Vaccination With Parenteral Toxoid B Protects Hamsters Against Lethal Challenge With Toxin A–Negative, Toxin B–Positive Clostridium difficile but Does Not Prevent Colonization

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Toxin A has historically been regarded as the primary virulence determinant in Clostridium difficile infection, but naturally occurring toxin A–negative, toxin B–positive (A−/B+) C. difficile strains are known to be virulent. To determine the role of toxin B in these strains, we immunized hamsters with a toxoid prepared from purified toxin B to determine whether they would be protected from lethal challenge with an A−/B+ strain of C. difficile.

After intraperitoneal vaccinations with toxoid B, 12 clindamycin-treated hamsters were challenged with 10⁶ spores of a toxin A–negative, toxin B–positive (A−/B+) Clostridium difficile strain, CF2. All 12 vaccinated hamsters were colonized within 2 days of CF2 challenge and remained symptom-free during a 30-day monitoring period, despite detection of fecal cytotoxicity (toxin B effect) in each animal. In contrast, only 1 of 10 unvaccinated hamsters survived the same challenge (P ≤ .0002). These data support the toxin B as an important virulence determinant in C. difficile.

C. difficile infection (CDI) is known to be toxin mediated, but the relative importance of the different toxins produced is still debated. Two large clostridial toxins (LCTs), toxins A and B, are implicated in the pathogenesis of CDI. There is a high degree of similarity in the amino acid sequence of these LCTs, and they are both single-unit toxins with receptor binding, translocation, and enzymatic domains [1]. LCTs mediate their toxic effects by monoglucosyltransferase activity on small guanosine triphosphatases of the Rho subfamily of proteins involved in actin/cytoskeleton organization. Despite similarities in structure and intracellular targets, they differ in activity when purified and analyzed by in vitro and in vivo toxin assays. Animal studies revealed that purified toxin A alone could induce many features of CDI when administered intragastrically to hamsters or injected into isolated rabbit ileal loops, whereas toxin B did not cause these effects unless it was coadministered with a subtoxic concentration of toxin A [2]. These data suggested that the toxins acted synergistically but that toxin A was the more important virulence factor that initiated the pathogenic process. However, toxin B is much more cytotoxic than toxin A for a variety of eukaryotic cell lines [3, 4], and the original diagnostic assay for C. difficile in clinical laboratories, the cell culture cytotoxicity assay, was primarily a measure of toxin B activity in patient fecal specimens [5].

Naturally occurring C. difficile strains that produce toxin B, but no functional toxin A, have been detected worldwide, but they were initially regarded as non-pathogenic because they did not produce toxin A and were frequently recovered from asymptomatic children
C. difficile against challenge with the CF2 strain of purified toxin B, to determine whether they would be protected we proposed to immunize animals with a toxoid prepared from

## METHODS

### Preparation of Toxoid

Purified toxin B was prepared from C. difficile strain VPI 10463 by D. Lyerly (TechLab) using diethylaminoethyl (DEAE) Sepharose CL-6B chromatography, followed by Sephacryl S-200 and S-300 gel filtration and immunoabsorption [18]. VPI 10463 was selected for vaccine development because it is an efficient producer of standard toxin B, whereas CF2 is a relatively poor toxin producer (D. Lyerly, personal communication). Toxin B (0.8 mg) was mixed with 37% formaldehyde and incubated overnight at 37°C with shaking, resulting in a final toxoid concentration of 0.396 µg/µL. The toxoid preparation was confirmed to be inactive by cytotoxicity assay (Bartel’s cytotoxicity assay for C. difficile toxin; Trinity Biotech). The readings were taken at 0, 24, and 48 hours. Cells treated with the toxoid showed no or little cell rounding compared with the positive control (purified toxin B not treated with formaldehyde).

### Vaccine Preparation

Monophosphoryl lipid A (0.5 mg/mL) and synthetic trehalose dicorynomycolate emulsion (0.5 mg/mL) (Ribi Adjuvant System; Sigma-Aldrich) were resuspended in sterile saline and mixed with the toxoid so that the final concentration of toxoid was 33 ng/µL. The initial vaccine dose was 10 µg toxoid (300-µL volume per injection). Booster doses (second and third vaccinations) were made at half-strength adjuvant and toxoid (5 µg toxoid), the negative control was made by adding 2 mL of sterile saline alone to the adjuvant vial, and comparable volumes were administered.

