

# A Novel Multivalent, Single-Domain Antibody Targeting TcdA and TcdB Prevents Fulminant *Clostridium difficile* Infection in Mice

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The incidence of *Clostridium difficile* infection (CDI) and associated mortality have increased rapidly worldwide in recent years. Therefore, it is critical to develop new therapies for CDI. In this study, we generated a novel, potently neutralizing, tetravalent, and bispecific antibody composed of 2 heavy-chain-only V<sub>H</sub> (V<sub>H</sub>H) binding domains against both TcdA and TcdB (designated “ABA”) that reverses fulminant CDI in mice infected with an epidemic 027 strain after a single injection of the antibody. We demonstrated that ABA bound to both toxins simultaneously and displayed a significantly enhanced neutralizing activity both in vitro and in vivo. Additionally, ABA was able to broadly neutralize toxins from clinical *C. difficile* isolates that express both TcdA and TcdB but failed to neutralize the toxin from TcdA<sup>-</sup>TcdB<sup>+</sup> *C. difficile* strains. This study thus provides a rationale for the development of multivalent V<sub>H</sub>Hs that target both toxins and are broadly neutralizing for treating severe CDI.

**Keywords.** *Clostridium difficile*; toxins; antibody; V<sub>H</sub>H; immunotherapy.

*Clostridium difficile* is the most common cause of nosocomial antibiotic-associated diarrhea and is the etiologic agent of pseudomembranous colitis [1]. *C. difficile* infection (CDI) is primarily caused by 2 large exotoxins, TcdA and TcdB. It is estimated that >500 000 cases of CDI occur annually in the United States, with the yearly mortality rate ranging from 3% to 17%, depending on the strains. The incidence of CDI-associated mortality among patients is increasing rapidly because of

the emergence of hypervirulent and antibiotic-resistant strains [2], and systemic complications are the major cause of death in *C. difficile*-associated disease [3].

Primary treatment for CDI involves the use of the antibiotics metronidazole and/or vancomycin. However, neither antibiotic is entirely effective, as high rates of recurrence occur despite initial successful treatment with these antibiotics [4]. The incidence of recurrence is estimated to be 20%–35%, after which there is an even greater probability (as high as 50%) of additional recurrences [5, 6]. Fidaxomicin, newly approved by the Food and Drug Administration, shows an improved efficacy over vancomycin at lowering the relapse rate [7]. Several experimental therapies are currently under development, including novel antibiotics [7, 8], probiotic and fecal transplant therapies [9, 10], novel vaccines [11, 12], and antibody-based therapies [13–16].

Camelidae species, including camels, llamas, and alpacas, produce both conventional immunoglobulin G (IgG) antibodies containing a heavy chain and light chain (IgG<sub>1</sub>) and unconventional IgGs (IgG<sub>2</sub> and IgG<sub>3</sub>)

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that contain only a heavy chain (HCAbs) [17]. HCAbs bind antigens only through the variable domain of the heavy chain, thus allowing easy cloning of the DNA encoding this binding domain, which is known as a single-domain antibody or heavy-chain-only  $V_H$  ( $V_{HH}$ ) [17]. Several groups have used  $V_{HH}$ s for treating toxin-mediated diseases [18, 19]. Recently Hussack et al reported the generation of anti-Tcd  $V_{HH}$ s from llamas that target the receptor-binding domains of the 2 toxins, some of which possessed toxin-neutralizing activity [20, 21]. Here, we report the isolation of panels of  $V_{HH}$ s that recognize and neutralize either TcdA or TcdB. We further constructed fusion proteins consisting of multiple-antitoxin  $V_{HH}$ s, which neutralize both TcdA and TcdB and alleviate fulminant disease symptoms in mice infected with *C. difficile*.

## MATERIALS AND METHODS

### Immunogens and Immunization

Two alpacas were immunized with purified full-length recombinant TcdA or TcdB (aTcdA and aTcdB) containing point mutations that inactivate the glucosyltransferase activity of these toxins [12, 22], as described previously [23, 24]. All animals were handled and cared for according to institutional animal care and use committee guidelines and in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animals were immunized subcutaneously up to 5 times at intervals of  $\geq 3$  weeks with 50–100  $\mu$ g of aTcdA or aTcdB with alum adjuvant (with CpG in primary immunization). Blood samples were collected before each immunization for IgG titer determination. Five days following the final boost, peripheral blood lymphocytes (PBLs) were harvested as the source of  $V_{HH}$  genetic material.

