins, toxins A and B (TcdA, 308 kDa; TcdB, 260 kDa), which act as glycosyltransferases and modify small GTPases within the host cell that are involved in actin polymerization and cytoskeleton structure [1–3]. TcdA alone is highly enterotoxic in animal models [4]. Although enterotoxicity has been difficult to demonstrate for TcdB, it is a potent cytotoxin for many cell lines, and both toxins contribute to the pathogenesis of CDAD [3]. A third toxin, binary toxin CDT, is found in some *C. difficile* strains [5–8]. Binary toxin CDT is unrelated to TcdA and TcdB but instead is related to the group of clostridial binary toxins that consist of 2 unlinked molecules, both of which are needed for toxic activity. CDTa is the enzymatic component, and CDTb is the receptor-binding component. This toxin is an ADP-ribosyltransferase and, like the large clostridial toxins, ultimately acts by disruption of the host's actin cytoskeleton [9].

In most surveys, binary toxin CDT genes are found in <10% of clinical *C. difficile* isolates [5, 7, 8, 10–14]. However, binary toxin genes were present in 65% of *C. difficile* clinical isolates recovered during a hospital outbreak of CDAD in Pittsburgh [15, 16], in which increased severity of CDAD was also noted, resulting in a large number of colectomies because of fulminant cases [17]. Recently, an epidemic *C. difficile* strain has been recognized as the cause of CDAD outbreaks in hospitals across the United States and in Quebec [18]. This strain (designated REA group BI) is characterized by restriction fragment–length polymorphism changes

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within the pathogenicity locus for the toxin A and B genes (toxinotype III), a deletion in the putative negative regulator for toxin production *(tcdC),* and the presence of binary toxin CDT genes [18].

Most binary toxin CDT–positive strains of *C. difficile* also produce TcdA and TcdB, and the potential contribution of CDT to the pathogenesis of CDAD has been difficult to demonstrate. Strains that produce binary toxin CDT but neither of the large clostridial toxins have recently been described [7, 19]. In this study, we have characterized 4 CDT-positive, large clostridial toxin–negative ($A^-B^-CDT^+$) *C. difficile* strains in 2 well-described animal models, to elucidate the role of binary toxin CDT in CDAD.

MATERIALS AND METHODS

Molecular analysis and toxin production. Four *C. difficile* strains, previously identified as $A^-B^-CDT^+$ [7, 19], were selected for testing in 2 animal models. Polymerase chain reaction (PCR) and restriction digestion of the amplified products of the pathogenicity locus (PaLoc) were used to confirm the toxinotype. The PaLoc is a 19.6-kb segment of the chromosome that encompasses the genes for toxins A and B (*tcdA* and *tcdB*) and 3 additional genes (*tcdR, tcdE,* and *tcdC*) [20]. Ten fragments covering the whole PaLoc (fragments PL1-4, A1-3, and B1-3) were amplified as described elsewhere [21, 22] (see also: http://www.mf.uni-mb.si/mikro/tox), and the restriction patterns of amplified regions were compared with strains of all other known toxinotypes. Primers Lok1 and Lok3 are outside the PaLoc, and Lok3-Lok1 PCR was used to confirm the absence of PaLoc [23]. Southern blot hybridization using probes F25 (2168 bp at the 3′ end of *tcdB*) and 5660 (2360 bp at the 3′ end of *tcdA*) [24] was used to confirm the results of the PCRs (table 1).

REA typing was also used to determine relatedness of the strains. *Hin*dIII restriction analysis of whole-cell genomic DNA was performed as described elsewhere [25]. Isolates showing ≤6 visible restriction band differences (a similarity index of >90%) are placed within the same REA group, which is designated by letter. Isolates with identical restriction patterns are assigned a specific REA type, which is designated by number (table 1).

The binary toxin genes, *cdtA* and *cdtB,* are located outside of the PaLoc and were detected by PCR, as described elsewhere [7].

Lack of TcdB and TcdA production was confirmed for all 4 $A^-B^-CDT^+$ strains. TcdB production was tested using 5-day-old culture supernatants in a standard cell culture assay (Cytotoxicity Assay for *C. difficile* Toxins; Bartels) and TcdA production by immunoassay (*C. difficile* Tox-A Test; TechLab).

