Journal of Infectious Diseases Advance Access published May 5, 2014

MAJOR ARTICLE

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_H (V_HH) 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 $TcdA^-TcdB^+$ *C. difficile* strains. This study thus provides a rationale for the development of multivalent V_HHs that target both toxins and are broadly neutralizing for treating severe CDI.

Keywords. *Clostridium difficile*; toxins; antibody; V_HH; 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_1) and unconventional IgGs (IgG_2 and IgG_3)

Received 12 November 2013; accepted 18 March 2014.

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The Journal of Infectious Diseases

DOI: 10.1093/infdis/jiu196

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_HHs for treating toxin-mediated diseases [18, 19]. Recently Hussack et al reported the generation of anti-Tcd V_HHs 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_HHs that recognize and neutralize either TcdA or TcdB. We further constructed fusion proteins consisting of multiple-antitoxin V_HHs, 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 ≥3 weeks with 50–100 μg 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_HHs 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_HHs and 11 unique TcdB-binding V_HHs were identified. Selected V_HH coding sequences were cloned into the pET32b expression vector (Novagen) for cytosolic expression of V_HHs 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_HHs having the most potent neutralizing activity and the highest binding affinity to distinct, nonoverlapping epitopes targeting each toxins were chosen for inclusion within a multimeric, multivalent antibody. For TcdA, V_HHs 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_HHs 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 κ-chain leader (for protein secretion) and a $His_{(6)}$-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 μg/mL recombinant TcdA or 0.5 μg/mL TcdB [22] overnight at 4°C and incubated with 50 μL bacterial supernatants or purified V_HHs. 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_HHs 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_HHs 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 (2.0×10^4 cells/well) were seeded in 96-well plates for 24 hours before the addition of toxin and V_HH agents. Serially diluted V_HHs 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_HH 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

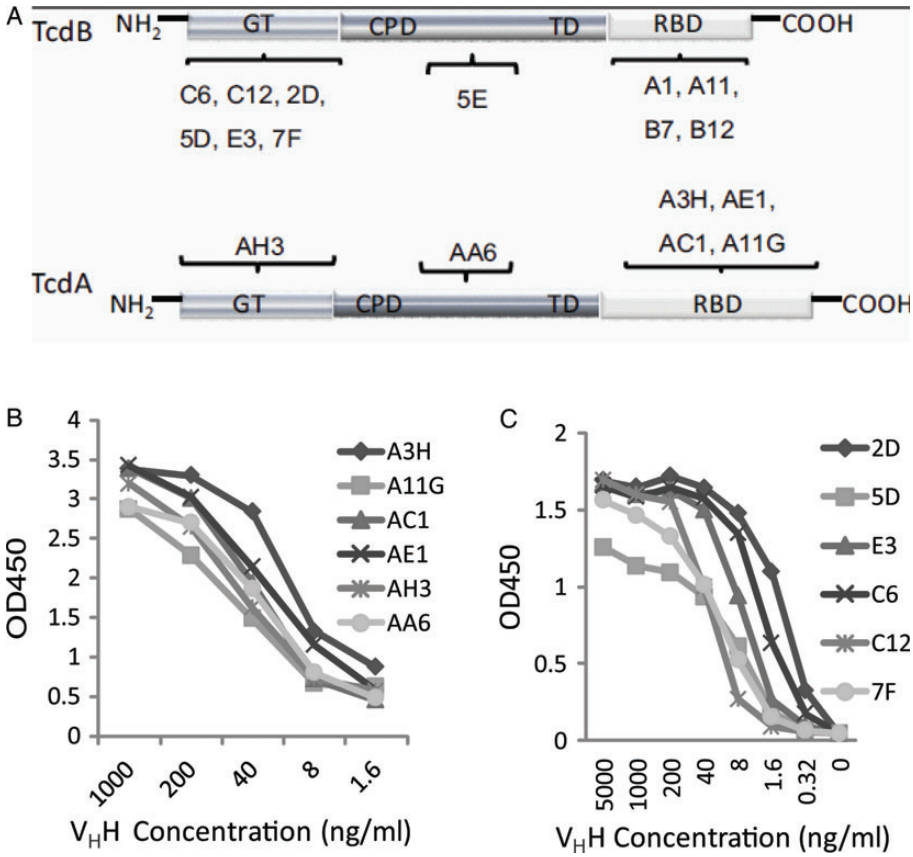

Figure 1. Binding of specific heavy-chain-only antibody (V_HH) to TcdA and TcdB. *A*, Diagram illustrates specific binding of individual V_HHs to the glucosyltransferase domain (GTD), cysteine protease domain (CPD), translocation domain (TD), or receptor-binding domain (RBD) in TcdA or TcdB. *B* and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