### Construction of $V_{HH}$ Libraries and Screening

Two  $V_{HH}$  phage display libraries were generated to obtain  $V_{HH}$ s recognizing TcdA and TcdB. The libraries were produced from PBLs obtained from a aTcdA- or aTcdB-immune alpaca (described above), using methods previously described [25, 26]. Panning for  $V_{HH}$ -displayed phage was done as described previously [25, 26] or by pull-down methods, using biotinylated TcdA or TcdB. For pull-down selection, the recombinant TcdA and TcdB [22] proteins were biotinylated using the Pierce EZ-Link NHS-PEG4 Biotin kit (Pierce Biotechnology, Rockford, IL) per the manufacturer's instructions.  $V_{HH}$  clones were sequenced, and those with distinctly homologous complementarity-determining region sequences were considered to be clonally related; only the  $V_{HH}$  in each group that had the most potent toxin-neutralizing activity or that produced the strongest enzyme-linked immunosorbent assay (ELISA) signal was pursued further. Six unique TcdA-binding  $V_{HH}$ s and 11 unique TcdB-binding  $V_{HH}$ s were identified. Selected  $V_{HH}$  coding sequences

were cloned into the pET32b expression vector (Novagen) for cytosolic expression of  $V_{HH}$ s fused to thioredoxin in *Escherichia coli* Rosetta-gami 2 (DE3)pLacI cells (Novagen).

### Generation of the $V_{HH}$ Heterotetramer AH3/E3/E3/AA6ABA (ABA)

$V_{HH}$ s having the most potent neutralizing activity and the highest binding affinity to distinct, nonoverlapping epitopes targeting each toxin were chosen for inclusion within a multimeric, multivalent antibody. For TcdA,  $V_{HH}$ s AH3 and AA6 were selected for their potent neutralizing activity. For TcdB, 2 copies of the E3  $V_{HH}$  were selected, because E3 is a potent TcdB-neutralizing  $V_{HH}$  targeting the well-conserved glucosyltransferase domain with particularly high affinity. To generate ABA, the coding sequences of individual  $V_{HH}$ s were amplified and fused under the cytomegalovirus promoter of a pSEC91 plasmid. DNA encoding a flexible linker sequence ( $[(G_3S)_4]$ ) was installed between each of the 4  $V_{HH}$ -coding sequences. Both an immunoglobulin  $\kappa$ -chain leader (for protein secretion) and a His<sub>(6)</sub>-tag (for purification) were added to the N-terminus of the tetramer. The insert was sequenced to ensure that the proper sequence was obtained, and the final construct was transfected into HEK293 cells. ABA purified from cell culture supernatants of ABA-secreting stable 293 clones displayed a single dominant band during sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) after GelBlue (Pierce) staining. The purified ABA showed no toxicity to mice after intravenously injection of doses up to 10 mg/kg.

### ELISAs

Microplates were coated with 0.5  $\mu$ g/mL recombinant TcdA or 0.5  $\mu$ g/mL TcdB [22] overnight at 4°C and incubated with 50  $\mu$ L bacterial supernatants or purified  $V_{HH}$ s. After washes, horseradish peroxidase (HRP)–conjugated anti-E-tag antibody was added to plates, followed by analysis by a standard ELISA. For competition ELISA, serial dilutions of  $V_{HH}$ s were mixed with serial dilutions of ABA before adding to plates coated with TcdA or TcdB. After incubation and washes, the binding of monomer  $V_{HH}$ s was measured by adding a biotinylated anti-thioredoxin  $V_{HH}$  generated by us, followed by HRP-conjugated streptavidin. To determine whether ABA is capable of binding the 2 toxins simultaneously, plates were coated with TcdA or TcdB before adding serial dilutions of ABA. After washes, serial dilutions of TcdB or TcdA, respectively, were added to the wells. After extensive washing, mouse monoclonal antibodies against TcdB or TcdA (List Biological Laboratories, Campbell, CA), respectively, were added to the wells before the addition of HRP-conjugated antimouse antibodies for detection.

### In Vitro Neutralizing Assays

Mouse colonic epithelial CT26 cells and African green monkey kidney Vero cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA)

with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/mL penicillin G, and 40 µg/mL streptomycin sulfate. Subconfluent CT26 or Vero cells ( $2.0 \times 10^4$  cells/well) were seeded in 96-well plates for 24 hours before the addition of toxin and  $V_{\text{H}}\text{H}$  agents. Serially diluted  $V_{\text{H}}\text{H}$ s and toxins were premixed using toxin at a concentration of 0.2 ng/mL for TcdB or 10 ng/mL for TcdA and then added to each well. In some experiments, 10-µL bacterial supernatants from 11 *C. difficile* strains were mixed with ABA (10 µg/mL) before addition to the Vero cell monolayer. This panel of strains was kindly provided by Dr Trevor Lawley and represent an assortment of genetically and geographically diverse clinical isolates [27, 28], Bacterial supernatant added without ABA acted as a control. After incubation for 24 hours, cells were observed under a phase-contrast microscope, and the percentage of cells that were rounded was assessed.