Binary toxin production was tested in all 4 strains by M. Popoff (Pasteur Institute, Paris), using a Western blot assay and polyclonal anti–iota toxin (Ia and Ib) antibodies, as described elsewhere [7, 26]. In addition, we tested one of the $A^-B^-CDT^+$ strains (strain IS58) for binary toxin in 2 additional Western blot assays, one using a monoclonal antibody (1D11) to recognize the C-terminal region of Ib and pro-Ib [27] and the other using polyclonal anti–*Clostridium spiroforme* antibody [28]. Supernatants were tested with and without trypsinization and were compared with supernatants from *Clostridium perfringens* type E strain NCIB 10748. For trypsinization, trypsin (Sigma; 2.5 mg/mL final concentration) and supernatant were coincubated at 37°C for 30 min. Ten microliters of each sample was loaded onto a reduced 4%–12% Bis-Tris SDS-PAGE gel and run for 35 min at 200 V. Transfer was done for 2 h at 100 V. Detecting monoclonal antibody 1D11 was used at a concentration of 10 μg/mL and probed with horseradish peroxidase–conjugated goat anti–mouse IgG (Sigma) at a dilution of 1:5000. Detecting polyclonal anti–*C. spiroforme* antibody was used at a dilution of 1:5000 and was probed with horseradish peroxidase–conjugated rabbit anti–goat IgG (Sigma) at a dilution of 1:5000. BioFX One Step tetramethylbenzidine substrate was used for developing.

To explain the molecular basis for endogenous protease activation of Ib (*C. perfringens* type E iota toxin) and the re-

Table 1. Molecular characterization of $A^-B^-CDT^+$ strains and results of the rabbit ileal loop and hamster challenge experiments.

			Toxinotyping				Hybridization		Ileal loop	Hamster challenge	
Isolate	Origin	REA type	PaLoc[a]	B1 *Hin*cII/*Acc*I[b]	A3 *Eco*RI[b]	Toxinotype	F25 *(tcdB)*	5660 *(tcdA)*	Fluid accumulation, volume-to-length ratio, median ± SD[c]	Colonization	Disease[d]
IS58	No data	AA1	+	−	5	XIa	−	+d	0.9 ± 0.4	7/10	0/10
3126	Asymptomatic patient	AA1	+	−	8	XIb	−	+	1.3 ± 0.3	ND	ND
R11402	Diseased foal	AA3	+	−	8	XIb	ND	ND	1.2 ± 0.2	8/10	0/10
6009	Patient with diarrhea	CS1	−	−	−	tox$^-$	−	−	1.0 ± 0.4	8/10	0/10

NOTE. ND not done; +, amplified, present; −, not amplified, not present; d deletion compared to VPI10463.

[a] Presence or absence of large clostridial toxin region PaLoc (pathogenicity locus) was detected with Lok3-Lok1 polymerase chain reaction.

[b] Restriction patterns of *tcdB* fragment B1 and *tcdA* fragment A3 (type according to Rupnik et al. [22]).

[c] Median volume-to-length ratios for negative controls were <0.3.

[d] Disease manifestations, as seen in positive controls (hamsters challenged with $A^+B^+CDT^-$ strains), include diarrhea ("wet tail") and/or death.