### Animals and Immunization Regimens

This study was approved by the Hines VA Institutional Animal Care and Use Committee. The 7–8-week-old, 90–110-g specific-pathogen-free Syrian hamsters (Charles River Laboratories) were housed individually, and animal husbandry was performed as described elsewhere [15]. In these experiments, we followed our established hamster protocol, in which hamsters are pretreated with clindamycin, challenged with C. difficile spores, and monitored for colonization and disease [15]. Colonization in this model is defined as repeated recovery of C. difficile from hamster feces [15]. Our previous experience indicates that clindamycin-treated hamsters who have positive fecal cultures after challenge with virulent strains of C. difficile remain colonized until they succumb to infection, as in the case of A+/B+ strains, or until the end of the experiment, as in the case of the A−/B− strain, CF2 [15].

Before the vaccination experiment, C. difficile spore inoculum was optimized using a separate group of 10 hamsters. Considering the incomplete colonization and lower mortality seen when hamsters were challenged with CF2 using our standard inoculum of 100 spores [15], we empirically increased the inoculum to 10^6 spores. Other than the spore inoculum, the same protocol was followed, including clindamycin administration, timing of CF2 challenge, and the 2-month postchallenge monitoring [15]. CF2 spores were prepared as described elsewhere [15], using similar growth, harvesting, and titering methods.

Twenty animals were used for the vaccination study. One hamster was euthanized on day 0, and serum was collected for preimmune serum control. Eighteen hamsters received intraperitoneal toxoid-adjuvant vaccine, and 2 hamsters were vaccinated with adjuvant alone. All hamsters were observed twice
daily for the first 4 days. Thereafter, the observations were reduced to once daily. Hamsters were monitored for signs of wet tail and altered activity. If hamsters were unable to ambulate, retain sternal recumbency, or eat or drink or if they lost ≥15% of their weight (they were weighed 3 times weekly), they were euthanized. The cages were changed on day 7 or 8, and fecal pellets were obtained the following day. The second vaccination was administered on day 14, and hamsters were placed in cages containing fresh bedding. Fecal pellets from the cages were collected the next day, and the hamsters were monitored twice daily for 3 days and once a day thereafter. A third vaccination was administered on day 21, and observations were recorded as described above. Three vaccinated hamsters were euthanized on day 25 (preclindamycin treatment), and serum samples and fecal pellets were collected. The remaining 15 toxoid-vaccinated hamsters were given clindamycin orogastrically on day 26 to predispose them to CDI. The 2 adjuvant control hamsters were also treated with clindamycin. On day 31, 3 hamsters (after vaccine, 5 days after clindamycin) were euthanized. Twelve hamsters (toxoid vaccine plus clindamycin) and the 2 control hamsters (adjuvant alone plus clindamycin) were challenged orogastrically with 10^6 spores of the A−/B+ C. difficile strain CF2 (isolate 5340) [8]. Hamsters were monitored for 30 days after CF2 challenge, and all surviving hamsters were euthanized on day 61 (35 days after clindamycin). Serum samples and fecal pellets were collected.

**Cytotoxicity of Hamster Fecal Pellets**

Sterile phosphate-buffered saline (PBS; 5 μL) was added to 1 mg of dry fecal pellet weight, with each sample containing 3 pellets. The PBS-pellet mix was incubated on ice for 1.5 hours, then vortexed to homogenize. A 100-mg sample was used for cytotoxicity testing in the Bartel’s cytotoxicity assay. Diluent (0.5 mL) from the assay kit was added to 100 mg of fecal PBS homogenate. The sample was vortexed thoroughly and centrifuged for 10 min at 6000 g. The supernatant was passed through a 0.45-μm filter before use. For each sample, 2 × 100-μL reactions were prepared. Diluent (100 μL) was added to one reaction, and 100 μL of antitoxin was added to the other. The controls were prepared according to instructions in the kit insert: (1) toxin control (100 μL of toxin and 100 μL of diluent); (2) antitoxin control (100 μL of antitoxin and 100 μL of diluent); (3) neutralization control (100 μL of toxin and 100 μL of antitoxin); and (4) blank (cell viability control) (200 μL of diluent). The reaction mixture was incubated at room temperature for 30 minutes. Fifty microliters of each reaction mixture was added to a tissue culture well in the assay kit and incubated at 35°C–37°C. Cell rounding was recorded at 24 and 48 hours.