### Systemic Challenge

Six-week-old female CD1 mice (Charles River Labs) were maintained in a pathogen-free animal biosafety level 2 facility. All mice used in the experiments were housed in groups of 5 per cage

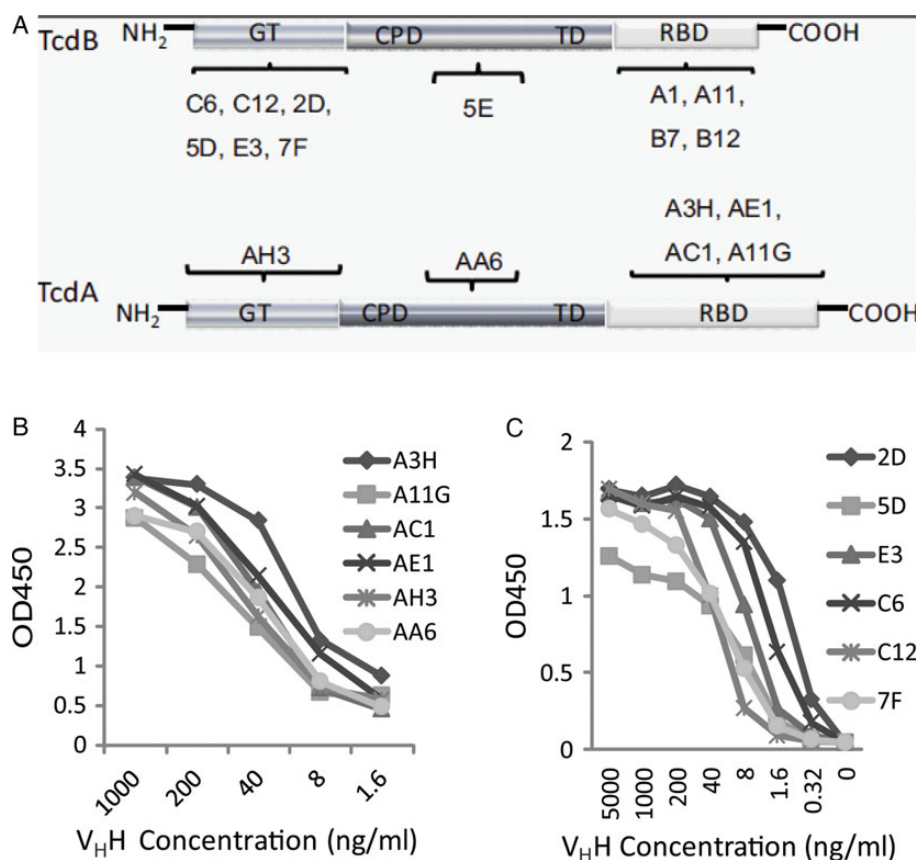
under the same conditions. Food, water, bedding, and cages were autoclaved. Mice (5 per group) were administered  $V_{\text{H}}\text{H}$  monomers or ABA by intraperitoneal injection 1 hour before intraperitoneal challenge of a mixture of TcdA and TcdB (25 ng/mouse of each toxin). Mice were monitored hourly for signs of illness, including hunched posture, ruffled coat, and rapid breathing. Animals that became moribund were euthanized.

### CDI Challenge

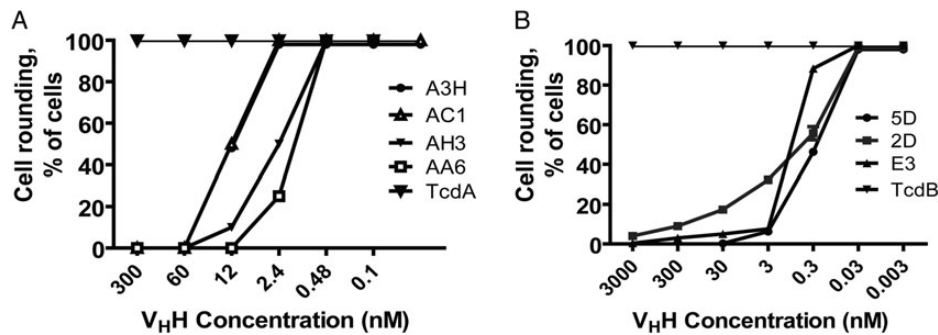
C57BL/6 mice were orally administered  $10^5$  *C. difficile* spores from the UK1 (BI/NAP1/027) strain after receiving antibiotic treatment, as previously described [12]. Mice (10 per group) were administered with ABA by intraperitoneal injection 24 hours after spore challenge. Mouse weights were measured daily, and the development of disease symptoms was monitored twice daily. Animals that became moribund or lost >20% of their body weight were euthanized.

### Statistical Analysis

Data were analyzed by Kaplan–Meier survival analysis with a log-rank test of significance, analysis of variance, and 1-way



**Figure 1.** Binding of specific heavy-chain-only antibody ( $V_{\text{H}}\text{H}$ ) to TcdA and TcdB. *A*, Diagram illustrates specific binding of individual  $V_{\text{H}}\text{H}$ s to the glucosyltransferase domain (GTD), cysteine protease domain (CPD), translocation domain (TD), or receptor-binding domain (RBD) in TcdA or TcdB. *B* and *C*, Enzyme-linked immunosorbent assay analysis of serially diluted anti-TcdA (*B*) or anti-TcdB (*C*)  $V_{\text{H}}\text{H}$ s in microtiter plates coated with 0.5 µg/mL of TcdA or TcdB, respectively.