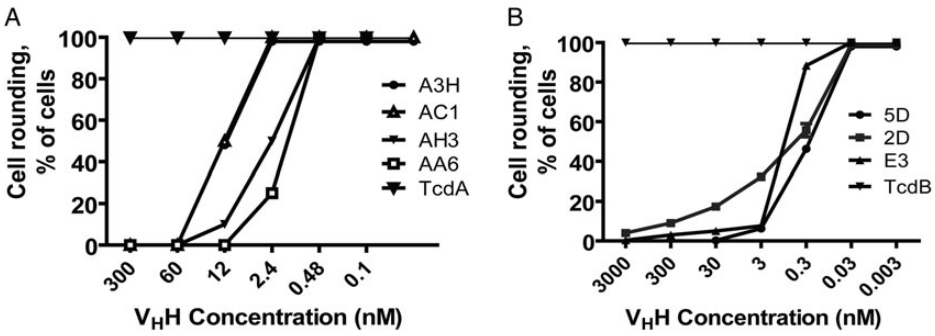

Figure 2. Neutralizing activities of anti-Tcd heavy-chain-only antibodies (V_HHs). CT26 cells were exposed to either 10 ng/mL TcdA (*A*) or 0.2 ng/mL TcdB (*B*) premixed with a serial dilution of each anti-TcdA or anti-TcdB V_HH, respectively, and then added to cells for 24 hours. Morphological changes 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_HHs

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

Generation of a Multivalent V_HH 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 3*A*). The selection of these V_HHs 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 1*B*). ABA successfully competed for the Tcd binding by its individual component V_HHs, AH3, AA6, and E3, in a dose-dependent manner but failed to block AC1 binding to TcdA or B12 binding to TcdB (Figure 3*B*), thus indicating that each of the component V_HHs 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 4*A* and 4*B*). 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_{50}) of around 10 pM, compared with the individual V_HHs, which had ED_{50} values of around 3 nM (Figure 5*A*). The neutralizing activity of ABA against TcdB was comparable to that of E3 (Figure 5*B*). 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 $TcdA^-TcdB^+$ strains (ribotype 017), suggesting that the glucosyltransferase domains (GTDs) from these strains are significantly different from those from the tested $TcdA^+TcdB^+$ 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

