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Antibodies for Treatment of *Clostridium difficile* Infection

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Antibodies for the treatment of *Clostridium difficile* infection (CDI) have been demonstrated to be effective in the research and clinical environments. Early uncertainties about molecular and treatment modalities now appear to have converged upon the systemic dosing of mixtures of human IgG1. Although multiple examples of high-potency monoclonal antibodies (MAbs) exist, significant difficulties were initially encountered in their discovery. This minireview describes historical and contemporary MAbs and highlights differences between the most potent MAbs, which may offer insight into the pathogenesis and treatment of CDI.

Clostridium difficile infection (CDI) is a global problem especially affecting patients in hospitals and long-term health care facilities. Prior antibiotic usage, age greater than 65 years, and comorbidities are considered to be major risk factors (1). Symptoms of CDI include diarrhea, fever, and inflammation of the bowel sometimes including pseudomembranous colitis (PMC) or fulminant colitis ("mega colon"). Diarrhea can be prolific, ~3 to 15 bowel movements per day and prolonged, for up to 30 days (2). These factors lead to increased hospital stays of an average of 6 days (3), driving up the cost per case and collectively resulting in a substantial health care burden variably estimated to be up to $4.8 billion in the United States alone (4). Furthermore, crude 30-day mortality rates have been reported to range from 2.8% to 29.8%, about half of which appears to be directly attributable to CDI (5, 6).

WHY ANTIBODIES?

Pathogenic effects of *C. difficile* are caused by large protein exotoxins, predominantly TcdA and TcdB. Nontoxigenic strains do not cause disease in hamsters or humans, and indeed, they have been administered to human volunteers without causing diarrhea (7–10). Strains encoding either TcdA or TcdB alone cause CDI in hamsters, but the presence of both toxins was found to be more potent than either alone (7). Indeed, a minority (typically ~5%) of strains causing CDI in humans are A^-B^+, suggesting that TcdB alone is capable of causing symptoms in susceptible humans (1). The role of a third toxin, *C. difficile* binary toxin (CDT), in causing CDI remains contentious (11–14), partly because the assessment of patient outcome is confounded by the increased morbidity and mortality associated with the epidemic 027/NAP1/BI clone.

Humoral immunity is a well-understood mechanism for the neutralization and clearance of toxin activities, and hence, neutralizing antibodies were an obvious choice for early researchers who wanted to develop potential treatments for CDI. More recent research has provided additional insights into the potential value of using monoclonal antibodies (MAbs) to treat CDI. MAbs offer a long serum half-life, typically in the order of 14 to 21 days for human IgG1 (15). This is significant with respect to the typical duration of a primary episode and the temporal proximity of an initial potential recurrence. MAbs also offer logistical benefits compared to oral antibiotics in terms of dosing frequency and ensuring patient compliance. Collectively, these features mean that a single infusion or subcutaneous injection could offer sustained "therapeutic" and "prophylactic" protection against primary and potential recurrent infections, respectively. Neutralized antibody-antigen complexes have also been shown both to educate (16, 17) and vaccinate (18) host immune systems, and antigen-Fc fusion vaccines have been shown to offer mucosal protection (19). Consequently, there is an interesting possibility of significant long-term clinical benefit from MAbs with the right properties.

Early on in the development of neutralizing antibodies, several key questions needed to be answered. Were polyclonal antibodies essential to achieve protection? Would MAbs be able to provide complete protection? Was it sufficient to neutralize TcdA alone or would TcdB need to be neutralized as well? Since CDI toxin effects are observed substantially in the gut, would the antibody isotypes generally found to be more effective in the gastrointestinal tract (IgA or IgM) be required? Would oral dosing rather than parenteral dosing be necessary? If an IgG was parenterally dosed, could this protect the gut tissue from actions apparently occurring in the gut lumen? Could complete protection be achieved in an animal model, and how relevant are these models and protection levels to treatment in humans? Finally, could production science and the changing regulatory environment deliver effective antibody regimens?