**Cytotoxicity of Hamster Fecal Pellets and REA Typing of C. difficile Isolates**

Part of the fecal pellet-PBS suspension prepared as above was inoculated onto selective sodium taurocholate fructose agarose plates containing cycloserine and cefoxitin (TCCFA) and incubated anaerobically for 48 hours. C. difficile colonies were subcultured onto blood agarose plates or cultured for a second time on TCCFA plates if isolated colonies could not be picked. C. difficile colony morphology was checked on the blood agarose plates, and a single colony was inoculated into 30 mL of brain-heart infusion medium. The brain-heart infusion culture was allowed to grow overnight anaerobically, bacteria were harvested, and DNA was extracted and subjected to HindIII restriction digestion [19]. The restriction patterns were compared with the known pattern for REA type CF2 to confirm colonization with the same strain used to inoculate the hamster.

**In Vitro Serum Neutralization Assay**

Bartel’s cytotoxicity assay was used to measure the ability of serum from vaccinated hamsters to neutralize the cytotoxic effects of toxin B in vitro. One hundred picograms of purified toxin B was added per well. Serum samples from vaccinated hamsters (3 hamsters euthanized after vaccination and before clindamycin administration, 3 hamsters euthanized after clindamycin administration, and 12 vaccinated, clindamycin-treated hamsters euthanized 30 days after challenge with CF2) and from nonvaccinated and adjuvant-only inoculated hamsters were diluted 1:12.5, 1:25, and 1:50 in PBS and added to the 100 pg of toxin B, then allowed to incubate for 30 minutes at room temperature, before addition of 50 μL of the mixture to the tissue culture cells. Cell survival was recorded after 24-hour incubation at 35°C, and the cytopathic effect was recorded and scored as follows: ½+ indicated ~25% cell rounding, +, ~50% rounding, and ++, ~75% rounding.

**In Vitro Assays of Toxin B and Supernatant From Strain CF2**

Purified Toxin B was also prepared from C. difficile strain CF2 (isolate 5340, [15]) and the A+/B+ strain B1 (isolate 832, [15]) by D. Lyerly using DEAE Sepharose CL-6B chromatography, followed by Sephacryl S-200 and S-300 gel filtration and immunoabsorption [18]. The purified toxins were then added to a monolayer of fibroblast cells, and the cells were photographed at 48 hours to record morphology.

Supernatants from these same strains (CF2 and B1) were also tested for potential disruption of intestinal epithelial cell barrier function in a polarized cell monolayer tight-junction assay [20]. Cultured human intestinal epithelial (T84) cells were grown to confluence on 0.33-cm² collagen-coated permeable supports (Transwell; Corning). Supernatants from 48-hour broth cultures of strain B1 (neat) and strain CF2 (10-fold concentration) were then added to the apical surface of the monolayer, and transepithelial electrical resistance (TER) was measured at 2-hour intervals by passing 25 μA of current and measuring the resulting voltage deflection and applying Ohm’s law (V = IR) to calculate resistance. Only monolayers expressing resistance >400 Ω cm² at baseline were used. Experiments were repeated in quadruplicate.
RESULTS

Hamster Challenge With High Spore Inoculum
All 10 hamsters challenged with 10^6 CF2 spores became colonized (compared with 6 of 10 challenged with 100 spores), and 9 of the 10 hamsters died (compared with 3 of 10 challenged with 100 spores) (Figure 1). Hamsters colonized with the higher inoculum died at a mean of 4.7 days (range, 3–12 days) after challenge, compared with a mean of 10 days (range, 7–13 days) after challenge with the lower inoculum of CF2, as reported elsewhere [15].