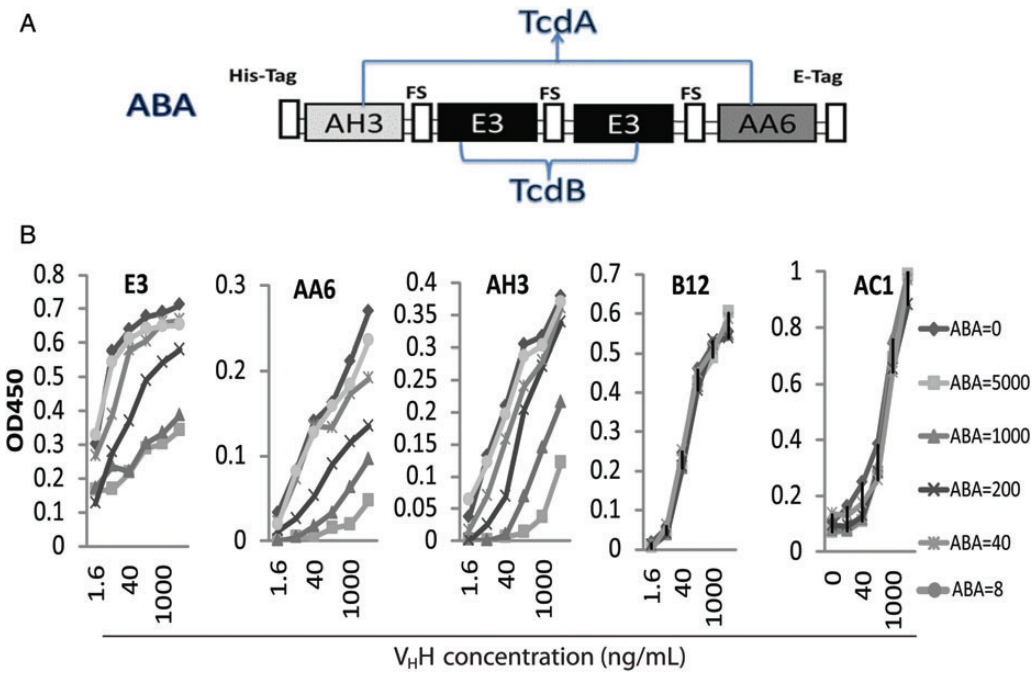

Figure 3. Generation of bispecific ABA against TcdA and TcdB. *A*, Diagram of the ABA fusion protein. AH3/E3/E3/AA6 heavy-chain-only antibody (V_HH) subunits were separated by a flexible linker sequence (FS). A His(6)-tag and E-tag were genetically fused at the N-terminal and C-terminal, respectively, 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_HH 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_HH was used for detection.

individual component V_HH 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 6*A*). Treatment with ABA at a dose of 1 mg/kg significantly prevented the severe weight loss associated with CDI (Figure 6*A*). More importantly, mice treated with ABA were significantly protected from mortality induced by *C. difficile* challenge, compared with the

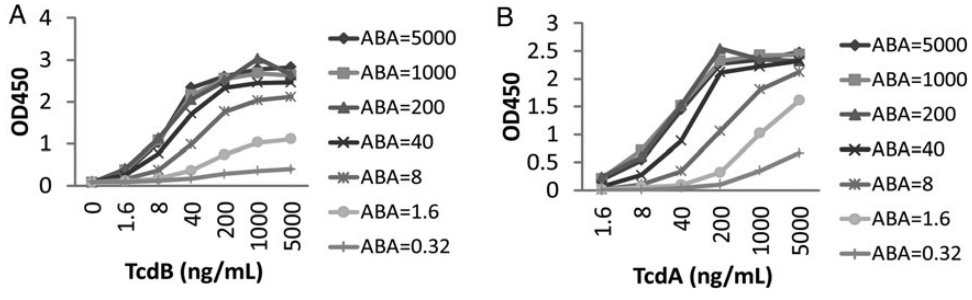

Figure 4. ABA binds to TcdA and TcdB simultaneously. Serially diluted ABA (ng/mL) was added to microplate wells coated with TcdA (*A*) or TcdB (*B*). After incubation and washes, serially diluted TcdB (*A*) or TcdA (*B*) were added to the plates. The binding of toxins was determined by detecting TcdB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