HUMAN ANTIBODY RESPONSES

Studies of human immune responses against *C. difficile* and its antigens and toxins have given strong, if not decisive, support for the clinical potential of therapeutic MAbs. Nevertheless, these studies have been beset with technical and other challenges. For example, there have been differences in the purity of toxin preparations used by different researchers. Some researchers have studied TcdA alone, while others studied both TcdA and TcdB. Notably, data from both toxin binding enzyme-linked immunosorbent assay (ELISA) and functional (neutralization) assays have been sparse, with most opting for the simpler toxin binding ELISA alone. It is clear, however, when looking across published studies that not all patients have preexisting toxin neutralizing antibodies

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and that neither the titer nor number of serum-positive patients universally increase after a spontaneous or drug-induced cure—something that has also been witnessed in animal models (20). It would also appear that high toxin binding titer does not necessarily coincide with high neutralization titer (21, 22). Uninfected and infected individuals have been shown to have highly variable levels of antitoxin antibodies, both in terms of the prevalence (0 to 87%) of antibodies and in serum titers (22–25). There have also been suggestions that serum responses may be nondurable (26) or weaker in the elderly (22, 24, 27), observations that are at least consistent with the phenomena known as "immune senescence" (28). Interestingly, an association has been drawn between higher levels of anti-TcdB antibodies and lower levels of disease (22, 23, 27, 29), although it is not clear whether this is due to a central role of anti-TcdB in preventing recurrence or simply that TcdB is more immunogenic than TcdA in humans. Lack of antibody responses have been linked with more severe disease (23), while toxin binding (i.e., nonneutralizing) serum responses do not alter patient outcomes (30, 31). In particular, one study suggested that low IgG2 and IgG3 responses (but not IgG1 or IgG4) to TcdA (but not TcdB) were associated with recurrence (32). Higher levels of anti-TcdA IgG (but not anti-TcdB IgG) were observed in asymptomatic carriers than in diarrheal patients (25), and patients with one episode of diarrhea had higher levels of anti-TcdA IgG (but not anti-TcdB IgG) than those with recurrent diarrhea (33). Collectively, these data suggest that neutralizing IgG could have roles in protection. In apparent contrast to these findings are data suggesting that asymptomatic carriers have higher levels of anti-TcdB than anti-TcdA (27). Perhaps the most meaningful data come from a phase II human trial where low serum levels of anti-TcdA and anti-TcdB were associated with recurrence (34). In the same study, the parenteral administration by infusion of a mixture of two neutralizing human IgG1 anti-TcdA and TcdB resulted in a large (72%) reduction in the recurrence rate, directly demonstrating the ability of IgG to modulate disease. It is not clear at this time whether larger phase III trials will hence demonstrate modulation of primary disease through improved statistical power, or indeed whether any MAbs, even those shown to be significantly more potent, will be able to modulate primary infections.

EXPERIMENTAL ANIMAL STUDIES

Some of the key questions regarding the use of antibodies were answered in studies using vaccination/immunization and hence polyclonal sera and are summarized in Table 1. Oral, nasal, rectal, muscular, and subcutaneous immunizations were shown by several researchers to elicit predominantly IgG rather than secretory IgA (sIgA) responses; further, serum IgG antibodies were capable of conferring full protection (35–37). Notwithstanding this, Ig of various types (bovine IgG, chicken IgY, and mouse and human IgG) could also confer protection when administered orally in sufficient doses (38–40), and the observation that IgG found in serum could be transported into stool, provided a clear link between the two in diseased animals (36, 37). In fact, serum IgG has been shown in multiple studies of circulating enterotoxins to be the predominant protective response in vaccinated animals (41). However, oral administration of immunoglobulin is beset by substantial acid and enzymatic degradation throughout the gastrointestinal tract, which will likely cause a dosing/cost problem which will be difficult to overcome for MAbs produced using traditional ("high-cost") CHO cell culture manufacturing (42).

Early works focused solely upon the activity and neutralization of TcdA ("enterotoxin") and did not also investigate neutralization of TcdB ("cytotoxin"). However, it is now clear from the prevalence and clinical impact of A^-B^+ strains, studies using engineered *C. difficile* strains in hamsters (7, 43) and the relative impotence in humans of anti-TcdA treatment alone (29) versus that of concomitant anti-TcdA and anti-TcdB treatments (34), that it is prudent for future therapeutics to neutralize both TcdA and TcdB. The importance of the neutralization of binary toxin expressed by some strains has not been evaluated in detail. Recent experiments support that binary toxin enhances the activity of TcdA and TcdB, but it is a somewhat ineffective toxin alone. $A^-B^-C^+$ strains showed no toxicity in *in vitro* assays; 6/9 infected hamsters had no symptoms, while 3/9 had relatively mild and atypical symptoms compared to those caused by TcdA or TcdB alone (43). In another study, $A^-B^-C^+$ strains did not cause diarrhea or death in hamsters (44), although binary toxin causes lipid raft formation (45) and is associated with disease recurrence in patients (14, 46).