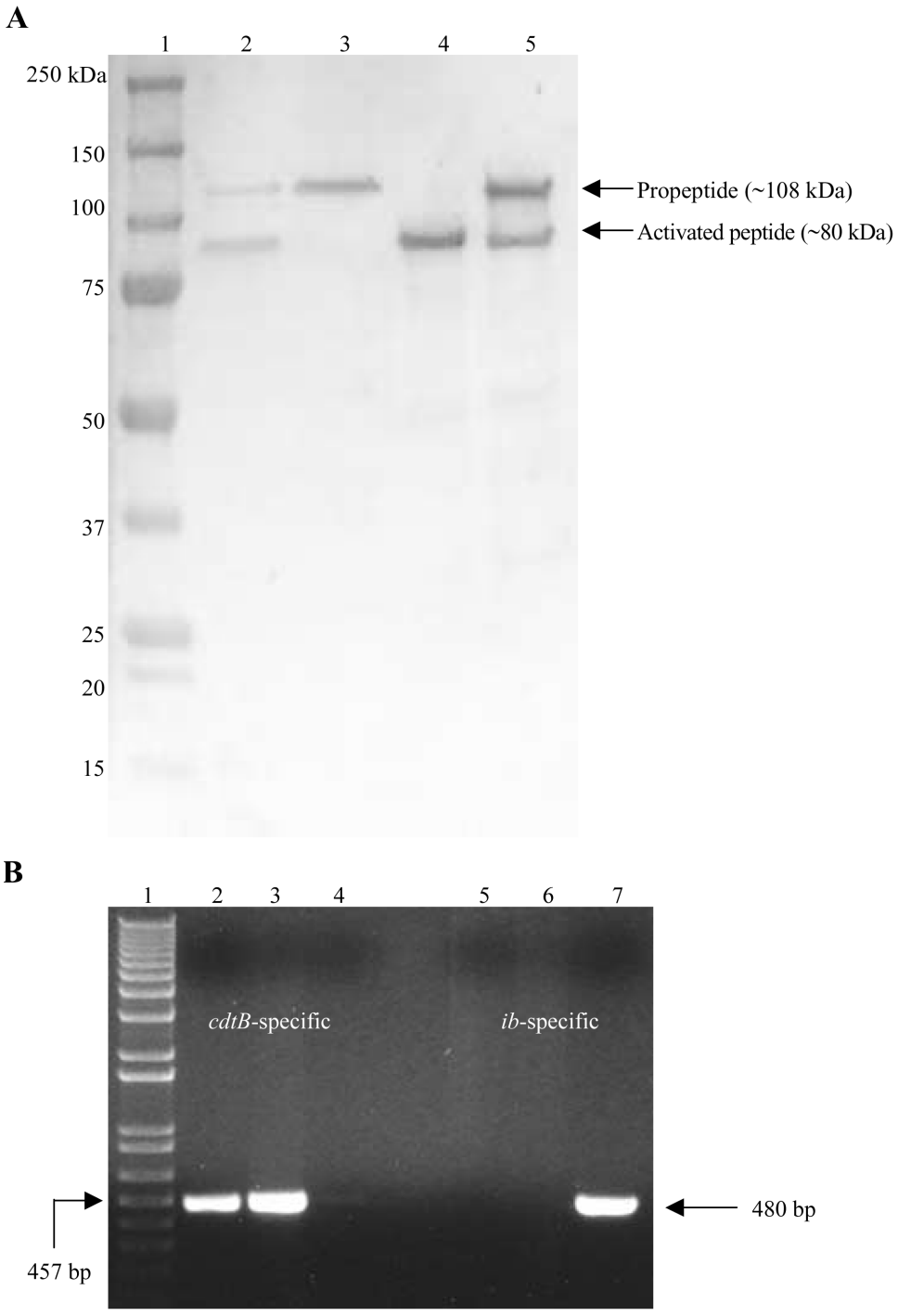

Figure 1. Activation of the binding components of binary toxin CDT and iota toxin and amplification of their corresponding genes. *A,* Western blot using monoclonal antibody 1D11 to detect propeptide and activated binary toxin B components in $A^-B^-CDT^+$ *Clostridium difficile* and *Clostridium perfringens* type E strains. *Lane 1,* Precision Plus Protein Standards (BioRad); *lane 2,* $A^-B^-CDT^+$ *C. difficile* strain IS58 after trypsinization; *lane 3,* strain IS58 without trypsin; *lane 4, C. perfringens* type E NCIB 10748 after trypsinization; *lane 5, C. perfringens* type E NCIB 10748 without trypsin. *C. difficile* strain IS58, but not the *C. perfringens* strain, requires exogenous trypsin to activate its binary toxin B component. Similar results were obtained using a Western blot assay with polyclonal anti–*Clostridium spiroforme* antibodies (results not shown). *B,* Polymerase chain reaction (PCR) of binary toxin genes from $A^-B^-CDT^+$ *C. difficile* strain and *C. perfringens* type E strain. A *cdtB*-specific PCR was used in lanes 2–4, and an *ib*-specific PCR was used in lanes 5–7. *Lane 1,* 1 kb Plus DNA ladder; *lanes 2* and *5, C. difficile* strain IS58 ($A^-B^-CDT^+$); *lanes 3* and *6, C. difficile* strain Pitt 45 ($A^+B^+CDT^+$); *lanes 4* and *7, C. perfringens* type E NCIB 10748. The *ib*-specific PCR amplifies an Ala:Ala clip site coding region that allows for activation of the protein by endogenous protease.

