

An update on antibody-based immunotherapies for *Clostridium difficile* infection

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Abstract: *Clostridium difficile* continues to be one of the most prevalent hospital-acquired bacterial infections in the developed world, despite the recent introduction of a novel and effective antibiotic agent (fidaxomicin). Alternative approaches under investigation to combat the anaerobic Gram-positive bacteria include fecal transplantation therapy, vaccines, and antibody-based immunotherapies. In this review, we catalog the recent advances in antibody-based approaches under development and in the clinic for the treatment of *C. difficile* infection. By and large, inhibitory antibodies that recognize the primary *C. difficile* virulence factors, toxin A and toxin B, are the most popular passive immunotherapies under investigation. We provide a detailed summary of the toxin epitopes recognized by various antitoxin antibodies and discuss general trends on toxin inhibition efficacy. In addition, antibodies to other *C. difficile* targets, such as surface-layer proteins, binary toxin, motility factors, and adherence and colonization factors, are introduced in this review.

Keywords: antibody, *Clostridium difficile*, immunotherapy, toxin

Introduction

Clostridium difficile is one of the most prevalent hospital-acquired bacterial infections in the developed world, with symptoms ranging from mild diarrhea to colitis and death.^{1,2} Reducing the rate and duration of *C. difficile* infection (CDI) are critical goals for health care providers due to the enormous cost associated with CDI. This is a considerable challenge, given that aging populations are particularly susceptible to CDI. While broad-spectrum antibiotics and the more recent narrow-spectrum antibiotic fidaxomicin have shown some efficacy toward containing CDI, novel therapeutics are desired.²⁻⁵ There are a number of treatments under development for CDI, including but not limited to vaccines, fecal transplantation therapy, antibiotics, probiotics, and antibody-based immunotherapy.^{4,6-8} With the focus of this review on chronicling the recent advances in monoclonal antibody (mAb)- and single-domain antibody (sdAb)-based immunotherapy, we direct readers to the excellent reviews highlighting other CDI therapies under development.⁹⁻¹²

Before discussing the present antibody-based therapeutics under development for CDI, it is important to understand the mechanisms of CDI, host colonization, and associated virulence factors. CDI often begins with a patient on broad-spectrum antibiotics being exposed to *C. difficile* spores. Other risk factors for potential CDI include age, gastrointestinal (GI) surgery, inflammatory bowel disease, and immunosuppression.² In general, patients on antibiotics have modified GI microbiota populations,

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allowing for *C. difficile* spores that travel to the lower GI tract an opportunity to begin their colonization process and transformation into vegetative cells.^{2,12} At this point, it is thought that the main *C. difficile* virulence factors toxin A (TcdA) and toxin B (TcdB) (Figure 1A–F) are transcribed

and secreted from the bacteria through a mechanism that requires the holin-like protein TcdE.^{13–17} Individuals who possess circulating antitoxin antibodies or those who mount a rapid and effective response are often only asymptomatic carriers or experience less severe CDI with a lower risk of

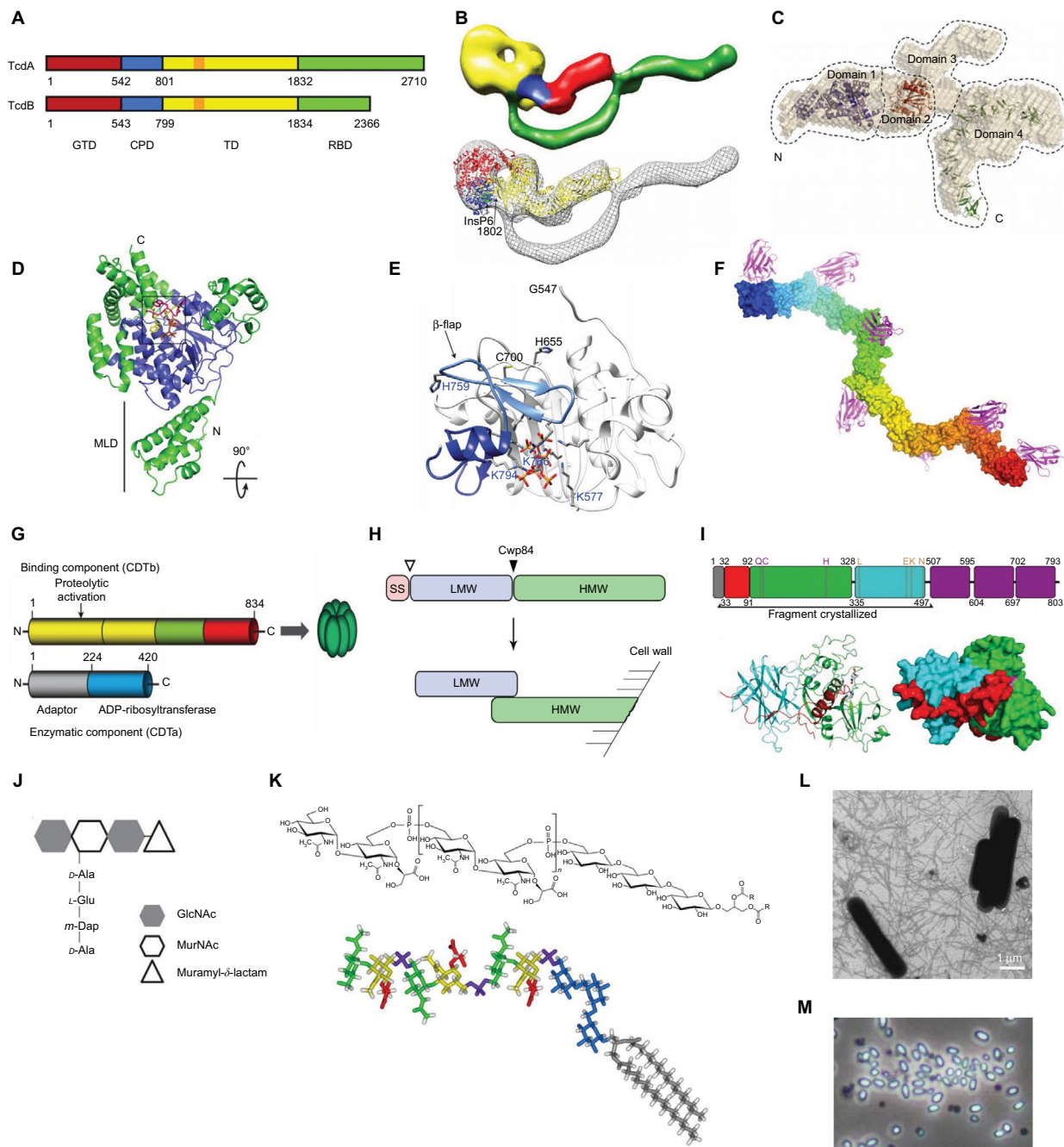


Figure 1 Validated and potential *C. difficile* targets for antibody-based immunotherapy of CDI.