**Figure 2.** Neutralizing activities of anti-Tcd heavy-chain-only antibodies (V<sub>H</sub>Hs). CT26 cells were exposed to either 10 ng/mL TcdA (A) or 0.2 ng/mL TcdB (B) premixed with a serial dilution of each anti-TcdA or anti-TcdB V<sub>H</sub>H, respectively, and then added to cells for 24 hours. Morphological changes in cells were observed under a phase-contrast microscope, and the percentage of cell rounding was assessed.

analysis of variance, followed by Bonferroni posttests, using the Prism statistic software program. Results are expressed as mean ± standard error of mean.

## RESULTS

### Identification and Characterization of Anti-Tcd V<sub>H</sub>Hs

Six anti-TcdA V<sub>H</sub>Hs and 11 anti-TcdB V<sub>H</sub>Hs were identified and expressed in *E. coli* and purified using nickel affinity chromatography (Supplementary Figure 1A). The domain location of the binding epitopes of each V<sub>H</sub>H in their targeted toxin (Figure 1A) was determined using Western blotting and ELISA of the V<sub>H</sub>Hs against recombinant toxin fragments [29] or toxin chimeras [30]. The relative affinities of the V<sub>H</sub>Hs for the different toxins was assessed by ELISA (Figure 1B), and the K<sub>D</sub>s for the toxins were determined by surface plasmon resonance (SPR) analysis (Supplementary Table 1). We further evaluated the ability of the V<sub>H</sub>Hs to neutralize the cytotoxic activities of TcdA or TcdB on cultured cells. Four V<sub>H</sub>Hs against TcdA (Figure 2A) and 3 V<sub>H</sub>Hs against TcdB (Figure 2B) had strong neutralizing activity, capable of neutralizing toxin-mediated cytopathic effects at nanomolar concentrations.

### Generation of a Multivalent V<sub>H</sub>H Agent That Neutralizes Both TcdA and TcdB

Since it has been found necessary to neutralize both TcdA and TcdB to achieve optimal protection against CDI [15, 30], we generated ABA (Figure 3A). The selection of these V<sub>H</sub>Hs was based on their high toxin affinities, their potent toxin neutralizing activities, and their binding to more well-conserved regions on these toxins. Purified ABA from stably transfected HEK293 cells displayed a 66-kDa band on SDS-PAGE (Supplementary Figure 1B). ABA successfully competed for the Tcd binding by its individual component V<sub>H</sub>Hs, AH3, AA6, and E3, in a dose-dependent manner but failed to block AC1 binding to TcdA or B12 binding to TcdB (Figure 3B), thus indicating that each of the component V<sub>H</sub>Hs in ABA remained functional.

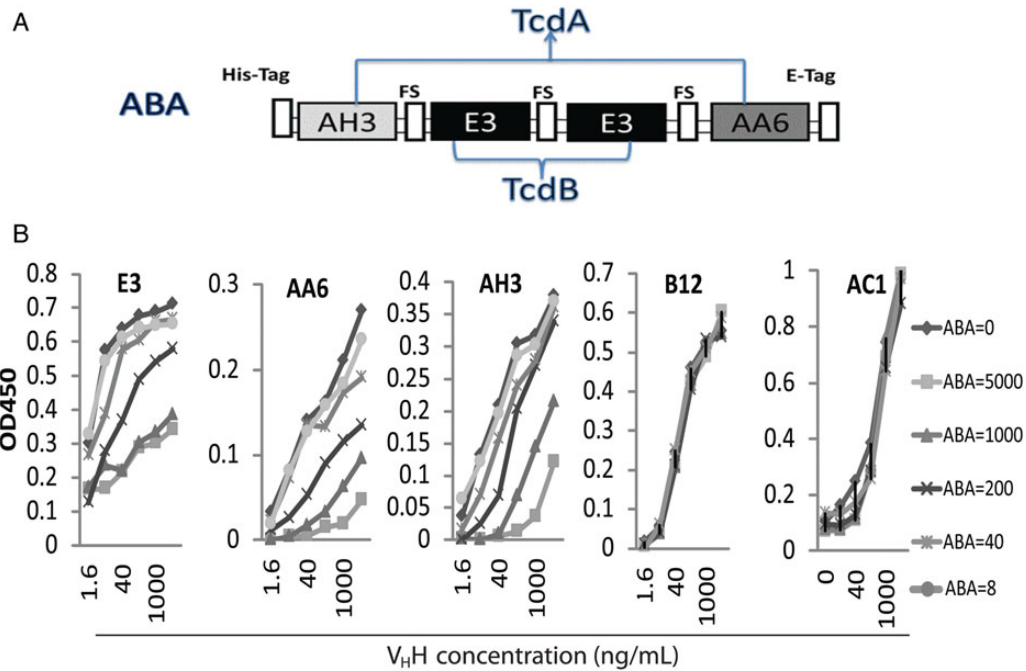
Additionally, in an ELISA, ABA demonstrated the capacity to bind to both toxins at the same time in a dose-dependent fashion, regardless of whether TcdA or TcdB was bound to the plate (Figure 4A and 4B). Thus, ABA is able to bind TcdA and TcdB simultaneously and is thus bispecific to both toxins. This finding was confirmed by SPR binding experiments in which ABA was coupled to a surface onto which TcdA and TcdB were titrated (Supplementary Figure 2). The maximal response value of the ABA surface was twice as high for both TcdA titrations as for the TcdB titration, suggesting that ABA is trispecific to 3 distinctive epitopes, with AH3 and AA6 subunits binding simultaneously to TcdA molecules along with E3 subunits binding to TcdB.