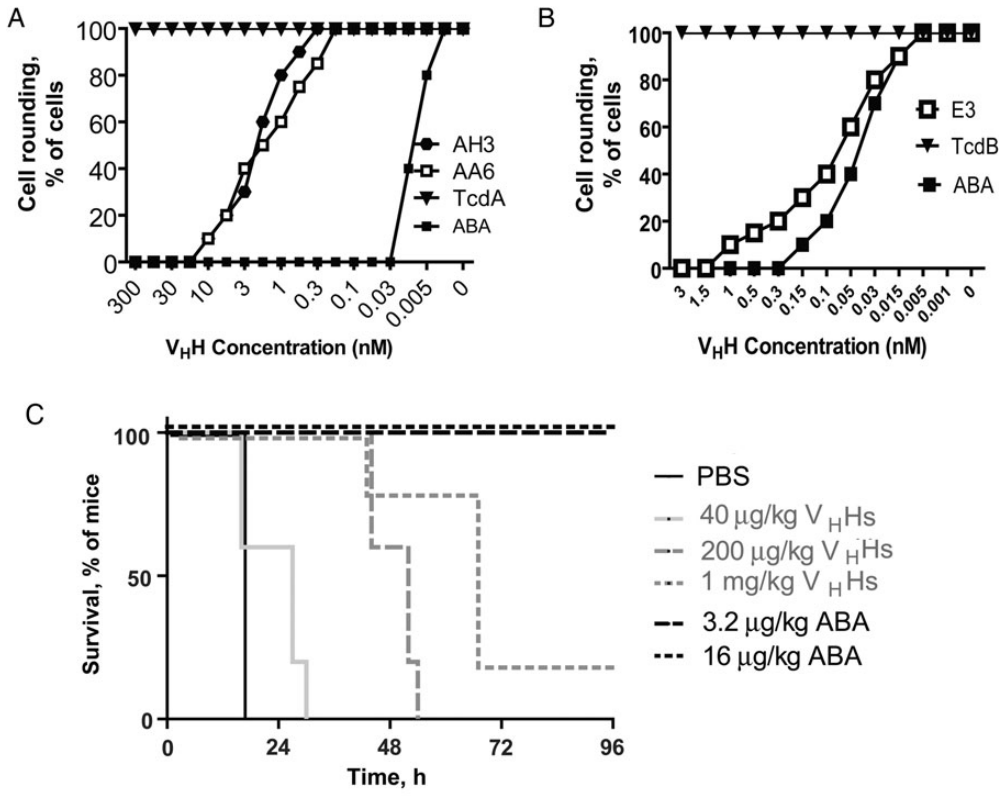

Figure 5. In vitro and in vivo neutralization of TcdA and TcdB due to ABA. *A* and *B*, CT26 cells were exposed to serially diluted E3, AH3, AA6, or ABA together with either 10 ng/mL TcdA (*A*) or 0.2 ng/mL TcdB (*B*) for 24 hours. Morphological changes in cells were observed under a phase-contrast microscope, 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_HH) 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.

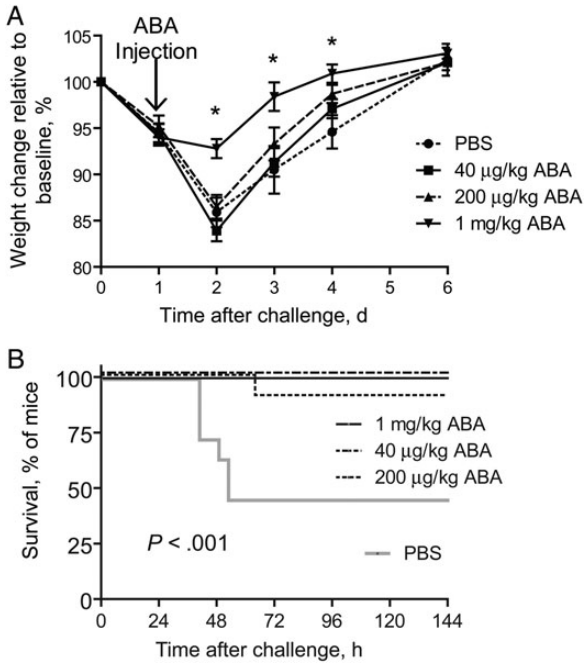

Figure 6. Therapeutic efficacy of ABA against *Clostridium difficile* infection in mice. C57BL/6 mice were pretreated with antibiotics before being challenged with UK1 spores. Mice were injected intraperitoneally with 1 mg/kg of ABA 24 hours after infection. Mouse weight change (*A*) and survival (*B*) 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 6*B*). 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 6*B*).