ANTIBODIES FOR USE IN HUMANS

Although hyperimmune polyclonal sera have been used for parenteral administration with other infectious diseases, issues with constancy/scale of supply and immunogenicity (typically horse or sheep sera) make them unusable on a wide scale. Intravenous polyclonal immunoglobulin (IVIG) has also been administered to CDI patients but with variable and debatable effect (reviewed in reference 47). Hence, studies of passive immunization to protect against CDI evolved to the identification of monoclonal antibodies that were capable of completely neutralizing toxin. For the sake of manufacturing simplicity, it would be desirable to identify a single MAb capable of neutralizing both TcdB and TcdA. MAb mixtures are likely more expensive than single MAbs to pass through early stage manufacturing and clinical development, although the "cost of goods" is thought to be broadly similar after the product has been launched (48). Also, antibody mixtures carry greater regulatory uncertainties and practical risks especially in the areas of coproduction versus coformulation versus coadministration than single MAbs do. Several researchers identified MAbs that could bind to both toxins, but they did not neutralize them (49–51). Subsequently, a concerted effort to identify cross-neutralizing MAbs using a combination of bioinformatics sequence analysis and varied immunization strategies resulted in several MAbs that could bind both toxins but neutralize only one (52). One recent preliminary report suggested that a cross-neutralizing MAb had been identified (53). Despite this recent report, research efforts have resorted to generating individual MAbs against both TcdA and TcdB, with the aim of coadministration or even coformulation into a single product. Although gaining increasing acceptance with the biopharmaceutical industry and regulators alike (54), the concept of antibody mixtures was in its relative infancy during the pioneering work of Babcock et al. (55). The Massachusetts Biological Laboratory (MBL) and Medarex first generated the anti-TcdA MAb CDA1, which was tested alone in human clinical studies (29, 56) before also identifying and testing the anti-TcdB MAb, MDX-1388 (55) in combination with CDA1. They immunized transgenic mice that expressed human antibody genes with toxoid A and B prepared by the UDP-dialdehyde method and discovered neutralizing MAbs using visual cell rounding and agglutination assays. They mapped the epitope/

TABLE 1 Antibodies used in the study of *Clostridium difficile* infection interventions