### ABA Demonstrates Strong Neutralizing Activity

We next determined the neutralizing activity of ABA to prevent toxin-mediated cytopathic effects on cultured cells. ABA had substantially enhanced neutralizing activity against TcdA-induced cytopathic effects on CT26 cells, with a concentration of half-maximal activity (ED<sub>50</sub>) of around 10 pM, compared with the individual V<sub>H</sub>Hs, which had ED<sub>50</sub> values of around 3 nM (Figure 5A). The neutralizing activity of ABA against TcdB was comparable to that of E3 (Figure 5B). Further experiments demonstrated that ABA was capable of preventing the rounding of Vero cells induced by culture supernatants from 11 genetically and geographically diverse clinical *C. difficile* isolates that express both functional TcdA and TcdB (Table 1) [27, 28]. Interesting, ABA failed to neutralize TcdB in the culture supernatants from 2 TcdA<sup>-</sup>TcdB<sup>+</sup> strains (ribotype 017), suggesting that the glucosyltransferase domains (GTDs) from these strains are significantly different from those from the tested TcdA<sup>+</sup>TcdB<sup>+</sup> strains.

We further assessed the *in vivo* neutralizing activity of ABA by evaluating its capacity to protect mice from systemic toxicity. Mice were completely protected against a lethal challenge of mixed TcdA and TcdB (25 ng of each per mouse) after ABA treatment at concentrations as low as 3.2 μg/kg, whereas the





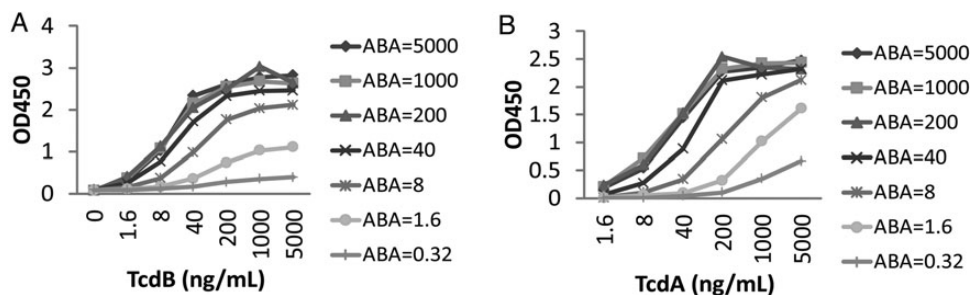
**Figure 3.** Generation of bispecific ABA against TcdA and TcdB. *A*, Diagram of the ABA fusion protein. AH3/E3/E3/AA6 heavy-chain-only antibody ( $V_{\text{H}}\text{H}$ ) subunits were separated by a flexible linker sequence (FS). A His(6)-tag and E-tag were genetically fused at the N-terminal and C-terminal, respectively, of the ABA molecule. *B*, Checkerboard Enzyme-linked immunosorbent assay analysis of ABA competition with individual components. Serially diluted ABA (ng/mL) was mixed with different serially diluted thioredoxin/ $V_{\text{H}}\text{H}$  fusion proteins targeting TcdA (AH3, AA6, or AC1) or TcdB (E3 or B12) and added to microplates coated with TcdA and TcdB, respectively. Biotinylated anti-thioredoxin  $V_{\text{H}}\text{H}$  was used for detection.

individual component  $V_{\text{H}}\text{H}$  combination failed to show complete protection at a dose as high as 1 mg/kg ( $P = .009$ ; Figure 5C). Thus, the *in vivo* neutralizing activity of ABA is at least 300-fold more potent than that of the mixture of the individual components.