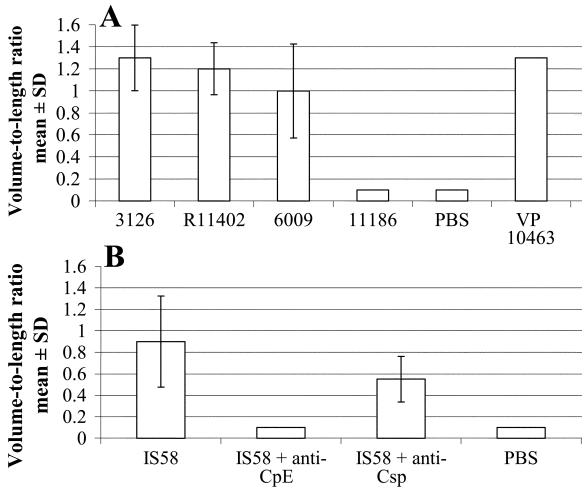

Figure 2. Rabbit ileal loop assay results for supernatants from binary CDT toxin–positive, large clostridial toxin–negative strains and neutralization of enterotoxic response using antisera raised against other binary toxin–positive clostridia. *A,* Mean (±SD) volume-to-length ratios for $A^-B^-CDT^+$ strains 3126, R11402, and 6009 (1.3 ± 0.3, 1.2 ± 0.2, and 1.1 ± 0.4, respectively) are compared with the mean ratios for the 2 negative controls, $A^-B^-CDT^-$ strain 11186 (0.1 ± 0) and PBS alone (0.1 ± 0), and the positive control, $A^+B^+CDT^-$ strain VPI 10463 (1.3). The VPI 10463 control (which was not run in quadruplicate) reflects the activity of toxin A and, therefore, did not require concentration or trypsin. The enterotoxic activity of toxin A is a reason that the role of binary toxin CDT has been difficult to establish in $A^+B^+CDT^+$ strains. $P \leq .005$ for all 3 $A^-B^-CDT^+$ strains compared with either negative control (Student's *t* test). *B,* Mean (±SD) volume-to-length ratios for $A^-B^-CDT^+$ strain IS58 (0.9 ± 0.4) compared with the results for the same strain after incubation of the supernatant with anti–*Clostridium perfringens* type E strain NCIB 10748 (CpE; 0.1 ± 0) or anti–*Clostridium spiroforme* strain NCTC 11493 (Csp; 0.5 ± 0.2) antisera: PBS alone, 0.1.

quirement for exogenous trypsin activation of CDTb (*C. difficile* binary toxin), the following PCR assays were designed. The forward primer for the first assay (*ib*-specific), 5′-GTAAGATTTTTTAGCGCAGCC-3′, was based on the reported sequence for *ib* (GenBank accession number X73562) and included the GCAGCC sequence coding for the Ala:Ala clip site recognized by endogenous clostridial protease. The forward primer for the second assay (*cdtB*-specific), 5′-CCAAAGTTGATGTCTGAT-3′, was based on the reported sequence for *cdtB* (GenBank accession number L76081) and matched the region in *ib* as closely as possible but did not contain the GCAGCC sequence. The reverse primers for both assays (5′-CAGCAGTTGAATTATCTGTTG -3′) were identical. The predicted *ib* amplicon was 480 bp, and the *cdtB* amplicon was 457 bp. Thermocycling conditions were as follows: 30 cycles at 94°C for 30 s, at 57°C for 60 s, and at 72°C for 60 s, and a holding temperature of 4°C.

Rabbit ileal loops. *C. difficile* isolates 3126, R11402, and 6009 were grown in brain-heart infusion dialysis sacs [4] at 37°C for 3 days. Culture supernatants were concentrated 10-fold by precipitation at 4°C for 4 h using ammonium sulfate (70% saturation) and dialysis in 10 mmol/L Tris buffer (pH 7.5) at 4°C, to remove residual ammonium sulfate [28]. Concentrates were dispensed through a filter (0.22 μm) into glass tubes and were stored under nitrogen at 4°C. Aliquots (4.5 mL) were trypsinized with 500 μL of 1% trypsin [29] to activate CDTb and then were concentrated 10-fold at 4°C (B15 Minicon; Millipore). Ileal loops were inoculated with 500 μL of test samples containing 450 μL of 100× culture fluid.

The ileal loop assay was performed as described elsewhere [30]. After review and approval of the protocol by the Virginia Polytechnic Institute's Animal Care Committee, New Zealand White rabbits (>12 weeks old) were fasted overnight and were anesthetized. After laparotomy, the ileum was identified, and 7 ileal loop segments (7–10 cm) were constructed by ligatures in each rabbit. After inoculation of the loops with test samples, the ileum was returned to the abdominal cavity, and the abdomen was closed. Each isolate supernatant was tested in 4 loop segments. Sixteen hours after surgery, rabbits were killed, the ileum was removed, and the length and volume of fluid within the loop segments was recorded. Volume-to-length (V:L) ratios of >0.3 reflect enterotoxic activity. *C. difficile* strain VPI 10463 ($A^+B^+CDT^-$) was used as a positive control (0.5 mL of filtered supernatant, not concentrated, not trypsinized). Two negative controls were used: *C. difficile* strain 11186 ($A^-B^-CDT^-$) supernatant prepared as described above, and PBS (also treated with trypsin).