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_HH 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_HH 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_HHs 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_HHs 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_HHs, all neutralizing anti-TcdB V_HHs recognize the N-terminal GTD; none of the 5 V_HHs 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 $TcdA^+TcdB^+$ 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_HHs, 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 $TcdA^+TcdB^+$ 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 $TcdA^-TcdB^+$ 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 $TcdA^-TcdB^+$ 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 $TcdA^{-}TcdB^{+}$ strains. Our data demonstrated that ABA failed to neutralize TcdB from these 2 $TcdA^{-}TcdB^{+}$ NAP9/CF/017 strains (Table 1), supporting the previous finding that the glucosyltransferase of TcdB from the $TcdA^{-}TcdB^{+}$ strains is significantly different from that of the VPI 10463 strain [39, 41]. Although ABA is able to broadly neutralizing toxins from $TcdA^{+}TcdB^{+}$ *C. difficile* strains, it is unable to neutralize TcdB from the tested $TcdA^{-}TcdB^{+}$ 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_HHs 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_HHs may induce an anti-V_HH antibody response and reduce the efficacy of subsequent treatments should patients develop multiple recurrent CDIs. Although V_HHs are not particularly immunogenic, their potential immunogenicity can be further reduced via the humanization of the V_HH scaffold [46]. In addition, a number of approaches are now available to improve the serum half-life of V_HHs, 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_HH antibody is able to rapidly alleviate fulminant CDI in mice. Our study thus demonstrates the feasibility of designing multivalent and bispecific V_HH 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 posted materials are not copyedited. 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

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Financial support. This work was supported by National Institute of Allergy and Infectious Diseases and National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (awards R01AI088748, R01DK084509, and R56AI99458 to H. F. and contract N01-AI-30050 and award U54 AI057159 to C. B. S.).

Potential conflicts of interest. All authors: No reported conflicts.

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.

References

1. Cloud J, Kelly CP. Update on *Clostridium difficile* associated disease. Curr Opin Gastroenterol **2007**; 23:4–9.
2. Kelly CP, LaMont JT. *Clostridium difficile*–more difficult than ever. N Engl J Med **2008**; 359:1932–40.
3. Siemann M, Koch-Dorfler M, Rabenhorst G. *Clostridium difficile*-associated diseases. The clinical courses of 18 fatal cases. Intensive Care Med **2000**; 26:416–21.
4. Huang H, Fang H, Weintraub A, Nord CE. Distinct ribotypes and rates of antimicrobial drug resistance in *Clostridium difficile* from Shanghai and Stockholm. Clin Microbiol Infect **2009**; 15:1170–3.
5. Zar FA, Bakkanagari SR, Moorthi KM, Davis MB. A comparison of vancomycin and metronidazole for the treatment of *Clostridium difficile*-associated diarrhea, stratified by disease severity. Clin Infect Dis **2007**; 45:302–7.
6. Johnson S. Recurrent *Clostridium difficile* infection: a review of risk factors, treatments, and outcomes. J Infect **2009**; 58:403–10.
7. Louie TJ, Miller MA, Mullane KM, et al. Fidaxomicin versus vancomycin for *Clostridium difficile* infection. N Engl J Med **2011**; 364:422–31.
8. Butler MM, Shinabarger DL, Citron DM, et al. MBX-500, a hybrid antibiotic with in vitro and in vivo efficacy against toxigenic *Clostridium difficile*. Antimicrob Agents Chemother **2012**; 56:4786–92.
9. Gough E, Shaikh H, Manges AR. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. Clin Infect Dis **2011**; 53:994–1002.
10. Brandt LJ, Aroniadis OC, Mellow M, et al. Long-term follow-up of colonoscopic fecal microbiota transplant for recurrent *Clostridium difficile* infection. Am J Gastroenterol **2012**; 107:1079–87.
11. Tian JH, Fuhrmann SR, Kluepfel-Stahl S, Carman RJ, Ellingsworth L, Flyer DC. A novel fusion protein containing the receptor binding domains of *C. difficile* toxin A and toxin B elicits protective immunity against lethal toxin and spore challenge in preclinical efficacy models. Vaccine **2012**; 30:4249–58.
12. Wang H, Sun X, Zhang Y, et al. A chimeric toxin vaccine protects against primary and recurrent *Clostridium difficile* infection. Infect Immun **2012**; 80:2678–88.
13. Kelly CP, Pothoulakis C, Vavva F, et al. Anti-*Clostridium difficile* bovine immunoglobulin concentrate inhibits cytotoxicity and enterotoxicity of *C. difficile* toxins. Antimicrob Agents Chemother **1996**; 40:373–9.