Hamster Challenge After Toxoid B Immunization
All 12 toxoid B–vaccinated hamsters that were challenged with 10^6 CF2 spores became colonized within 1–2 days. All 12 vaccinated animals remained colonized until the end of the experiment, and none died (Figure 2). In addition, none of the vaccinated hamsters developed wet tail or had noticeable changes in activity. The 2 control hamsters given adjuvant alone died 6 and 13 days after CF2 challenge, respectively (Figure 2). REA typing results of C. difficile isolates recovered from fecal pellets collected after CF2 challenge confirmed that hamsters were colonized with CF2. REA type T7 was recovered from 1 hamster (No. 246), indicating that cocolonization with T7 (a nontoxigenic C. difficile strain) and REA typing of the isolate from another hamster (No. 252) failed to produce a restriction pattern, but cytotoxicity was demonstrated in the fecal pellets from both of these hamsters, indicating that CF2 was present. In summary, all 12 vaccinated hamsters survived challenge with 10^6 colony-forming units of CF2, compared with only 1 of 10 unvaccinated hamsters (P ≤ .0002, Fisher exact test).

Cytotoxicity of Hamster Fecal Pellets
Fecal pellets from all 12 hamsters immunized with toxoid B and 1 of the hamsters immunized with adjuvant alone and subsequently challenged with CF2 were cytotoxic (Table 1). Fecal pellets were obtained 10 days after CF2 challenge. The second hamster immunized with adjuvant alone died before the fecal pellets could be collected.

Neutralization of fecal cytotoxicity was demonstrated by anti–C. difficile toxin antibody in all 12 specimens from the vaccinated and CF2-challenged hamsters and 1 hamster that received adjuvant only, followed by CF2 challenge (Table 1). Neutralization was demonstrated for 6 of the fecal specimens when incubated at the initial dilution with antitoxin. Neutralization was demonstrated in the other 7 fecal specimens when these specimens were diluted 1:25 with buffer before incubation with antitoxin.

In Vitro Serum Neutralization
Except for 1 specimen (from hamster 248), all the other serum samples from vaccinated, clindamycin-treated, CF2-challenged hamsters neutralized 100 pg of toxin B at 1:12.5 and 1:25 dilutions (Table 1). These samples were obtained at the end of the experiment, at the time of euthanasia. The control serum specimen from the nonvaccinated hamster did not neutralize toxin B. Antiserum alone had no effect on the test cells.

In Vitro Effects of Toxin B and Supernatant From Strain CF2
Purified toxin B from A-/B+ strain CF2 demonstrated a noticeably different cytopathic effect on fibroblast cells than that due to purified toxin B from a standard A+/B+ strain (REA type B1) shown by more profound cell rounding and detachment with the variant toxin B from CF2 (data not shown). Supernatants from these strains also showed differences in the TER of polarized T84 cells over time (Figure 3). The TER drop was nearly complete at 6 hours with supernatant from strain B1 (A+/B+). However, the supernatant from strain CF2 demonstrated only a 32% decrease in resistance by 12 hours.

DISCUSSION
The role of the 2 LCTs in C. difficile pathogenesis has been a subject of ongoing debate [21]. Construction of isogenic
mutants of *C. difficile* have recently been reported by 2 groups, and the *tcdA* mutants (toxin B–only producing) constructed from a standard toxigenic strain by both investigators were virulent in hamsters [16, 17]. Our results showing protection of hamsters by toxoid B vaccination using CF2, a toxin B–variant strain of *C. difficile*, support a major and independent role for toxin B in the pathogenesis of CDI, specifically due to A2/B1 CF strains.