Next, neutralization of the supernatant enterotoxic activity from the $A^-B^-CDT^+$ strain IS58 was tested using polyclonal antisera raised against other binary toxin–producing clostridia. Fresh sac culture fluids were concentrated 50-fold in Minicons in a refrigerator and then were trypsinized. After antitoxins or buffer was added, each loop was inoculated with 1 mL of test samples containing 900 μL of 50× culture fluid. IS58 supernatant was also preincubated with 100 μL of goat antiserum [28] raised to toxoid preparations of either *C. spiroforme* strain NCTC 11493 or *C. perfringens* type E strain NCIB 10748 30 min at room temperature. Neutralization experiments were tested in duplicate.

Hamster challenge assay. Antimicrobial susceptibility of the $A^-B^-CDT^+$ strains used in hamster experiments was tested by a modification of the E-strip method (AB Biodisk) using clindamycin and erythromycin E-strips, as described elsewhere [31].

Young adult Syrian golden hamsters, 90–100 g each, were individually maintained in sterilized cages. The spore inocula used for the challenge studies were prepared as described elsewhere [31]. Before treatment and inoculation, a sample of the hamster's fecal pellets was cultured using selective TCCFA (taurocholate, cycloserine, cefoxitin, and fructose agar) media to exclude prior *C. difficile* colonization. Each hamster received a single dose of