14. Lowy I, Molrine DC, Leav BA, et al. Treatment with monoclonal antibodies against *Clostridium difficile* toxins. N Engl J Med **2010**; 362:197–205.
15. Roberts A, McGlashan J, Al-Abdulla I, et al. Development and evaluation of an ovine antibody-based platform for treatment of *Clostridium difficile* infection. Infect Immun **2012**; 80:875–82.
16. Marozsan AJ, Ma D, Nagashima KA, et al. Protection against *Clostridium difficile* infection with broadly neutralizing antitoxin monoclonal antibodies. J Infect Dis **2012**; 206:706–13.
17. Wesolowski J, Alzogaray V, Reyelt J, et al. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. Med Microbiol Immunol **2009**; 198:157–74.
18. Adams H, Brummelhuis W, Maassen B, et al. Specific immuno capturing of the staphylococcal superantigen toxic-shock syndrome toxin-1 in plasma. Biotechnol Bioeng **2009**; 104:143–51.
19. Tremblay JM, Mukherjee J, Leysath CE, et al. A single VHH-based toxin neutralizing agent and an effector antibody protects mice against challenge with Shiga toxins 1 and 2. Infect Immun **2013**; 81:4592–603.
20. Hussack G, Arbabi-Ghahroudi M, Mackenzie CR, Tanha J. Isolation and characterization of *Clostridium difficile* toxin-specific single-domain antibodies. Methods Mol Biol **2012**; 911:211–39.
21. Hussack G, Arbabi-Ghahroudi M, van Faassen H, et al. Neutralization of *Clostridium difficile* toxin A with single-domain antibodies targeting the cell receptor binding domain. J Biol Chem **2011**; 286:8961–76.
22. Yang G, Zhou B, Wang J, et al. Expression of recombinant *Clostridium difficile* toxin A and B in Bacillus megaterium. BMC Microbiol **2008**; 8:192.
23. Maass DR, Sepulveda J, Pernthaner A, Shoemaker CB. Alpaca (Lama pacos) as a convenient source of recombinant camelid heavy chain antibodies (VHHs). J Immunol Methods **2007**; 324:13–25.
24. Tremblay JM, Kuo CL, Abeijon C, et al. Camelid single domain antibodies (VHHs) as neuronal cell intrabody binding agents and inhibitors of Clostridium botulinum neurotoxin (BoNT) proteases. Toxicon **2010**; 56:990–8.
25. Mukherjee J, Tremblay JM, Leysath CE, et al. A novel strategy for development of recombinant antitoxin therapeutics tested in a mouse botulism model. PLoS One **2012**; 7:e29941.
26. Vance DJ TJ, Mantis NJ, Shoemaker CB. Stepwise Engineering of Heterodimeric Single Domain Camelid VHH Antibodies that Passively Protect Mice from Ricin Toxin. J Biol Chem **2013**; 288:36538–47.
27. Stabler RA, Dawson LF, Valiente E, et al. Macro and micro diversity of *Clostridium difficile* isolates from diverse sources and geographical locations. PLoS One **2012**; 7:e31559.
28. He M, Sebaihia M, Lawley TD, et al. Evolutionary dynamics of *Clostridium difficile* over short and long time scales. Proc Natl Acad Sci U S A **2010**; 107:7527–32.
29. He X, Sun X, Wang J, et al. Antibody-enhanced, Fc gamma receptor-mediated endocytosis of *Clostridium difficile* toxin A. Infect Immun **2009**; 77:2294–303.
30. Wang H, Sun X, Zhang Y, et al. A chimeric toxin vaccine protects against primary and recurrent *Clostridium difficile* infection. Infect Immun **2012**; 80:2678–88.
31. Castagliuolo I, Sardina M, Brun P, et al. *Clostridium difficile* toxin A carboxyl-terminus peptide lacking ADP-ribosyltransferase activity acts as a mucosal adjuvant. Infect Immun **2004**; 72:2827–36.
32. Olling A, Goy S, Hoffmann F, Tatge H, Just I, Gerhard R. The repetitive oligopeptide sequences modulate cytopathic potency but are not crucial for cellular uptake of *Clostridium difficile* toxin A. PLoS One **2011**; 6: e17623.