Notes: (A–F) *C. difficile* toxin A (TcdA) and toxin B (TcdB). (A) A schematic of TcdA and TcdB.¹⁵⁵ (B) A proposed global structure of TcdA.^{155,156} (C) A proposed global structure of TcdB.¹⁵⁷ (D) A crystal structure of TcdA GTD.¹⁵⁸ (E) A crystal structure of TcdA APD, including the CPD.¹⁵⁶ (F) A model of TcdA RBD cocrystal structure in complex with A20.1 V_H.⁷⁸ (G) A schematic of *C. difficile* binary toxin CDT.⁸⁶ (H) A schematic of *C. difficile* SLPs¹⁰⁴; arrows denote SS and Cwp84 cleavage sites. (I) A crystal structure of Cwp84.^{159,160} (J) A schematic of *C. difficile* spore peptidoglycan complex.¹⁶¹ (K) *C. difficile* LTA.¹¹³ (L) A photograph of *C. difficile* (630 strain) showing flagella.¹⁶² (M) A photograph of *C. difficile* (R20291 strain) spores (courtesy of Susan Logan, NRC, Canada).

Abbreviations: *C. difficile*, *Clostridium difficile*; CDI, *C. difficile* infection; GTD, glucosyltransferase domain; APD, autoprocessing domain; CPD, cysteine protease-domain; RBD, receptor-binding domain; SLPs, surface-layer proteins; SS, signal sequence; LTA, lipoteichoic acid; TD, translocation domain; MLD, membrane localization domain; LMW, low-molecular weight SLP subunit; HMW, high-molecular weight SLP subunit.

recurrent CDI.^{2,18–20} On the other hand, individuals who fail to respond quickly to the toxins develop symptoms of CDI, which include diarrhea and colitis. Both TcdA and TcdB are glucosyltransferase-containing multi-domain proteins that enter host epithelial cells, undergo an acid-induced conformational change, and release their glucosyltransferase domain (GTD; Figure 1D) inside the cell to inactivate GTPases, such as Rho, Rac, and Cdc42.^{14,21} GTPase inactivation causes a cascade of downstream effects, culminating in a loss of epithelial barrier function, proinflammatory responses, and toxins reaching underlying germinal centers.^{22,23} Individuals who eventually restore their natural GI tract microbiota and/or who mount an effective antitoxin immune response clear the infection, while those who fail to do so are prone to rounds of relapsing CDI.² Given the importance of these two toxins in manifesting the severe symptoms associated with CDI, antibody-based immunotherapies have largely focused on targeting the toxins.

Conventional wisdom implies that the use of antibodies as therapeutic agents against bacterial infections is a logical choice, given the immune system, including antibodies, has evolved to combat bacterial infections. A stream of antibacterial antibodies in various stages of development, including many antibodies in various phases of clinical development, is in line with this idea and a testament to the optimism and confidence drug developers have in antibodies as effective antibacterial agents.^{24–26} The infections targeted by antibodies include, but are not limited to, CDI, hospital-acquired pneumonia, ventilator-associated pneumonia, and Shiga toxin-associated *Escherichia coli*-induced hemolytic uremic syndrome, caused by bacteria such as *C. difficile*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *E. coli*. In many cases such as in CDI, the antibody targets are toxins.

In this review, we chronicle the recent advances in antibody-based immunotherapy for treating CDI. Antitoxin approaches dominate the current immunotherapy pipeline with bezlotoxumab, which successfully passed a recent Phase III clinical trial, leading the way.²⁴ We discuss other antitoxin antibody approaches in development and in particular take a detailed look at the toxin epitopes targeted by these antibodies. We review nontoxin-based *C. difficile* targets that show promise for potential antibody targeting (Figure 1G–M). Finally, we propose next-generation antibody formats for *C. difficile* targeting, including multiple specificities that go beyond targeting one type of *C. difficile* virulence factor. These novel formats may show promise as GI-targeting oral therapeutics, opening up a novel and potentially very

efficacious delivery route to disrupt *C. difficile* before it can effectively establish infection.

Antibody-based immunotherapies

We previously documented various passive antibody-based immunotherapies under development for the treatment of CDI.⁶ Since then there have been numerous advances and additional formats of antibodies characterized, including key Phase III clinical trial data from the actoxumab/bezlotoxumab program. While there are other antibody-based approaches that have shown efficacy in treating CDI in animals, namely, polyclonal antibody preparations^{27–30} and intravenous (IV) immunoglobulin therapy,³¹ the focus of this review is on mAbs and sdAbs.

mAbs are a widely successful class of antibodies with at least 45 antibodies approved to treat a range of indications, including cancer, autoimmune disorders, cholesterol, and infectious disease (Figure 2A). Hybridoma technology allows for the isolation of murine mAbs, but these often require conversion into semihuman (chimeric) or humanized mAbs before use in human beings. More recent techniques using transgenic animals with fully human antibody repertoires or recombinant selection systems allow for the isolation of fully human mAbs. mAbs, specifically the immunoglobulin G1 (IgG1) isotype, offer long serum half-lives of up to 21 days³² and high target affinity and specificity, making them ideal antitoxin agents for use systemically.⁸ This long half-life suggests that a single infusion of IgG1 could offer protection of patients from CDI, or in the case of patients with CDI, this could reduce the chance of relapse.^{8,33} While the development of mAbs as antitoxin and antimicrobial agents is well documented, concerns over their potential costs and widespread adoption are warranted.^{24,34,35}

sdAbs provide attractive therapeutic modalities against CDI. Defined as autonomous variable domains of antibodies with antigen-binding capabilities, sdAbs may be Ig V_H domains, Ig V_L domains, V_HH domains derived from *Camelidae* heavy-chain IgGs, or V_{NAR} domains derived from cartilaginous shark IgNAR antibodies (Figure 2).^{36–38} Some of their unique features compared to mAbs and other recombinant antibody fragments such as fragment antigen binding and single-chain variable fragment (scFv)³⁹ include their single-domain nature; small size (13–15 kDa); high chemical, thermal, and proteolytic stability; high refoldability; high aggregation resistance; high-level expression in microorganisms; high modularity; tissue-penetrating properties; ability to access cryptic epitopes (eg, cavities in receptors, enzymes, toxins, and infectious agents);