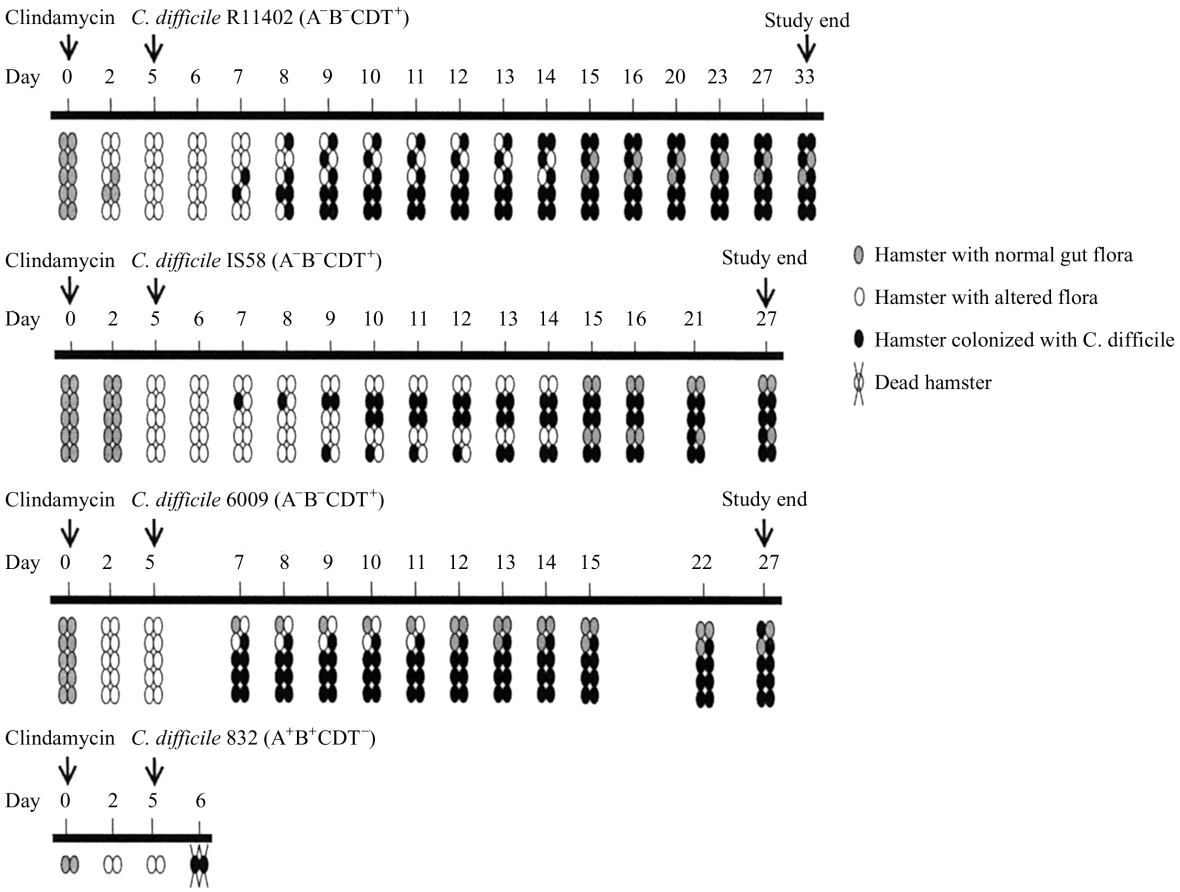

Figure 3. Hamster model of *Clostridium difficile* infection: hamsters ($n = 10$/group) were challenged with $A^-B^-CDT^+$ *C. difficile* strains 5 days after treatment with clindamycin. Presumed normal intestinal flora was indicated by small violaceous colonies on culture of fecal samples with taurocholate, cycloserine, cefoxitin, fructose agar (TCCFA); altered flora was indicated by completely negative results of fecal cultures with TCCFA. Positive control hamsters challenged with the $A^+B^+CDT^-$ strain 832 are shown for comparison.

clindamycin (30 mg/kg) orogastrically 5 days before challenge with *C. difficile* spores. An inoculum of 100 cfu/hamster was administered intragastrically to groups of 10 hamsters, which were then monitored for onset, duration, and outcome of colonization by serial culture of fecal pellets and observation for disease symptoms for 3–4 weeks after *C. difficile* administration. At the end of the observation period, colonization with the strain originally administered was confirmed by REA typing and toxinotyping using one *C. difficile* colony isolated from the TCCFA culture of cecal contents from each of 3 or 4 hamsters per group. Ceca from these same hamsters were placed in 10% formalin for subsequent histological examination.

Two hamsters were administered *C. difficile* strain 832 ($A^+B^+CDT^-$, REA type B1, toxinotype 0) and served as positive controls. Strains of B1 REA type have been associated with a high rate of CDAD versus asymptomatic colonization in human patients, and this particular strain was highly virulent in our hamster model [31]. Negative controls included 2 hamsters given clindamycin but not challenged with *C. difficile,* as well as 2 hamsters given neither clindamycin nor *C. difficile.* Preserved cecal tissue was embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Blinded samples were examined microscopically by a pathologist (Dr. Hidejiro Yokoo, Lakeside VA Hospital, Chicago).

RESULTS

$A^-B^-CDT^+$ strain characterization. REA typing and toxinotyping showed good correlation (table 1). Strains IS58, R11402, and 3126 belonged to the same REA group (AA) and the same, unique toxinotype (XI). Toxinotype XI is characterized by a truncated PaLoc with only a portion of the 3′ end of *tcdA* and *tcdC* still present [19, 32]. Strain 6009 has a markedly different REA pattern (REA group CS); it also lacks the entire PaLoc and therefore does not have a toxinotype designation per se. Southern blot hybridization with *tcdA* and *tcdB* probes confirmed the PCR results. All 4 strains—IS58, R11402, 3126 and 6009—were confirmed to lack production of the large clostridial toxins TcdA and TcdB but were positive for binary toxin CDT by Western blot (table 1).

Supernatant from strain IS58 demonstrated a band of ~108 kDa when tested in the monoclonal antibody 1D11 Western blot assay (figure 1*A*), which is consistent in size with the propeptide (unactivated) form of CDTb. After trypsinization, IS58 supernatant showed a band of ~80 kDa in this assay, which is consistent with activated CDTb (figure 1*A*). In contrast, supernatant from *C. perfringens* type E strain NCIB 10748 showed a band of ~80 kDa with and without trypsinization, which is consistent with activated Ib. Similar results were obtained in the polyclonal anti–*C. spiroforme* Western blot assay.

Next, 2 PCR assays were used to amplify similar regions of the binary toxin genes from *C. perfringens* and *C. difficile,* one that included the coding region for the clip site recognized by endogenous clostridial protease (*ib*-specific) and one that did not amplify this region (*cdtB*-specific). DNA from strain IS58 and *C. difficile* strain Pitt 45 ($A^+B^+CDT^+$), but not from the *C. perfringens* type E strain, was amplified in the *cdtB*-specific assay (figure 1*B*). In contrast, only DNA from the *C. perfringens* type E strain was amplified in the *ib*-specific assay (figure 1*B*). These results provide a molecular explanation for exogenous activation of CDTb compared with the endogenous activation of the otherwise highly similar binding component (Ib) of *C. perfringens* iota toxin.