Antibody type	Antibody no.	Antibody name/type	Method	Summary	Notes	Reference(s)
Polyclonal antibodies/ exptl vaccines	1	Polyclonal	Toxoid immunization	Immunization of hamsters with toxoid A and/or toxoid B resulted in a protective antitoxin polyclonal response	100% protection with toxoid A^+B; ~23% protection with toxoid A or B alone. In animals with serological response, reciprocal reduction in cecal TcdA or TcdB concentration or both in the presence of antisera.	59
	2	Polyclonal, subdomain specific	Peptide vaccination	Hamsters were vaccinated with the majority of the C-terminal domain of TcdA and challenged with a $TcdA^+B^+$ strain. Hamsters with higher serum titers survived.	Immunized sequence is (presumed to be) derived from VPI10463; the infection challenge strain was VPI7698, which produces moderate levels of toxin. Notable that sera against TcdA alone (presumed) protected against a strain expressing both TcdA and TcdB regarding survival but not onset of diarrhea.	81
	3	Polyclonal IgG, bovine	Orogastric dosing	Hyperimmune IgG fraction from bovine colostrum protected ~66% of hamsters when given alone or protected 100% hamsters when given with infant formula milk.	900 mg/day of IgG was given daily starting 48 h prior to infection. >10-fold-higher neutralization of TcdB than of TcdA.	38
	4	Immunization of hamsters by various routes		Hamsters were immunized with toxoid, inactivated culture filtrates, or formalin-killed cells and immunized with cholera toxin or RIBI adjuvants by one of eight different combinations of route of administration.	Data suggested that *C. difficile* may have some ability to "evade" host immune response when encountered in the gastrointestinal (GI) tract and that TcdB in particular may be difficult to raise antibody responses against.	82
	5	Polyclonal, bovine colostrum	Orogastric dosing	Cows were immunized with inactivated TcdA or culture filtrate. Anti-filtrate colostrum IgG neutralized the *in vitro* effects of TcdA or culture filtrate (TcdA and TcdB).		39
	6	Polyclonal	Vaccine	Oral immunization of rabbits with attenuated *Vibrio cholerae* strains secreting TcdA subdomains	Resulted in serum IgG that protected against TcdA challenge in a ligated ileal loop model.	35
	7	Polyclonal	Toxoid immunization	Immunization with TcdA or TcdA and TcdB, but not TcdB alone, protected hamsters against infection. Neutralizing antibodies could be transferred to infants by immunized biological and foster mothers.	Purified TcdA or TcdB alone could be lethal when dosed orogastically (o.g.). Hamster antitoxin IgG was detected in cecal homogenates.	36, 60
	8	Polyclonal, subdomain specific	Orogastric dosing of IgY	IgY was raised against subdomains spanning full-length toxin sequence. Antibodies against the C-terminal domain were the most effective. Anti-TcdA alone conferred ~70% protection to hamsters, but anti-TcdA and anti-TcdB conferred 100% protection.	Weight loss was measured as marker of morbidity. Protection of hamsters was dose dependent but required large doses, up to 320 mg/day. Anti-TcdA was sufficient for prophylactic protection, but both anti-TcdA and anti-TcdB were required for therapeutic protection. Protected animals survived rechallenge.	40
	9	Polyclonal	Toxoid immunization. Passive transfer of MAbs	Various sites of immunization and adjuvant conferred 20% to 100% protection of hamsters due to neutralizing anti-TcdA and anti-TcdB. Hamsters were protected by passively transferred hyperimmune hamster sera or mouse ascitic fluid but not goat polyclonal sera in a dose-dependent manner.	Demonstration of hamster weight loss after challenge. Demonstration that high serum titer or large parenteral serum doses were required for protection of hamsters. Protection was due to the IgG fraction, and autologous Fc domains may be required. Antitoxin IgG was not detected in feces.	37
	10	Polyclonal	Mouse immunization with part of TcdA C terminus	High binding titer and some neutralizing serum, but no mucosal antibody titer, was seen against TcdA.		83

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TABLE 1 (Continued)