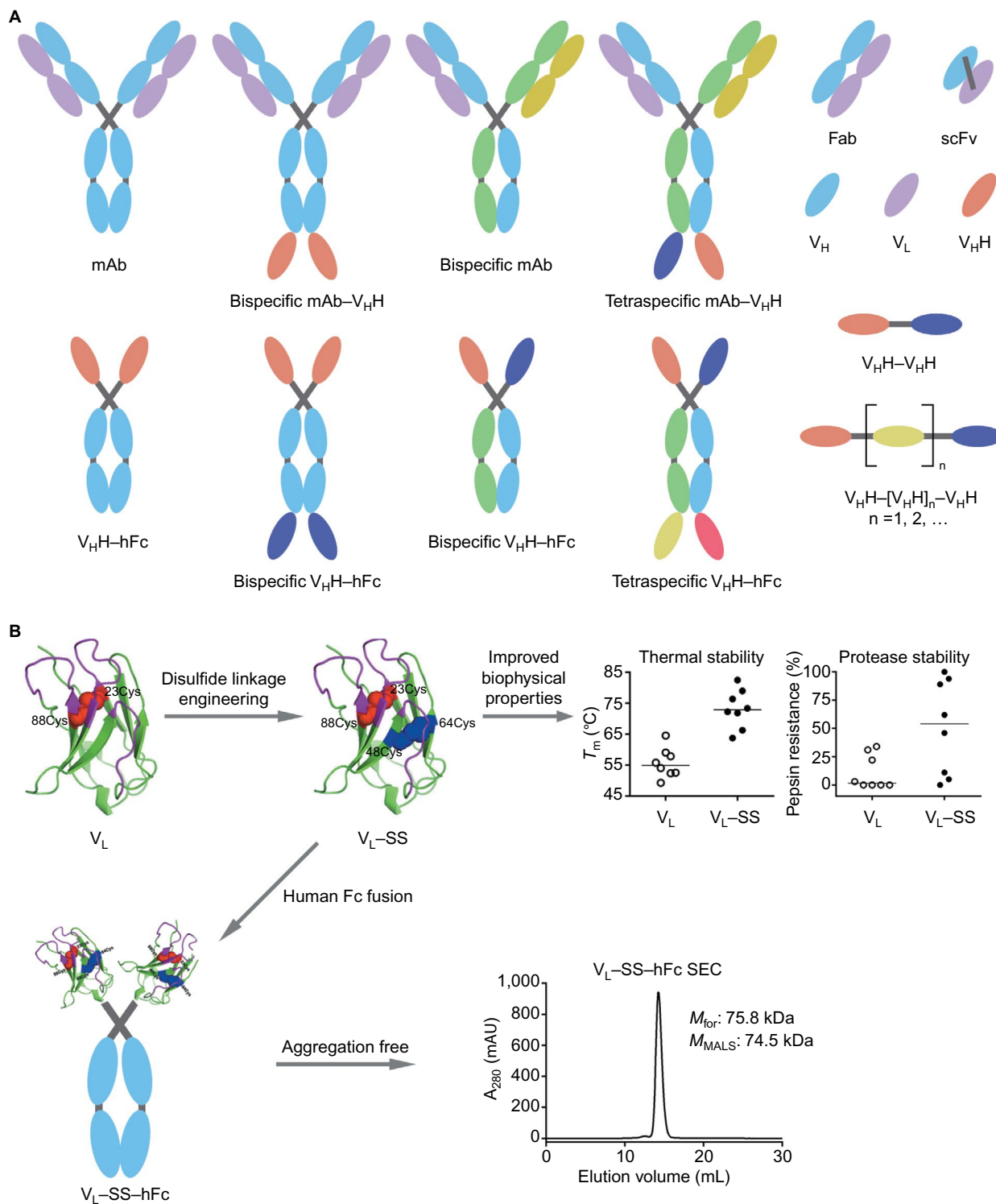


Figure 2 Potential antibody formats for CDI immunotherapy.

Notes: (A) Conventional mAbs and various multispecific targeting formats are now a reality. Antibody fragments such as Fab, scFv, V_H , V_L , and V_HH allow for modular assembly of multispecific affinity reagents. Several examples, but by no means an exhaustive collection of possible antibody formats, are shown. For more antibody formats, refer to Spiess et al.¹²⁰ (B) Engineering robust and efficacious sdAb (human V_H , human V_L , V_HH) therapeutics. Dramatic improvements to sdAb thermal and proteolytic stability, for example, by disulfide linkage engineering of human V_L domains, may allow for greater resistance to GI tract protease degradation and lead to more efficacious oral therapeutics targeting *C. difficile*. Furthermore, disulfide-engineered sdAbs (V_L -SS) can be efficiently turned into highly stable, aggregation-free, efficacious systemic therapeutics by fusion to hFc (V_L -SS-hFc).

Abbreviations: CDI, *C. difficile* infection; mAbs, monoclonal antibodies; Fab, fragment antigen binding; sdAb, single-domain antibody; GI, gastrointestinal; *C. difficile*, *Clostridium difficile*; hFc, human Fc; T_m , melting temperature; mAU, milli absorbance unit; M_{for} , formula molecular mass; M_{MALS} , molecular mass determined by MALS; MALS, multiangle light scattering.

amenability to in vitro selection and engineering approaches for robust domains that are resistant to proteases (eg, GI protease); and acidic pH-induced and heat-induced aggregation, denaturation, and degradation.^{36,38,40–52} In addition, sdAbs can be fully human (V_{Hs} and V_{Ls}) or readily humanized (V_{Hs} , V_{NARs}) to reduce/eliminate their potential immunogenicity.^{36,38,53–56} Moreover, high-affinity sdAbs with equilibrium dissociation constants ($K_{D,s}$) in the low nanomolar to picomolar range are readily obtainable.³⁶ The aforementioned features render sdAbs as efficacious therapeutics with low cost of goods.

Table 1 describes the various antitoxin antibodies isolated or characterized, and in particular, examines toxin target specificity, epitopes, and neutralizing potency and breadth. Table 1 is categorized by antitoxin antibody format, beginning with mAbs and then camelids (llama and alpaca) and human sdAbs.

Antitoxin A/B mAbs

The prime targets for therapeutic antibodies against CDI have been the toxin A/toxin B pair (Figure 1A–F). Owing to their remarkable specificity combined with the fact that they target toxins, antitoxin A and B antibodies are not expected to induce broad resistance among bacteria or disturb the healthy microbiota, as is the case with most conventional antibiotics, and presumably the reason for their ability to reduce the recurrence of CDI.