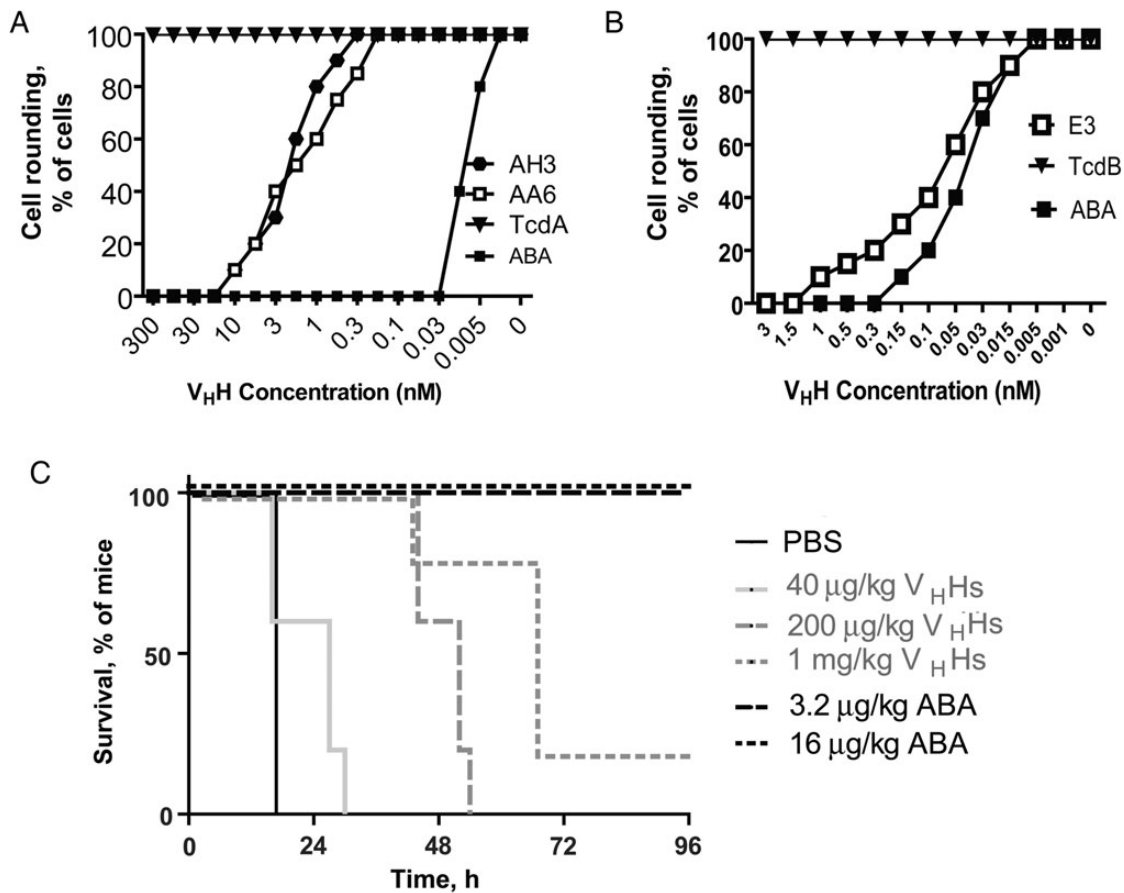
#### ABA Treatment Reverses Fulminant Disease Symptoms in Mice With CDI

Since ABA exhibits potent neutralizing activity against both toxins, we evaluated its therapeutic potential against fulminant

CDI, to which no effective treatment is available. After oral challenge of antibiotic-treated mice with *C. difficile* spores from the NAP1/027 strain, a single dose of ABA (1 mg/kg) or vehicle control was parenterally administered to mice. At the time of ABA treatment, a majority of mice had developed severe diarrhea and were experiencing weight loss (Figure 6A). Treatment with ABA at a dose of 1 mg/kg significantly prevented the severe weight loss associated with CDI (Figure 6A). More importantly, mice treated with ABA were significantly protected from mortality induced by *C. difficile* challenge, compared with the



**Figure 4.** ABA binds to TcdA and TcdB simultaneously. Serially diluted ABA (ng/mL) was added to microplate wells coated with TcdA (*A*) or TcdB (*B*). After incubation and washes, serially diluted TcdB (*A*) or TcdA (*B*) were added to the plates. The binding of toxins was determined by detecting TcdB (*A*) or TcdA (*B*), respectively, using toxin-specific mouse monoclonal antibodies.



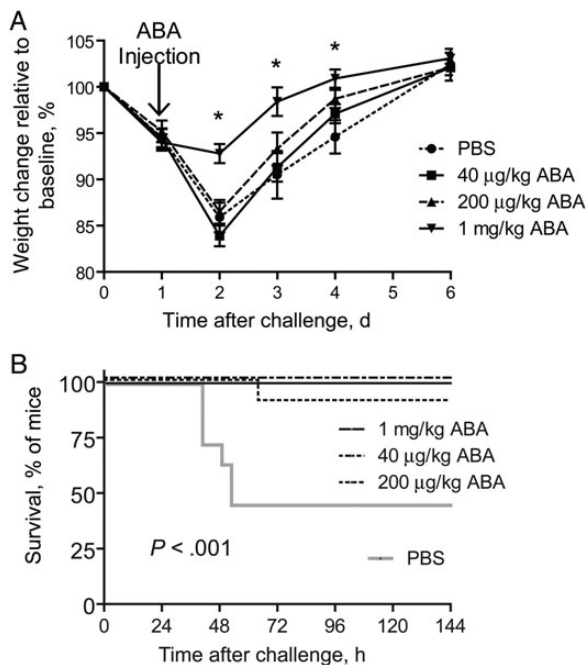
**Figure 5.** In vitro and in vivo neutralization of TcdA and TcdB due to ABA. *A* and *B*, CT26 cells were exposed to serially diluted E3, AH3, AA6, or ABA together with either 10 ng/mL TcdA (*A*) or 0.2 ng/mL TcdB (*B*) for 24 hours. Morphological changes in cells were observed under a phase-contrast microscope, and the percentage of cell rounding was assessed. *C*, For in vivo neutralization, mice were injected intraperitoneally with the indicated doses of ABA or a mixture of the individual heavy-chain-only antibody (V<sub>H</sub>H) components, followed 1 hour later by intraperitoneal inoculation with a mixture of TcdA and TcdB (25 ng each/mouse). Overall mouse survival was analyzed by Kaplan–Meier survival curves. Abbreviation: PBS, phosphate-buffered saline.