33. Babcock GJ, Broering TJ, Hernandez HJ, et al. Human monoclonal antibodies directed against toxins A and B prevent *Clostridium difficile*-induced mortality in hamsters. Infect Immun **2006**; 74:6339–47.
34. Hussack G, Arbabi-Ghahroudi M, van Faassen H, et al. Neutralization of *clostridium difficile* toxin A with single-domain antibodies targeting the cell-receptor binding domain. J Biol Chem **2011**; 286:8961–76.
35. Permpoonpattana P, Hong HA, Phetcharaburanin J, et al. Immunisation with Bacillus spores expressing toxin A peptide repeats protects against infection with toxin A+ B+ strains of *Clostridium difficile*. Infect Immun **2011**; 79:2295–302.
36. Leuzzi R, Spencer J, Buckley A, et al. Protective efficacy induced by recombinant *Clostridium difficile* toxin fragments. Infect Immun **2013**; 81:2851–60.
37. Lanis JM, Barua S, Ballard JD. Variations in TcdB activity and the hypervirulence of emerging strains of *Clostridium difficile*. PLoS Pathog **2010**; 6:e1001061.
38. Stabler RA, He M, Dawson L, et al. Comparative genome and phenotypic analysis of *Clostridium difficile* 027 strains provides insight into the evolution of a hypervirulent bacterium. Genome Biol **2009**; 10: R102.
39. Sambol SP, Merrigan MM, Lyerly D, Gerding DN, Johnson S. Toxin gene analysis of a variant strain of *Clostridium difficile* that causes human clinical disease. Infect Immun **2000**; 68:5480–7.
40. Kim J, Pai H, Seo MR, Kang JO. Clinical and microbiologic characteristics of tcdA-negative variant *Clostridium difficile* infections. BMC Infect Dis **2012**; 12:109.
41. Huelsenbeck J, Dreger S, Gerhard R, Barth H, Just I, Genth H. Difference in the cytotoxic effects of toxin B from *Clostridium difficile* strain VPI 10463 and toxin B from variant *Clostridium difficile* strain 1470. Infect Immun **2007**; 75:801–9.
42. Rupnik M, Wilcox MH, Gerding DN. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat Rev Microbiol **2009**; 7:526–36.
43. Steele J, Feng H, Parry N, Tzipori S. Piglet models of acute or chronic *Clostridium difficile* illness. J Infect Dis **2010**; 201:428–34.
44. He X, Wang J, Steele J, et al. An ultrasensitive rapid immunocytotoxicity assay for detecting *Clostridium difficile* toxins. J Microbiol Methods **2009b**; 78:97–100.
45. Steele J, Chen K, Sun X, et al. Systemic dissemination of *Clostridium difficile* toxins A and B is associated with severe, fatal disease in animal models. J Infect Dis **2012**; 205:384–91.
46. Vincke C, Loris R, Saerens D, Martinez-Rodriguez S, Muyldermans S, Conrath K. General strategy to humanize a camelid single-domain antibody and identification of a universal humanized nanobody scaffold. J Biol Chem **2009**; 284:3273–84.
47. Tijink BM, Laeremans T, Budde M, et al. Improved tumor targeting of anti-epidermal growth factor receptor Nanobodies through albumin binding: taking advantage of modular Nanobody technology. Mol Cancer Ther **2008**; 7:2288–97.
48. Coppieters K, Dreier T, Silence K, et al. Formatted anti-tumor necrosis factor alpha VHH proteins derived from camelids show superior potency and targeting to inflamed joints in a murine model of collagen-induced arthritis. Arthritis Rheum **2006**; 54:1856–66.
49. Harmsen MM, Van Solt CB, Fijten HP, Van Setten MC. Prolonged in vivo residence times of llama single-domain antibody fragments in pigs by binding to porcine immunoglobulins. Vaccine **2005**; 23:4926–34.
50. Harmsen MM, van Solt CB, Fijten HP, et al. Passive immunization of guinea pigs with llama single-domain antibody fragments against foot-and-mouth disease. Vet Microbiol **2007**; 120:193–206.