The most clinically advanced and characterized antibodies for the treatment of CDI are actoxumab and bezlotoxumab (Table 1). These fully human mAbs target TcdA and TcdB, respectively,⁵⁷ and are the only antibodies for the treatment of *C. difficile* to have been tested in clinical trials.^{24,58} Actoxumab (previously named MK-3415, CDA1, MDX-066, 3D8) recognizes the C-terminal cell-surface receptor-binding domain (RBD) of TcdA and appears to bind each TcdA protein twice.⁵⁹ The antibody was shown to potently neutralize TcdA in vitro.⁵⁷ The mechanism of TcdA neutralization is through direct toxin neutralization and is thought not to involve effector functions.⁶⁰ Bezlotoxumab (previously named MK-6072, CDB1, MDX-1388, 124-152) recognizes the C-terminal RBD of TcdB and appears to bind each TcdB protein twice.⁵⁹ Orth et al⁶¹ showed that the bezlotoxumab binding site on TcdB overlaps with the putative TcdB carbohydrate-binding region. The antibody was shown to potently neutralize TcdB in vitro.⁵⁷ In vivo, actoxumab, when combined with bezlotoxumab, was protective in multiple mouse and hamster models.^{57,60} In contrast, in a piglet model of CDI, prophylactic administration of bezlotoxumab alone or combined with actoxumab provided 100% protection

from systemic and GI tract CDI.⁶² Piglets given actoxumab alone showed a similar lack of efficacy compared to placebo, with a mortality rate of 67%, suggesting that much of the protection was driven by the anti-TcdB mAb.⁶² Interestingly, in the same piglet study, administration of alpaca antitoxin A polyclonal antibodies also showed a similar lack of efficacy compared to placebo. Clinically, in Phase II trials, the combination of both antibodies significantly reduced the rate of CDI relapse compared to standard-of-care antibiotic therapy.⁵⁸ In the recently completed Phase III trials, treatment of CDI with bezlotoxumab in conjunction with standard-of-care antibiotic therapy reduced CDI recurrence for 12 weeks compared to placebo.²⁴ Treatment with both mAbs provided no added efficacy, and actoxumab alone did not prevent *C. difficile* recurrence.²⁴ It is likely, based on the totality of the available data, that the contribution of antitoxin A antibodies to protection is related to the nature of the host species (human vs piglet vs rodent) rather than to the nature of the antibody itself.

The aforementioned mAbs do not reduce the severity of diarrhea during the initial CDI episode, time to resolution of diarrhea, or the duration of hospitalization for the initial episode,^{1,58} although Phase II and III clinical studies were not designed to assess these, leaving the door open for developing next-generation antibody therapeutics with improved and more complete efficacy. Ideally, such therapeutics should be produced at low cost, work with high efficacy, eliminate CDI recurrence, and reduce the severity of diarrhea, time to resolution of diarrhea, and duration of hospitalization. Additionally, the lack of efficacy of actoxumab in clinical trials does not necessarily demonstrate that targeting TcdB alone is sufficient to achieve full efficacy. Already a number of antitoxin A/B antibody combinations are in the early stages of development and claim to have improved preclinical efficacy in reducing CDI recurrence and severity of diarrhea relative to actoxumab/bezlotoxumab.^{59,63} Thus, the next-generation CDI therapeutics will most likely require combinations of antitoxin A and antitoxin B antibodies.

Recently, Anosova et al⁶³ presented data on a human mAb to TcdA and a pair of human mAbs to TcdB, isolated from healthy human donors using a high-throughput B-cell cloning strategy. The TcdA-neutralizing mAb A2 recognizes the C-terminal RBD of TcdA, possibly a linear epitope represented by the TGWQTI motif. In vitro, A2 neutralized TcdA from toxinotypes 0, III, and V in both Vero cell cytotoxicity and T84 transepithelial electrical resistance (TEER) assays. To target TcdB, the group isolated two mAbs, B1 and B2, which targeted the N-terminal GTD. B1 possibly recognizes

Table 1 Summary of mAbs and sdAbs targeting *C. difficile* toxin A and toxin B

Name	Antibody format	Specificity	Epitope location and stoichiometry (Ab:toxin)	Neutralizing in vitro?	Protective in vivo (model)?	References
Actoxumab (MK3415, CDA1, MDX-066, 3D8)	mAb (human IgG1)	TcdA	RBD (2:1)	Yes	Yes (mouse, hamster, piglet, and human) in combination with bezlotoxumab. No/ poor efficacy in piglets/human beings when used alone	24, 33, 57–60, 62
Bezlotoxumab (MK6072, CDB1, MDX-1388, 124-152)	mAb (human IgG1)	TcdB	RBD (2:1)	Yes, through partial blockage of the carbohydrate-binding pocket	Yes (mouse, hamster, piglet, and human) in combination with actoxumab. Efficacious in human beings when used alone	24, 57–62
A2	mAb (human IgG1)	TcdA	RBD (unknown). A2 possibly binds a linear epitope, including the TGWQTI motif	Yes, broad neutralization of TcdA from multiple <i>C. difficile</i> ribotypes	Yes (hamster) in combination with B1 and B2. No protection when used alone	63
B1, B2	mAb (human IgG1)	TcdB	GTD (unknown). B1 and B2 do not overlap. B1 possibly binds a linear epitope, including the SGRNK motif. B2 binds a conformational epitope	Yes, broad neutralization of TcdB from multiple <i>C. difficile</i> ribotypes	Yes (hamster) in combination with A2. Efficacious when B1 and B2 used alone or in combination with each other	63
CA997	mAb (humanized IgG1)	TcdA	RBD (≥12:1)	Yes, neutralized TcdA from multiple <i>C. difficile</i> ribotypes. More potent than actoxumab	Yes (hamster) in combination with CA1125 and CA1151. Greater protection than actoxumab/ bezlotoxumab combination	59
CA1125, CA1151	mAb (humanized IgG1)	TcdB	RBD (CA1125 = 1:1; CA1151 = 2:1). Nonoverlapping epitopes	Yes, a combination of both mAbs was strongly neutralizing and single antibodies were weakly neutralizing	Yes (hamster) when both were used in combination with CA997. Greater protection than actoxumab and bezlotoxumab combination	59
PA-50	mAb (humanized IgG1)	TcdA	RBD (multiple Abs:1). Epitope is broadly conserved throughout 027 ribotype strains. Does not overlap with actoxumab	Yes, broad neutralization of TcdA from multiple <i>C. difficile</i> ribotypes. More potent than actoxumab	Yes (hamster) in combination with PA-41. Greater protection than actoxumab and bezlotoxumab combination	64
PA-41	mAb (humanized IgG1)	TcdB	GTD (1:1). Epitope is conserved among 027 ribotypes	Yes, broad neutralization of TcdB from multiple <i>C. difficile</i> ribotypes. More efficacious than bezlotoxumab	Yes (hamster) in combination with PA-50. Greater protection than actoxumab and bezlotoxumab combination	64
PCG-4	mAb (mouse IgG2a)	TcdA	RBD (5–6:1). Recognizes amino acids 2097–2141 and 2355–2398	No; however, Demarest et al ⁶⁷ showed modest neutralization	Yes (hamster) neutralized the effects of TcdA given intragastrically	65–69, 74
3358, 3359	mAb (mouse IgG2a and IgG1)	TcdA	RBD (3358=14:1; 3359=9:1). Nonoverlapping epitopes. 3358 and PCG-4 bound overlapping epitopes	Yes, single mAbs were neutralizing and combinations were more potent	N/d	67
IG3, IB5, 2D4, 2C7, 4A4, 5D8	mAb (mouse)	TcdA	RBD (unknown). All recognized linear epitopes	Yes	Yes (mouse), 4A4 protected in TcdA IP challenge. 2C7 and 5D8 did not protect. Combinations of some improved protection	70
A1H3	mAb (mouse IgG2a)	TcdA	Unknown	Unknown. A1H3 enhances cell-surface recruitment of TcdA, enhancing toxicity	N/d	71–73