**Table 1. ABA Neutralization Against Toxins Secreted From Different *Clostridium difficile* Strains**

| Strain | Ribotype | REA Type | PFGE Type | Place/Date of Isolation/Source | ABA Neutralization |
|--------|----------|----------|-----------|--------------------------------|--------------------|
| R20291 | 027      | BI       | NAP1      | London/2006/human              | Yes                |
| CD196  | 027      | BI       | NAP1      | France/1985/human              | Yes                |
| 630    | 012      | R        |           | Zurich/1982/human              | Yes                |
| M120   | 078      | BK       | NAP7,8,9  | UK/2007/human                  | Yes                |
| BI-9   | 001      | J        | NAP2      | Gerding collection             | Yes                |
| Liv024 | 001      | J        | NAP2      | Liverpool/2009/human           | Yes                |
| Liv022 | 106      | DH       | NAP11     | Liverpool/2009/human           | Yes                |
| TL178  | 002      | G        | NAP6      | Belfast/2009/human             | Yes                |
| TL176  | 014      | Y        | NAP4      | Cambridge, UK/2009/human       | Yes                |
| TL174  | 015      | ...      | ...       | Cambridge, UK/2009/human       | Yes                |
| CD305  | 023      | ...      | ...       | London/2008/human              | Yes                |
| CF5    | 017      | CF       | NAP9      | Belgium/1995/human             | No                 |
| M68    | 017      | CF       | NAP9      | Dublin/2006/human              | No                 |

ABA was mixed with bacterial culture supernatants from 13 *C. difficile* strains and added to Vero cell monolayers. Cell rounding was observed under a light microscope. All supernatants alone caused cell rounding, whereas no cell rounding was observed when supernatants were mixed with ABA, except for CF5 and M68 strains.

Abbreviations: PFGE, pulsed-field gel electrophoresis; REA, restriction endonuclease analysis.



**Figure 6.** Therapeutic efficacy of ABA against *Clostridium difficile* infection in mice. C57BL/6 mice were pretreated with antibiotics before being challenged with UK1 spores. Mice were injected intraperitoneally with 1 mg/kg of ABA 24 hours after infection. Mouse weight change (A) and survival (B) were monitored and are plotted. The experiments were repeated twice with similar results (10 mice/experiment). Error bars denote standard errors of the mean. Abbreviation: PBS, phosphate-buffered saline.

phosphate-buffered saline control (Figure 6B). None of the mice in the 1 mg/kg or 40 µg/kg groups died, and only 1 mouse in the 200 µg/kg group died, whereas 60% of the mice in the vehicle control group died (Figure 6B).

## DISCUSSION

The incidence of CDI-associated mortality has increased rapidly in recent years [3], while treatment options against severe and fulminant CDI remain limited [2]. In this study, we generated a novel bispecific V<sub>H</sub>H antibody that is capable of neutralizing both TcdA and TcdB and reversing fulminant disease symptoms in mice with CDI. Our study thus provides evidence for the usefulness and efficacy of bispecific V<sub>H</sub>H antibodies in the treatment of fulminant CDI in patients.

The glucosyltransferase-deficient holotoxins aTcdA and aTcdB [12] were used to immunize alpacas to induce potent B-cell responses. These atoxic holotoxins both remain soluble and appear to maintain native conformations and thus should induce antibody responses that are similar to those of wild-type toxins. As expected, the majority of neutralizing anti-TcdA V<sub>H</sub>Hs recognize the C-terminal combined repetitive oligopeptides (CROPs), which has been established as the immunodominant

domain of TcdA [12, 31]. However, a recent study has shown that a truncated TcdA protein, without the CROPs, can cause cytotoxicity in cells [32]. Our 2 most potent neutralizing anti-TcdA V<sub>H</sub>Hs recognize the N-terminal GTD and the central translocation domain (TD), indicating that the toxins can also be effectively neutralized by blocking glucosyltransferase or the membrane insertion/cytosolic release of TcdA.

Unlike the anti-TcdA V<sub>H</sub>Hs, all neutralizing anti-TcdB V<sub>H</sub>Hs recognize the N-terminal GTD; none of the 5 V<sub>H</sub>Hs that bind to the CROPs of TcdB demonstrated neutralizing activity, despite their high binding affinities. This result is in line with our recent finding that the major neutralizing epitopes of TcdB are largely located in the N-terminal portion of the toxin rather than in the C-terminal CROP region [12]. The CROP region of TcdB has been the traditional immunogen choice for vaccine development and generation of neutralizing antibodies [33–35]. This notion now warrants revision because of convincing data that the N-terminal portion of TcdB is the most effective domain as an immunogen for vaccines and immunotherapies against CDI. Further support for this concept is found in recent studies in which TcdB CROPs failed to induce protective titers of neutralizing antibodies [34, 35], while the GTD fragment of TcdB led to the generation of highly protective neutralizing antibody responses [36].