Rabbit ileal loops. Inoculation of ileal loop segments with concentrated, trypsinized supernatants from all 4 $A^-B^-CDT^+$ strains showed marked nonhemorrhagic fluid responses (figure 2), whereas no fluid accumulation was observed in the negative controls ($A^-B^-CDT^-$ strain 11186 and PBS). A vigorous enterotoxic response to the $A^-B^-CDT^+$ strains was indicated by mean V:L ratios of 0.9–1.3, compared with mean V:L ratios of <0.3 for the negative controls. The visible character of the fluid in response to the $A^-B^-CDT^+$ supernatants was watery, pale brown, and moderately clear, with no obvious hemorrhage, in distinction to the hemorrhagic fluid response to the $A^+B^+CDT^-$ (strain VPI 10463) unconcentrated supernatant. The fluid response to one $A^-B^-CDT^+$ strain (IS58) was completely neutralized with anti–*C. perfringens* type E sera and partially neutralized with anti–*C. spiroforme* sera (figure 2*B*). The partial neutralization by *C. spiroforme* antiserum was likely due to the lower antitoxin titer than that of the *C. perfringens* antiserum, on the basis of crossed immunogel electrophoresis.

Hamster challenge. Three $A^-B^-CDT^+$ strains of different REA types (AA1, AA3, and CS1) and toxinotypes (XIa, XIb, and PaLoc-Neg) were selected for testing in the widely used hamster model of *C. difficile* disease. Inoculation of hamsters with strains R11402, IS58, or 6009 resulted in colonization of 70%–80% of the hamsters (table 1; figure 3). Onset of colonization was variable, ranging from 1 to 22 days after challenge (median, 3 days). Colonization onset after challenge with strain 6009 was similar to that after challenge with the control $A^+B^+CDT^-$ strain 832 but earlier than after challenge with the other $A^-B^-CDT^+$ strains (figure 3). Susceptibility to clindamycin, however, was similar for all 3 $A^-B^-CDT^+$ strains; MICs at 24 and 48 h were 1.5 and 2.0 μg/mL, 0.5 and 1.0 μg/mL, and 2.0 and 2.0 μg/mL for strains R11402, IS58, and 6009, respectively. In contrast, the MIC for strain 832 was >256 μg/mL. None of the hamsters showed signs of disease or died during the observation period (22–28 days) after administration of the $A^-B^-CDT^+$ strains. Control hamsters died shortly after or coincident with colonization after challenge with the $A^+B^+CDT^-$ strain 832. Cecal tissue from the $A^+B^+CDT^-$-challenged hamsters showed extensive mucosal hemorrhage and infiltration with polymorphonuclear white blood cells. No histological changes compared with noninfected hamsters were apparent in cecal tissue from the hamsters challenged with the $A^-B^-CDT^+$ strains.

All 4 negative control hamsters remained uncolonized with *C. difficile* and showed no signs of disease throughout the observation period (19 days). Fecal pellet cultures from the control hamsters that were neither given clindamycin nor challenged with *C. difficile* showed small violaceous colonies on the TCCFA plates, representing non–*C. difficile* bacterial flora that were able to grow on this relatively selective *C. difficile* media. Alteration of normal intestinal flora, as evidenced by complete clearing of the TCCFA fecal pellet cultures, was observed in the control hamsters that were given clindamycin but not challenged with *C. difficile.* Return of the small colonies on TCCFA fecal cultures and presumed restoration of the normal intestinal flora was noted ~14 days after clindamycin administration.

DISCUSSION

In contrast to the more defined roles of TcdA and TcdB in the pathogenesis of CDAD [3, 33], the contribution of binary toxin CDT has been difficult to determine because most CDT^+ *C. difficile* strains are also A^+B^+ [5, 26, 34]. Production of multiple toxins is characteristic of the clostridia [35], and because genetic manipulation and gene knockout studies are not yet possible in *C. difficile,* characterization of naturally occurring genetic variants has been important when ascribing potential virulence for particular toxins. We and other researchers have recently described strains of *C. difficile* that produce binary toxin CDT but do not produce either of the large clostridial toxins [7, 19]. The results of this present study in 2 animal models support a potential adjunctive role for binary toxin CDT.