(Continued)

Name	Antibody format	Specificity	Epitope location and stoichiometry (Ab:toxin)	Neutralizing in vitro?	Protective in vivo (model)?	References
A9, I41-2, C11	mAb (mouse)	TcdA	RBD (unknown). Recognized a region of RBD that covers amino acids 1964–2682	N/d	Yes (mice), ascites fluids injected IV protected mice from <i>C. difficile</i> challenge	74
G-2	mAb (mouse IgG1)	TcdA/B	Unknown, binds a shared epitope on TcdA and TcdB	No	N/d	65
A4.2, A5.1, A19.2, A20.1, A24.1, A26.8	Llama V _H H	TcdA	RBD (A4.2, A5.1, A19.2, A24.1 = unknown; A20.1 = 7:1; A26.8 = 1:1). A19.2 recognized a linear epitope; A4.2, A5.1, A20.1, A24.1, and A26.8 recognized conformational epitopes. A4.2, A5.1, and A26.8 shared overlapping epitopes, while A20.1 was nonoverlapping	Yes, V _H Hs neutralized TcdA. Enhanced neutralization efficacy with pair/triplet combinations	N/d. Administration of B. longum transformed with A20.1 or A26.8 resulted in gut expression (secretion) of both V _H Hs in mice	41, 75, 76, 78, 79
B5.2, B13.6, B15.5, B39	Llama V _H H	TcdB	RBD (B5.2, B13.6, B15.5 = unknown; B39 = 4:1)	No	N/d	76, 78
B4, B5, B12, B17	Human V _L	TcdB	RBD (unknown). V _L s bind epitopes within the C-terminal 80 amino acids	No	N/d	77
ABA (V _H H) ₄ , AH3-E3-E3-AA6	Alpaca V _H H (bispecific, tetrameric)	TcdA/B	TcdA GTD, AH3 V _H H; TcdA TD, AA6 V _H H; TcdB GTD, E3 V _H H (unknown)	Yes, tetramer broadly neutralized toxins A and B from eleven clinical isolates	Yes (mouse), protected from lethal IP systemic TcdA/B challenge. Tetramer protected mice infected with 027 ribotype strain	80
B2, E2, G3, D8	Llama V _H H	TcdB	RBD (unknown). Three epitopes targeted: B2, E2/G3, and D8	Yes, V _H H monomers (B2, G3, D8) were neutralizing. Combinations of V _H Hs did not improve neutralizing potency	Yes (hamster), using an oral administration model with <i>Lactobacilli</i> displaying B2 and G3 V _H Hs. Oral administration of V _H Hs (B2+G3+D8) not displayed on <i>Lactobacilli</i> was not protective	81

Abbreviations: mAb, monoclonal antibody; sdAb, single-domain antibody; *C. difficile*, *Clostridium difficile*; Ab, antibody; RBD, receptor-binding domain; GTD, glucosyltransferase domain; N/d, not determined; IP, intraperitoneal; IV, intravenous; B. longum, *Bifidobacterium longum*; TD, translocation domain.

a linear epitope containing residues SGRNK in a four-helix bundle near the N-terminus of the GTD, and B2 is thought to bind a conformational epitope that is within the GTD but distinct from the B1 mAb. The pair of TcdB mAbs neutralized toxinotypes 0, III, V, VIII, and X in Vero cell assays and T84 TEER assays. Using the hamster model of CDI, simultaneous intraperitoneal (IP) administration of all three mAbs protected the animals from mortality, following challenge with clinical *C. difficile* strain 630. Treatment of hamsters with A2 alone was not protective, while treatment with B1 alone, B2 alone, or B1 + B2 resulted in survival rates of 70%, 40%, and 50%, respectively.

In an earlier study, Davies et al⁵⁹ also showed strong protection against CDI in hamsters using a single anti-TcdA mAb and a pair of anti-TcdB mAbs. CA997 is a humanized mAb that binds the C-terminal RBD of TcdA and was estimated to bind at least 12 times per toxin. Using Caco-2 cells, CA997 neutralized TcdA from ribotypes 003, 027, and 078 and was more potent than actoxumab. In addition, CA997 inhibited TcdA-induced TEER loss in TEER assays, while actoxumab was poorly protective in the TEER assay. CA1125 and CA1151 are humanized TcdB mAbs that target nonoverlapping epitopes in the RBD and bind with valencies of one and two, respectively. In vitro, single mAbs were weakly neutralized in Caco-2 inhibition assays; however, when combined, potent TcdB neutralization was reported. In vivo, using the hamster CDI model, simultaneous IP administration of all three mAbs provided 100% protection at 11 days. At 28 days, the combo of three antibodies showed higher levels of protection than the combination of actoxumab and bezlotoxumab (82% vs 28% survival, respectively).

Elsewhere, PA-50 is a humanized anti-TcdA mAb that targets TcdA RBD at multiple sites.⁶⁴ This epitope is broadly conserved throughout *C. difficile* 027 ribotype strains and does not overlap with the TcdA epitope recognized by actoxumab. PA-50 was capable of TcdA neutralization from a broad range of *C. difficile* ribotypes (001, 002, 003, 012, 014, 017, 027, 078) and was more potent than actoxumab in vitro. The authors suggested a mechanism of toxin neutralization involving cooperative inhibition, possibly due to multivalent interactions with TcdA. Marozsan et al⁶⁴ also isolated a potent TcdB inhibiting antibody termed PA-41. PA-41 targets the N-terminal GTD of TcdB and is thought to recognize a single epitope that is conserved among 027 ribotypes. In vitro, PA-41 was capable of broad TcdB neutralization, inhibiting toxins from the same *C. difficile* ribotypes described for PA-50-based TcdA neutralization. The fact that PA-41 showed superior neutralizing efficacy compared to bezlotoxumab

suggests that targeting the GTD is an effective and potent area to target on TcdB. Using the hamster CDI model, PA-50 in combination with PA-41 resulted in long-term survival of hamsters compared to 0% survival for animals treated with the standard antibiotic vancomycin or, surprisingly, with a combination of actoxumab and bezlotoxumab.