Antibody type	Antibody no.	Antibody name/type	Method	Summary	Notes	Reference(s)
	11	Polyclonal, bovine WPC-40 "MucoMilk"	Bovine colostrum	A preparation (prepn) of 40% whey protein from cows immunized with inactivated bacteria and culture filtrate tested as an oral prepn in a human uncontrolled cohort study for the prevention of recurrence.	The immunoglobulin fraction comprised 10% of the whey prepn, and the ratio of sIgA to IgG was 100:1. Whey neutralized TcdA with a titer of ~3,100 and neutralized TcdB with a titer of ~330. Protection of hamsters was 80 to 90% when given orally for 3 days. None of the 16 patients had a recurrence; median follow-up period was 333 (range, 35 to 365) days.	84
	12	Polyclonal	Transcutaneous TcdA immunization	Transcutaneous immunization of mice with toxoid A elicited potent IgG and IgA responses. Sera were potently neutralizing (*in vitro*), and IgA was shown in feces.		85
	13	Polyclonal	DNA immunization with part of TcdA C terminus	High binding titer and protection against lethal challenge with TcdA		86
	14	Polyclonal	IVIG	Review of reports of use of intravenous (i.v.) pooled human immunoglobulin to treat protracted, recurrent or severe CDI.	Uncontrolled data suggest efficacy of pooled human immunoglobulin, particularly to treat patients with multiple recurrences of CDI.	47
	15	Polyclonal	Chimeric inactive toxin	Mice and hamsters were immunized with a glucosyl transferase inactive version of TcdB, which had its C-terminal cell binding domain switched to that of TcdA.	Mice and hamsters were protected against infection challenge. Antibody (Ab) responses were neither cross-neutralizing nor cross-protective. Ab responses were mostly IgG1 and IgG2a. TcdA was more immunogenic than TcdB.	67
	16	Polyclonal	Immunization with a hybrid C terminus comprised of approx half of the TcdA and most of the TcdB cell binding domains.	Mice, hamsters, and monkeys were immunized with hybrid C-terminal domain.	TcdA domains were more immunogenic (higher neutralizing titer and sooner) than TcdB domains in the presence and absence of adjuvant. Noninfected hamsters had antitoxin IgG in stool. Hamsters were protected 100% against lethality but not against symptoms.	87
	17	Polyclonal sera	Alpaca-derived sera	Mice were protected against bacterial challenge by either immunization with toxoid or passive immunization with alpaca-derived polyclonal sera.	Mice challenged with spores produced lower levels of antitoxin IgG response than those immunized with toxoid. Regardless, IgG responses were stronger than those of IgA, and anti-TcdA was stronger than anti-TcdB. Antitoxin antibodies, but not vancomycin, were required to protect mice against recurrent disease.	20
	18	Polyclonal, sheep IgG	Polyclonal IgG (pIgG) intraperitoneally (i.p.)	Generation of neutralizing titers (1/16,000 to 1/20,000) required four immunizations over 14 weeks.	Anti-TcdA and anti-TcdB mixtures conferred ~50 to 90% protection after a total of 75 mg of each antisera (25 mg per dose, "top-up" dosing) but poorly protective at 7.5 mg total dose. Administration of anti-TcdA sera alone slowed symptoms but ultimately offered no protection against death, while anti-TcdB alone had no protective effects.	61
	19	Polyclonal, alpaca IgG		Passive immunization of neutralizing polyclonal antibodies (PAbs) were shown to prevent serum toxemia caused by gut infections with *C. difficile*.	Both TcdA and TcdB can be found in serum of infected animals and cause elevation of serum proinflammatory cytokines. pIgG against TcdB alone was found to protect piglets from infection-related GI symptoms and toxemia, but pIgG against TcdA alone did not protect against disease (actually appeared to worsen outcomes).	76, 88
	20	Polyclonal	Subdomain-specific immunization of mice and hamsters.	Hamsters were fully protected when immunized with at least one subdomain from TcdA and one from TcdB, but neither alone was protective.	Antibody titer (in mice) was not predictive of neutralizing titer. TcdB-derived domains were generally less immunoreactive than TcdA-derived domains. The most effective domains for protection were found to be the C-terminal domain of TcdA and the N-terminal domain of TcdB. Sera were found to be weakly cross-reactive for toxin binding, but not cross-neutralizing. IgG antibodies were found in the gut lumen.	62

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TABLE 1 (Continued)