Supernatants from all 4 $A^-B^-CDT^+$ strains caused obvious enterotoxic effects in the ileal loop assay. The neutralization of this response with antisera raised against other binary toxin–producing clostridia suggests that binary toxin CDT was responsible for the enterotoxicity. The iota toxin produced by *C. perfringens* type E and the iota-like toxin produced by *C. spiroforme* are the suspected virulence factors for hemorrhagic enteritis in neonatal calves and scouring in rabbits, respectively

[36, 37]. Iota toxin, unlike binary toxin CDT, is activated by endogenous protease [38]. As demonstrated in figure 1, this difference is likely due an Ala:Ala clip site (encoded by the GCAGCC sequence in *ib*) that is present in iota toxin but not CDT. The nonhemorrhagic fluid response to the $A^-B^-CDT^+$ supernatants was similar to the fluid response seen with *C. spiroforme* in the ileal loop assay (R.J.C., unpublished data). In contrast, the fluid response seen with supernatants from $A^+B^+CDT^-$ strains is grossly hemorrhagic. Purified TcdA alone can reproduce this effect in the ileal loop assay, and TcdA administered to hamsters intragastrically results in diarrhea and death [4]. Unlike *C. difficile* binary toxin, *C. spiroforme* iota toxin requires neither concentration nor activation to elicit its in vitro biological activity [39], and *C. spiroforme* has been shown to cause fatal disease in antibiotic-treated hamsters [40]. The need for concentration of supernatants from these binary toxin–producing *C. difficile* strains is likely related to lower toxin production [26].

Despite the enterotoxicity observed in the ileal loop assay, none of the hamsters challenged with the $A^-B^-CDT^+$ strains developed diarrhea, died, or had histological changes noted in the cecum, yet 70%–80% of the hamsters became colonized after challenge. Control hamsters demonstrated disruption of the intestinal flora after administration of clindamycin and died after being given $A^+B^+CDT^-$ strains. The hamster is a widely accepted animal model for CDAD. Similar to human disease, disruption of normal intestinal bacterial flora with antibiotics enables colonization of the hamster with *C. difficile*. Disease following infection with $A^+B^+CDT^-$ strains is usually of short duration and fatal. Pathological findings (i.e., fluid cecal content, cecal dilatation, and proliferative mucosal changes accompanying inflammation and hemorrhage) are localized primarily to the cecum [41]. Similar to results obtained by other researchers using an $A^+B^+CDT^+$ strain [26], the supernatants from our $A^-B^-CDT^+$ strains needed concentration and trypsin to demonstrate biological effect (cytotoxicity or enterotoxicity). It is possible that the lack of disease in hamsters challenged with the $A^-B^-CDT^+$ strains is related to the low amount of toxin produced by these strains. In addition, hamsters may be less sensitive than other animals to binary toxin. The absence of disease following colonization of hamsters with the $A^-B^-CDT^+$ strains may not, however, be unexpected. Of the 9 $A^-B^-CDT^+$ strains reported in the literature, only 1 was recovered from a patient with diarrhea [7, 19]. We hypothesize that binary toxin CDT alone may not be sufficient to cause disease but that this toxin may play an adjunctive role in the pathogenesis of disease caused by A^+B^+ strains.

In multiple surveys of toxigenic clinical *C. difficile* isolates (A^+B^+), binary toxin has been seen in a low percentage (<10%) of strains [5, 7, 8, 10–14]. However, one study from a reference laboratory in Italy noted an increasing prevalence of binary toxin–positive strains ($A^+B^+CDT^+$) over time [42]: 0, 24%, and 45% of isolates collected prior to 1990, from 1991 to 1999, and from 2000 to 2001, respectively, had binary toxin genes. In one US hospital in which CDAD disease severity was striking, an unprecedented percentage of strains (65%) were positive for binary toxin CDT genes [15]. Although the analysis did not achieve significance, there was a suggestion of correlation between disease severity and binary toxin–positive strains, and a newly recognized epidemic strain (designated by REA as group BI) was recovered from many of these patients [16]. Another study showed that binary toxin–producing strains were more often community-acquired and more often represented the cause of hospitalization, suggesting an association with more severe disease [43]. The potential importance of binary toxin CDT is highlighted by the wide dissemination of the epidemic BI strain that has caused numerous US and Quebec hospital outbreaks of CDAD since 2001, often associated with increased disease severity [18]. This strain (designated by REA typing as group BI) is notable for genetic variations within the PaLoc and fluoroquinolone resistance as well as the presence of binary toxin CDT genes [18]. The relative contribution of binary toxin CDT to severity of disease seen in patients infected with these strains and the mechanism whereby binary toxin may be linked to the large clostridial toxins or some other unrecognized virulence factor needs further study.

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