A number of earlier mouse mAbs were isolated and capable of toxin neutralization. PCG-4⁶⁵⁻⁶⁹ is a TcdA targeting mouse IgG2a mAb that binds the RBD with a valency of five or six and recognizes epitopes in amino acids 2097–2141 and 2355–2398 (Figure 1A). In vitro, Lyerly et al⁶⁵ reported that the antibody failed to inhibit TcdA in Chinese hamster ovary (CHO) K1 cell-based assays; however, Demarest et al⁶⁷ reported modest in vitro TcdA neutralization with the antibody. In vivo, PCG-4 neutralized the effects of TcdA (diarrhea and death) when TcdA was administered intragastrically to hamsters and when TcdA was used in an intestinal loop model in rabbits. Demarest et al⁶⁷ characterized several mouse mAbs capable of TcdA inhibition. mAbs 3358 (IgG2a) and 3359 (IgG1) bound nonoverlapping epitopes in the TcdA RBD at up to 14 and nine sites, respectively. The authors revealed that 3358 and PCG-4 (expressed as a recombinant mouse/human chimeric antibody) contained overlapping TcdA RBD epitopes. Both 3358 and 3359 neutralized TcdA in CHO K1 cell-based assays, and the combination of the two resulted in increased neutralizing potency compared to individual mAbs. Interestingly, 3358 appeared to increase the detectable level of RBD on the surface of CHO cells, while 3359 inhibited RBD binding to the CHO cell surface. Elsewhere, Zhang et al⁷⁰ isolated a panel of TcdA-specific mouse mAbs (1G3, 1B5, 2D4, 2C7, 4A4, 5D8) that recognized linear RBD epitopes and were capable of TcdA inhibition in CHO and HT-29 cell-based assays. In mouse models with IP administration of TcdA, 50% of mice given mAb 4A4 survived, while 2C7 and 5D8 mAbs were not as strongly protective. When combined, 4A4/2C7 and 4A4/5D8 pairs were more protective than the 4A4 mAb alone. Another TcdA-specific mouse IgG2a mAb, A1H3, has been used in several studies⁷¹⁻⁷³ but is poorly characterized with respect to TcdA epitope and neutralizing potency. A1H3 enhanced TcdA-mediated cellular effects in murine macrophages and human monocytes through FcγR1-mediated endocytosis. A1H3 also facilitated cell-surface recruitment of TcdA, likely contributing to enhanced cytotoxicity. Corthier et al⁷⁴ characterized three TcdA-binding mouse mAbs (A9, 141-2, C11) produced from ascites. All bound to a region of the TcdA RBD covered by amino acids 1964–2682. Mice were protected from *C. difficile* challenge upon IV administration

of the antibodies. Finally, one of the first mouse mAbs (IgG1) isolated, G-2, which recognized both TcdA and TcdB, was not capable of in vitro TcdA or TcdB neutralization.⁶⁵

Antitoxin A/B sdAb fragments

In addition to antitoxin human(ized) and mouse mAbs under development, a number of sdAb fragments are being explored as alternative immunotherapeutic agents for treating CDI (Figure 2). The potential advantages of using sdAb fragments are described earlier (Antibody-based immunotherapies). One of the first sdAbs isolated against *C. difficile* toxins was from our laboratory.^{75–77} In this work, a number of llama V_HHs were isolated from an immune phage display library constructed after immunization with C-terminal RBD fragments from each toxin.⁷⁶ A number of TcdA-binding V_HHs were found to potently inhibit TcdA in cell-based assays, and combinations of various V_HHs significantly improved neutralizing potency. V_HHs A4.2, A5.1, A20.1, A24.1, and A26.8 recognized conformational RBD epitopes, while A19.2 bound a linear epitope. The A20.1 epitope was found to not overlap with those for A4.2, A5.1, or A26.8, which appeared to be overlapping, and none of the V_HHs prevented free trisaccharide from binding the RBD carbohydrate-binding pocket.⁷⁶ Cocystal structures of A20.1 and A26.8 in complex with a TcdA fragment confirmed this binding pattern, with A20.1 binding very close to the carbohydrate-binding pocket with a proposed valency of seven V_HHs per RBD based on modeling (Figure 1F).⁷⁸ A26.8 appeared to bind the extreme C-terminus of TcdA with a stoichiometry of 1:1.⁷⁸ Recently, Shkoporov et al⁷⁹ expressed A20.1 and A26.8 in *Bifidobacterium longum* and showed TcdA neutralization in vitro. The group went on to administer the probiotic bacteria to mice and confirmed the in vivo expression (secretion) of the V_HHs in the gut of mice. Several V_HHs and human V_Ls that bind the TcdB RBD have also been isolated and characterized,^{76–78} and despite some with very high affinity binding, none were capable of toxin inhibition in cell-based assays. This includes B5.2, B13.6, and B15.5 V_HHs⁷⁶; B39 V_HH⁷⁸; and B4, B5, B12 and B17 human V_Ls.⁷⁷ B39 has been proposed to bind to TcdB at four sites.⁷⁸ Interestingly, B5.2 showed cross-reactive binding to TcdA, but did not neutralize the toxin.

Yang et al⁸⁰ later reported the isolation of TcdA- and TcdB-specific alpaca V_HHs. The AH3 V_HH bound the N-terminal GTD of TcdA, the AA6 V_HH recognized the central translocation domain, and the E3 V_HH bound the GTD of TcdB. The group constructed a novel, tandem-linked molecule consisting of a string of four V_HHs (AH3–E3–E3–AA6), with the TcdA-targeting antibodies at the termini and

the TcdB-targeting antibody, represented twice, in the middle. The tetramer broadly neutralized both toxins from several clinical *C. difficile* isolates in cell-based (Vero and CT26) assays, including ribotypes 001, 002, 012, 014, 015, 023, 027, 078, and 106. The tetramer protected mice against a lethal systemic challenge of a mixture of TcdA/TcdB, at a tetramer concentration as low as 3.2 µg/kg, and reversed CDI in mice infected with the 027 strain after a single 1 mg/kg injection.

More recently, Andersen et al⁸¹ reported the isolation of four llama V_HHs against the TcdB RBD after immunization with whole TcdB toxin. The four V_HHs (B2, E2, G3, and D8) bind to three unique TcdB RBD epitopes (B2, E2/G3, and D8). As monomeric V_HHs, B2, G3, and D8 neutralized the cytopathic effects of TcdB on MA-104 cells. In combinations as doublet and triplet sets, the antibodies had no additive effect compared to the most protective singlet V_HH G3. The group expressed the antibodies on the surface of probiotic bacteria, and B2, G3, and D8 retained neutralizing potency. Interestingly, additional V_HHs that were nonneutralizing as monomers became neutralizing when surface displayed, presumably due to the large steric effects imparted by the probiotic bacteria. In a prophylactic, oral treatment hamster model of CDI, a combination of two strains of *Lactobacilli* (one displaying B2 and the other displaying G3) delayed the death of hamsters challenged with TcdA⁺ TcdB⁺ *C. difficile* spores, compared to zero survivors in the controls (infection-only group and non-V_HH-expressing *Lactobacillus* group). Half of the hamsters receiving the V_HH-expressing *Lactobacillus* survived until the end of the experiment at day 5. A mixture of B2, G3, and D8 dosed orally was not protective, possibly due to its degradation and/or transitory nature in the GI tract. Interestingly, Andersen et al⁸¹ also immunized llamas with whole TcdA, but failed to isolate any neutralizing antibodies, which is exactly the opposite of Hussack et al⁷⁶ who isolated TcdA neutralizers but not TcdB neutralizers.