Antibody type	Antibody no.	Antibody name/type	Method	Summary	Notes	Reference(s)
	21	Polyclonal	Subdomain-specific DNA immunization of mice and rabbits.	C-terminal domain of TcdA but not TcdB is immunogenic in mice. Central domains of TcdA and TcdB are not immunogenic. N-terminal domain of TcdB is immunogenic in mice.	TcdA antisera was fully protective against an i.p. challenge with purified TcdA. Protection against diluted culture filtrate require administration of both anti-TcdA (C-terminal) and anti-TcdB (N-terminal) sera.	63
Monoclonal antibodies	22	MAb, murine PCG-4 G-2	MAb + purified toxin	PCG-4 is a TcdA-specific neutralizer. G-2 is a nonneutralizing cross-reactive MAb against TcdA and TcdB	PCG-4 protected hamsters against the effects of purified o.g. TcdA. Shown to bind to the C terminus of TcdA at ≥2 epitopes. PCG-4 binds minimally to positions 2097 to 2193 (2078 to 2193) and 2279 to 2414, which map to CROP3 and CROP4. PCG-4 blocks the binding of TcdA to Caco-2 cells.	49, 78, 89, 90
	23	MAb, murine G2 various 5288; 1339, 1134, 1142	MAb	First reports of cross-reactive MAbs, specifically those which bind to both TcdA and TcdB; however, these do not neutralize either toxin.		49, 50, 51, 91
	24	MAb, murine 37B5	Mouse IgG2b MAb	37B5 neutralized enterotoxicity of TcdA in a rabbit ligated ileal loop assay but did not neutralize hemagglutination, mouse lethality, or cytotoxicity.	The apparent separation of enterotoxicity and hemagglutinin (HA) functionalities was in contrast to PCG-4, which neutralized both, and was suggestive of distinct epitopes for both. Seven (nonneutralizing) IgM MAbs cross-reacted to TcdA and TcdB and remained nonneutralizing when combined together.	92
	25	MAb, murine TTC8 2CV	MAb	TTC8 is a mouse MAb specific for the C terminus of TcdA and mapped to a minimal 30-amino-acid (aa) peptide that encodes a predicted epitope repeated eight times in the toxin. 2CV is a mouse MAb specific for the C terminus of TcdB and mapped to a minimal 140-aa peptide that contains a predicted unique epitope. TTC8 precipitates and neutralizes TcdA, 2CV neither precipitates nor neutralizes TcdB.	Suggestion that MAbs that bind oligoclonally are discoverable and might be more strongly neutralizing than those which bind at one epitope	79
	26	MAb, human CDA1	MAb, hIgG1	Human phase II clinical trial of anti-TcdA alone.	No effect on recurrence rate but a trend toward a delay in recurrence was observed. Low levels of patient anti-TcdB and infection with strain 027 were associated with recurrence.	29
	27	MAb, human MK-3415 (anti-TcdA also CDA1, Actoxumab, 3D8). MK-6072 (anti-TcdB also MDX-1388, CDB-1, 124–152, Bezlotoxumab)	2 doses of hIgG1 human-mouse MAb mixture, i.p. (hamsters), i.v. (humans)	A mixture of two MAbs both directed against the toxin C-terminal domains. Most advanced of all *C. difficile* antitoxin antibodies; partway through phase 3 clinical trials (MODIFY I and II). Anti-TcdA was first tested alone in humans without clinical effect before the addition of anti-TcdB. Phase II clinical trials showed a 72% reduction in recurrence rate, but no effect on the duration or severity of diarrhea.	Hamsters were dosed with a total of 200 mg of anti-TcdA or 200 mg of anti-TcdB or 200 mg of each prior to infection ("prophylactic dosing") with strain BI. Protection levels at day 2 postinfection were approximately 55%, 17%, and 94%, respectively; these rates declined to 5%, 0%, and 55%, respectively. MAbs subsequently shown by others to lack neutralizing capacity for toxins produced by strains of ribotype 027 and 078. Shown to reduce the production of inflammatory markers tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β) in peripheral blood mononuclear cells (PBMCs) in human colonic explants. MDX-1388 alone was found to protect piglets from infection related GI symptoms and toxemia, but CDA1 alone did not protect against disease (there was trend toward worse outcomes).	29, 34, 52, 55, 56, 58, 88, 93; clinical trials registered at ClinicalTrials.gov under registration nos. NCT01241552 and NCT01512239
	28	MAb, murine A1H3	mIgG2a	MAb that enhances the activity of TcdA on cells by ~1,000 times	A1H3 (*de facto* a nonneutralizing MAb) recruits TcdA to the cell surface via FcγRI and has been used to develop an extremely sensitive assay capable of demonstrating the presence of serum-borne toxin in a piglet model.	76, 94, 95

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TABLE 1 (Continued)