Because various *C. difficile* strains expressing different toxin isoforms are isolated from patients with CDI, it is important for therapeutic antibodies to target conserved toxin epitopes, thus conferring broad protection across clinically relevant isolates. Recent reports have shown that TcdA is relatively well conserved between historical and epidemic strains, whereas TcdB shows a significant degree of variability [37, 38]. The sequence variability in TcdB from TcdA<sup>+</sup>TcdB<sup>+</sup> strains is mainly found within the C-terminal CROPs and the adjacent region (88% identity between historical and epidemic strains), whereas the N-terminal GTD is more conserved and shows 96% amino acid sequence identity [37]. Unlike the anti-TcdB monoclonal antibody undergoing clinical trials [14], which binds to the CROP region [33], E3 binds to the GTD of TcdB. In addition, ABA consists of 2 distinct V<sub>H</sub>Hs, AH3 and AA6, that bind to the GTD and TD of TcdA, respectively. In fact, ABA was able to neutralize toxins a panel of genotypically diverse TcdA<sup>+</sup>TcdB<sup>+</sup> clinical isolates collected from different geographic locations [27, 28], including some BI/NAP1/027 strains that are responsible for recent outbreaks of CDI, thus demonstrating the broad efficacy range of this agent against those strains producing both TcdA and TcdB.

Although the majority of pathogenic *C. difficile* produce both TcdA and TcdB, some clinical isolates produce only TcdB [39]. The TcdA<sup>-</sup>TcdB<sup>+</sup> strain infections occur in sporadic form in Europe and North America but are more frequent in East Asian countries [40]. Sequence analysis of the *TcdB* gene from TcdA<sup>-</sup>TcdB<sup>+</sup> strains identified multiple point mutations

in GTD regions, with only 84% homology with that of laboratory strain VPI 10463 [39]. Since E3 binds to the GTD of TcdB and neutralizes its activity, we examined the ability of ABA to neutralizing TcdB from 2 TcdA<sup>-</sup>TcdB<sup>+</sup> strains. Our data demonstrated that ABA failed to neutralize TcdB from these 2 TcdA<sup>-</sup>TcdB<sup>+</sup> NAP9/CF/017 strains (Table 1), supporting the previous finding that the glucosyltransferase of TcdB from the TcdA<sup>-</sup>TcdB<sup>+</sup> strains is significantly different from that of the VPI 10463 strain [39, 41]. Although ABA is able to broadly neutralizing toxins from TcdA<sup>+</sup>TcdB<sup>+</sup> *C. difficile* strains, it is unable to neutralize TcdB from the tested TcdA<sup>-</sup>TcdB<sup>+</sup> ribotype 017 strains and, thus, is unlikely to be therapeutically effective against CDI caused by these strains. Therefore, further improvement via the introduction of V<sub>H</sub>Hs that are able to also neutralize TcdB from the 017 strains is desirable.

CDI causes a wide spectrum of clinical symptoms and outcomes, including mild diarrhea, fulminant disease, and death [2, 42]. A single dose of ABA was able to significantly protect mice against weight loss and fulminant CDI after *C. difficile* spore challenge. We have previously reported that toxins are released into the bloodstream of animals experimentally infected with *C. difficile*, a feature associated with severe and fulminant CDI in several animal models [43–45]. The potent neutralizing activity of ABA may allow for the rapid neutralization of circulating toxins liberated from the intestines of mice with fulminant CDI. In fact, a single injection of ABA of only 3.2 µg/kg provided full protection against lethal systemic toxin challenge in mice.

In this study, we effectively used a 1-mg/kg dose of ABA to treat CDI in mice, compared with the 50-mg/kg and 10-mg/kg doses of human monoclonal antibodies that have been used in hamsters [33] and patients [14], respectively, with CDI. The serum half-life of ABA in its current form is likely to be short because of its smaller size and its lack of an Fc domain. Thus, either multiple doses of the antibody in its current form or further modifications to increase bioavailability may be necessary to treat chronic infections. Multiple doses of V<sub>H</sub>Hs may induce an anti-V<sub>H</sub>H antibody response and reduce the efficacy of subsequent treatments should patients develop multiple recurrent CDIs. Although V<sub>H</sub>Hs are not particularly immunogenic, their potential immunogenicity can be further reduced via the humanization of the V<sub>H</sub>H scaffold [46]. In addition, a number of approaches are now available to improve the serum half-life of V<sub>H</sub>Hs, such as genetic fusion with albumin-binding domains [47, 48] or immunoglobulins [49], as well as PEGylation [50], thereby enhancing their therapeutic potential.

In summary, we report here that a novel bispecific V<sub>H</sub>H antibody is able to rapidly alleviate fulminant CDI in mice. Our study thus demonstrates the feasibility of designing multivalent and bispecific V<sub>H</sub>H antibodies against both toxins with significant enhanced therapeutic efficacy to reduce the morbidity and mortality associated with this debilitating disease.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

**Disclaimer.** The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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