Antibodies to other *C. difficile* targets

While the primary targets of numerous mAbs and sdAbs is *C. difficile* toxins A and B, many other *C. difficile* virulence factors such as binary toxin, surface-layer proteins (SLPs), and flagella are being used as possible targets for immunotherapy (Figure 1). One of the oldest immunotherapies investigated for CDI is IV immunoglobulin therapy, in which human polyclonal mixtures of antibodies are infused into CDI patients.³¹ The exact composition of these mixtures is unknown, although they likely contain antitoxin antibodies and potentially antibodies to *C. difficile* virulence factors/surface proteins. Analysis of sera from patients with CDI⁸²

has led to a number of novel *C. difficile* targets being explored as potential vaccines.^{12,83,84} Successful vaccine targets could conceivably drive the development of antibodies specific to these targets and guide the next generation of immunotherapies for CDI.

There are emerging opinions that a “total cure” against CDI may have to include antibodies to other targets (Figure 1) in addition to those against toxins A and B.¹ In line with this new emerging trend, a vaccine candidate includes TcdA, TcdB, and components of *C. difficile* binary toxin CDT.^{85–87} Similar to toxins A and B, CDT was shown to cause death in mice and hamsters, and the aforementioned combination vaccine approach protected mice when challenged with native toxins A, B, and CDT.⁸⁵ Unger et al⁸⁸ isolated llama sdAbs against CDTa and CDTb and found several sdAbs to neutralize cytotoxicity in vitro. As the antitoxin antibodies do not prevent the initial *C. difficile* colonization step, complementing them with antibodies that target spores and prevent or eliminate their carriage or dissemination appears attractive. Antibodies that target cell-surface components involved in the colonization and adherence to gut tissues of hosts, such as SLPs,^{83,87,89–95} flagella,^{83,87,96–98} and Cwp84,^{83,87,98,99} are other promising complementary therapeutics. A number of experiments, including some in vivo animal studies, strongly suggest that targeting SLPs by antibodies is a viable therapeutic approach against CDI,^{83,94,95,100–103} and interestingly, SLP-specific sdAbs have been shown to inhibit the motility of *C. difficile* in in vitro assays.¹⁰⁴ Several studies support flagella as a therapeutic target,^{96,98,101} including one immunization study where flagellin (FliC)- and flagellin filament cap protein (FliD)-immunized mice showed a significant decrease in *C. difficile* colonization¹⁰¹ and another where orally administered purified FliD-specific IgY protected hamsters from CDI when challenged with *C. difficile* strain 630.⁹⁸ Moreover, mice vaccinated rectally with a combination of FliD, a flagella preparation, Cwp84, and cell wall extract showed a significant reduction in *C. difficile* colonization,¹⁰¹ and rectal as well as oral vaccination with Cwp84 partially protected hamsters from lethality.^{105,106} PSII polysaccharides, abundantly expressed on the surface of all *C. difficile*, are also attractive targets for therapeutic antibodies.^{83,107–109} In support of this view, studies point to PSII as a beneficial vaccine,^{83,108,110–112} and interestingly, PSII–toxin conjugates appear to be a promising vaccine for targeting both cell-surface PSII and toxins A and B.¹¹¹ Finally, a related promising antibody target is the surface-exposed lipoteichoic acid polymer, which has been

shown to be conserved in the majority of *C. difficile* strains and easily accessible to antibodies.^{113,114}

Future perspectives and conclusion

Going forward, much will be learned from the late-stage clinical data produced from actoxumab/bezlotoxumab and from vaccine trials underway, to determine if the biologics/vaccine route is an effective one for CDI therapy. Another major consideration is the optimal delivery route (eg, systemic or oral administration) and the target population of antibody-based or vaccine-based CDI therapies. For now, the main route for antibody-based therapies is systemic delivery; however, improved delivery systems to the GI tract coupled with stabilized antibody formats may open the possibility for oral delivery. The target population for antibody-based therapies will likely be at-risk groups, including the elderly, the immunocompromised, those admitted to hospitals for antibiotics, and those admitted to hospitals during a CDI outbreak. Improving our understanding of *C. difficile* biology, host–pathogen interactions, and the identification of novel virulence factors will provide a source of new targets for immunotherapy, with an end goal of not only reducing CDI recurrence but also rapidly clearing primary CDI and its effects or even preventing them entirely.

With respect to antibody-based immunotherapy, an attractive alternative to the combination therapy approach (eg, administering two antibodies each with distinct specificity) is to combine the specificity of individual component antibodies in a therapeutic cocktail into one bispecific or multispecific antibody construct.^{115–120} The approach, in particular the bispecific one, has been applied to therapeutic antibodies against cancer and inflammatory and infectious diseases. Bispecific antibodies are in general more efficacious than their parental individual antibodies but similar in efficacy to combined antibody pairs. The costs associated with resources for manufacturing, clinical studies, and regulatory reviews are substantially lower in the bispecific or multispecific antibody approach than the combination therapy approach since the number of antibodies to be developed and approved is reduced in the former scenario. However, issues such as manufacturability and the stability of bispecific and multispecific antibodies still need to be resolved. A good example of an antibacterial bispecific antibody is MEDI3902 (BiS4 α Pa), which is a dual-targeting antibody that is protective in a lethal pneumonia mouse model, whereas a monotherapeutic is not protective.^{121,122} Figure 2A proposes a number of bispecific

and multispecific antibody formats as the next generation of CDI therapeutics.