The toxin B produced by REA group CF, toxinotype VIII strains has been shown to be a functional hybrid of 2 other LCTs, TcdB-10463 and TscL-1522 [14]. This variant toxin, designated TcdB-1470, produces a qualitatively different cytopathic effect on fibroblasts than that produced by TcdB-10463 (eg, wild-type toxin B), specifically, complete cell rounding without arborization [14]. This effect has been shown to be due to inactivation of R-Ras by TcdB-1470 [22], and there was speculation that this variant toxin B might be replacing the function historically ascribed to toxin A. However, the recent data showing virulence for mutants of a wild-type, toxin B–producing strain with toxin A gene deletion, as well as our data showing protection in hamsters vaccinated with a wild-type toxoid B and challenged with a TcdB-1470 variant strain, suggest that toxin B, regardless of its Rho substrate targets, is sufficient to cause disease. Other investigators using subcutaneously transplanted human intestinal xenografts in immunodeficient mice have concluded that toxin B, like toxin A, is a potent inflammatory enterotoxin for human intestine [23].

Our data also support a possible synergistic or additive role for toxin A, in that using comparable inoculum size, A2/B1 CF, toxinotype VIII strains demonstrated lower colonization efficiency and lower mortality in hamsters than A+/B+ strains [15]. The difference in TER drop using A2/B+ and A+/B+ strain supernatants in our polarized monolayer tight-junction assay (Figure 3) parallels the virulence differences in the hamster studies. Further work is needed to understand how these LCTs may work in concert to initiate *C. difficile* disease.

Although we demonstrated 100% protection from mortality in hamsters vaccinated with toxoid B and challenged with CF2, all of the hamsters were colonized and had cytotoxicity (primarily toxin B manifestation) demonstrated in their feces. Toxin-neutralizing antibodies were present in the serum of all but 1 of the hamsters and were probably of the immunoglobulin G class based on the route of vaccination. It is not clear whether these neutralizing serum antibodies are the mechanism of protection or whether they are a surrogate for another protective mechanism. The mechanism by which the serum neutralizing antibodies may confer protection against a mucosal pathogen, such as *C. difficile*,

### Table 1. Toxin B–Mediated Cytotoxicity of Hamster Feces 10 Days After CF2 Challenge and Neutralization of Cytotoxicity With Hamster Serum Samples After Toxoid B Vaccination and 30 Days After CF2 Challenge

<table>
<thead>
<tr>
<th>Hamster No.</th>
<th>Hamster feces cytotoxicity</th>
<th>Toxin B neutralization (toxin B plus hamster serum sample)</th>
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<td>Feces only</td>
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CF2 is a toxin A–negative, toxin B–positive (A−/B+) *Clostridium difficile* strain. For the scoring, 0 indicates no cell rounding, +, ~50% rounding, and ++, ~75% rounding.

- In hamster 246, follow-up fecal pellet culture and *C. difficile* isolate typing showed cocolonization with a nontoxigenic strain.
- Hamster 263 was given adjuvant only.
- Hamster 265 was not immunized.

Figure 3. Drop in transepithelial electrical resistance elicited by exposure of T84 cell monolayers to supernatants from *Clostridium difficile* restriction endonuclease analysis (REA) type CF2 (toxin A negative, B positive [A−/B+]) and REA type B1 (toxin A positive, B positive [A+/B+]).

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is not clear, but these antibodies may gain access to gut lumen as a result of some mild inflammation of the mucosa [24] caused by limited epithelial disruption mediated by localized toxin B activity, or there may be an independent means of transport of serum immunoglobulin G to the gut lumen. Regardless, our data support toxin B as an independent virulence factor for C. difficile disease and suggest that disease symptoms after human infection with CF, toxinotype VIII strains is due to toxin B.

Notes

Financial support. This work was supported by the Veterans Administration Merit Review Research program (S. J., D. N. G.).

Potential conflict of interest. S. J. has served on the advisory boards of Optimer, ViroPharma, Astellas, Pfizer, Cubist, and Bio-K. D. N. G. holds patents for the treatment and prevention of CDI, licensed to ViroPharma and is a consultant for Merck, ViroPharma, Optimer, Cubist, Hospira, The Medicines Company, Pfizer, Astellas, and Actelion and holds research grants from Merck, Eurofins Medinet, GOJO, Optimer, Sanofi-Pasteur, and ViroPharma. G. V. is a consultant for ViroPharma.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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