The current trend for administering antitoxin antibodies relies on systemic delivery by injection. Presumably, the systemically delivered antibodies should traverse the mucosal barrier into the GI tract to be effective, and a recent study indicates that they do so by a toxin-mediated, FcRn-independent paracellular transport mechanism through the compromised (leaky) gut wall barrier in infected animals.¹²³ The need for crossing the barrier may be a bottleneck to efficacy by preventing the delivery of sufficient quantities of antibody drugs to the site of infection. This drawback does not exist with orally administered antibody therapeutics as they bypass the requirement for traversing the mucosal barrier and can directly reach the site of infection. Other advantages of oral therapy include patient compliance; simplicity of administration; reduced cost, partly due to less stringent manufacturing requirements than injectable antibodies; enhanced potency and specificity; needle-free nature; higher safety; immune-tolerant nature, which allows repeated ingestion of therapeutic antibodies without compromising safety and eliminates the need to humanize antibodies; minimized systemic drug exposure and its subsequent unwanted side effects; and low dosage requirements.^{124–126} Studies with bovine immune Ig preparations^{6,7,30,124,127} and IgA preparations^{6,7,124,128} have shown that an oral antibody-mediated approach against CDI is possible. However, significant challenges facing oral antibody therapeutics include the presence of the protein-degrading enzymes, such as pepsin, trypsin, chymotrypsin, carboxypeptidase, and elastase, in the stomach and intestine; the denaturing acidic character of the gastric fluid (pH 1–3.5); the transient presence of therapeutic antibodies in the GI tract; and the requirement for repeated dosing leading to increased costs.^{6,124,125} However, these challenges are not insurmountable. To begin, one may utilize certain classes of antibodies that are more resistant to proteases such as IgA,^{6,7,125} IgY,^{6,7,125,129} and sdAbs.^{6,37,38,41,130} Furthermore, advances in protein engineering allow for the creation of robust antibodies that are resistant to the GI tract proteases and acidic environment of the stomach. Their stability can further be enhanced by applying strategies such as decoy proteins, pH modulation, masking protease active sites, enzyme inhibition, and encapsulation.^{124,125} As for the transient presence of oral therapeutic antibodies, theoretically, the GI half-life of antibodies can be improved by fusing them to GI retaining molecules. Moreover, engineered probiotic bacteria (eg, lactic acid bacteria) displaying or secreting recombinant antibody fragments present a promising solution: residing

within the microbiota of the intestine, they can “administer” antibodies to the lower GI tract for prolonged periods of time.^{81,126,131–138} Additional advantages of the approach include the generally-regarded-as-safe nature of the carrier bacteria, cost-effective production, long shelf life (eg, in lyophilized form), simple distribution logistics, ease of administration, and possibility of engineering probiotic bacteria that surface display recombinant antibodies with high avidity and multiple specificity for improved efficacy. The approach has shown promising results in *in vivo* protection studies with several GI viral and bacterial pathogens, including *C. difficile*, where the target antigen was TcdB.^{81,126,131–136,138–140} Another promising oral therapy approach takes advantage of the ability of the gut’s enteroendocrine cells to produce and release large quantities of proteins into the gut environment in an intermittent or continuous fashion and a natural, nontoxic, and biocompatible polymer that serves as a protective carrier of the drug-encoding nucleotides into the enteroendocrine cells (<http://www.engeneinc.com/>).¹⁴¹ Both of the latter oral therapeutic approaches bypass the adverse GI tract conditions (presence of proteases and low acidic pH) and the need for purified antibodies.

With regard to systemic applications, unlike mAbs, sdAbs have very fast half-lives as low as 5 minutes (in mice) due to their small size, being below the renal filtration molecular mass cut-off, and lack of Ig Fc region.^{142–150} Several approaches can be applied to sdAbs for half-life extension.^{142,151,152} In particular, two popular recombinant approaches can be efficiently applied to therapeutic sdAbs, thanks to the highly modular nature of sdAbs. These include their tandem fusion to a second sdAb that is specific to human serum albumin or fusion to human IgG Fc region, with the latter format emulating the overall structure of naturally occurring *Camelidae* heavy-chain antibodies (Figure 2).^{38,56,142–144,148–150,153,154} In these cases, the half-life extension is conferred directly (ie, sdAb–Fc fusion) or indirectly, through serum albumin (ie, sdAb–sdAb fusion) binding to FcRn and through an increase in size (ie, sdAb–Fc fusion). Modularity of sdAbs also allows efficient generation of bispecific or multispecific recombinant antibodies against *C. difficile* targets, including toxins A and B. The options include fusing different sdAbs in tandem to make dimers, trimers, or tetramers or fusing sdAbs to mAbs to construct sdAb–mAb hybrid fusions (Figure 2). In the case of dimers and trimers, one may include a human serum albumin-specific sdAb to confer sufficient serum half-life, but in the case of a tetramer, this augmentation may not be necessary as its size surpasses the renal filtration molecular

mass cut-off giving it decent half-life for sufficient levels of in vivo efficacy. In fact, a bispecific antitoxin A/antitoxin B tetramer was shown to be protective of death in CDI-inflicted mice.⁸⁰ This also supports the finding that Fc-mediated host effector functions are not needed for in vivo protection of antitoxin A/antitoxin B antibodies and direct toxin neutralization will serve the purpose.⁸⁰ The increase in size and avidity in the fusion constructs may further enhance the neutralization potency of sdAbs.

The stability of sdAbs against the denaturing acidic environment and proteases of the GI tract, their superior and efficient folding, and the fact that they can be engineered into robust molecules make sdAbs attractive oral therapeutics against GI diseases such as CDI. One simple yet general stability engineering approach is disulfide engineering of sdAbs. The approach appears to be universally applicable to all V_Hs, V_Ls, and V_HHs and results in antibodies with very high thermostability, resistance to low acidic (stomach) pH, and resistance to GI proteases (Figure 2B).^{36,41,49,130} As an example, disulfide engineering applied to antitoxin A V_HHs (A4.2, A5.1, A20.1, and A26.8) rendered them highly thermostable and acidic pH and pepsin resistant without significantly compromising their neutralization capabilities.^{41,76} In addition, these robust domains can also be efficiently turned into aggregation-free sdAb-human Fc molecules (Figure 2B; example given for V_Ls). With respect to probiotic antibody-mediated oral therapy applications, sdAbs are the ideal therapeutic antibody format, both in secreted and cell-surface display modes, and several studies have shown the protection capability of V_HH displaying *Lactobacilli* in animal models of infections.^{79,81,131,133,135–138,140} Owing to their stability and superior folding and expression properties, sdAbs are produced feasibly and efficiently by engineered host probiotic bacteria at higher levels than scFvs, in both secretion and cell-surface display formats, resulting in higher therapeutic efficacies.^{79,131,133} Interestingly, the aforementioned antitoxin A A20.1 and A26.8 V_HHs were shown to be secreted in a functional form by the probiotic bacterium *B. longum* at a much higher efficiency than an scFv.⁷⁹

In conclusion, antibodies are poised to become a critical tool for health care professionals to use in their fight against CDI; however, concerns over their cost and widespread adoption by physicians remain valid. Efforts to obtain more efficacious antitoxin antibodies and antibodies to novel *C. difficile* targets, as well as the search for alternative antibody delivery routes (eg, GI delivery), could provide low-cost options for treating primary CDI and preventing cases of relapse.

Disclosure

The authors report no conflicts of interest in this work.

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