Antibody type	Antibody no.	Antibody name/type	Method	Summary	Notes	Reference(s)
	29	MAb, murine 3358 and 3359	mIgG	Murine MAbs against the C-terminal domain of TcdA, given i.p. in hamster model.	Modestly neutralizing MAbs, which became more complete neutralizers as a combination. One neutralizing MAb (3358) increased binding of TcdA to cells, while another (3359) blocked TcdA binding to cells, suggestive of different modes of neutralization. Both MAbs were shown to bind to TcdA multiple times (14 and 8 times, respectively). The combination offered some delay in disease onset in the hamster model, but zero protection at ca. day 6 when infected with an A^+B^+ strain. Patent engineered enhanced stability into MAbs in order to facilitate o.g. dosing.	42, 80
	30	MAb, llama	Llama V_HH	Immunized llamas used for "phage display discovery of V_HH domains"	Anti-TcdA V_{HH} were partially neutralizing *in vitro*, anti-TcdB V_HH were nonneutralizing *in vitro*. Combinations of 2 or 3 V_HH conferred more-complete neutralization. No data for infection models. Molecular basis for multiple binding events shown with X-ray crystallography	77, 96
	31	MAb, humanized PA-50 (anti-TcdA), PA-41 (anti-TcdB)	2 doses of hIgG1 humanized mouse MAb mixture, i.p. (hamsters)	A mixture of two MAbs, anti-TcdA (PA-50) directed against the toxin C-terminal domains and anti-TcdB (PA-41) directed against the catalytic N-terminal domain.	Both MAbs had low pM activities against their respective toxins *in vitro* and shown to neutralize toxin from nine different ribotypes (20 strains). Levels of protection in hamsters were 90 to 100% at day 39 postinfection, but MAb was dosed both pre- and postinfection ("top-up/therapeutic" dosing), making direct comparison with the data of Babcock et al. (55) not possible.	58, 66
	32	MAb, humanized CA997 (anti-TcdA) CA1125 (anti-TcdB) CA1151 (anti-TcdB)	3 doses of hIgG1	A mixture of three MAbs, one anti-TcdA (CA997) and two anti-TcdB (CA1125, CA1151), all targeting the C-terminal domains	MAbs were high-affinity toxin binders (pM) and high-potency (low ng/ml) neutralizers of R0003, R027, and R078 toxins *in vitro*. MAbs demonstrated multiple binding events to toxin "oligoclonality". CA997 protected against TEER loss *in vitro*. Hamster challenge with R012 showed 100% protection to day 11, 82% protection to day 28 and were shown to be superior to the CDA1/MDX-1388 mix.	52
		MAb, murine 4A4 (anti-TcdA) 2C7 (anti-TcdA)	2 doses of IgG	A mixture of two MAbs for detection of and protection against TcdA.	The most potent MAb (4A4) conferred 50% protection to mice challenged i.p. with purified TcdA. Combination with 2C7 resulted in ~90% protection.	97
	33	MAb, human	Epstein-Barr virus (EBV) immortalized human B cell	EV029105a (anti-TcdA) and EV029104 (anti-TcdB) are both human IgG1 directed against the C-terminal domains.	EV029105a (0.15 nM affinity) was a more potent neutralizer than CDA1 (3G8) comparator, while EV029104 was less potent than MDX1388. The MAb pair was 100% protective of hamsters to day 10 against R012 challenge when given as three 50-mg/kg doses on days −1, 0, and 1 and were shown to be superior to the CDA1/MDX-1388 mix.	98

binding location retrospectively using toxin subdomain binding studies, and hence, this represents an example of "blind" MAb discovery. They found neutralizing MAbs that bound to both the N- and C-terminal domains but selected MAbs that bound to the C-terminal domain for *in vivo* studies. In part, this was based on the desire to block the first event in toxin activity, namely, cell binding. It has subsequently been shown that the C-terminal cell binding domains also have the potential to exert some deleterious effect directly onto cells, such as inducing the secretion of proinflammatory cytokines (57). The reported affinities of CDA1 (TcdA) and MDX-1388 (TcdB) were 1.4 nM and 164 pM, respectively. Babcock et al. (55) discovered that very large doses of MAbs were required to confer any protection to hamsters (a total of 200 mg of both CDA1 and MDX-1388/kg of body weight); this issue of potency has been substantiated by others studying alternative MAbs (52, 58). The need for, and potential significance of, these very large doses remains enigmatic, since it has been shown that the concentration in serum, pharmacokinetics (PK), and access to healthy gut endothelium of human MAbs in hamsters are "normal" with serum half-lives in the typical range of 2 to 4 days for self IgG in rodents (52). MDX-1388 alone offered no protection in the hamster model, while CDA1 conferred partial protection (~50%) that was short-lived and nondurable (~2 days). In contrast, the combination of both MAbs conferred considerable protection at day 2 (94%), which persisted to day 5 and beyond for 55% of the hamsters. These data, suggesting that both toxins need to be effectively neutralized to protect hamsters from microbial challenge, have been substantiated by multiple other groups (40, 58–63). Of note, CDA1 and MDX-1388 are poor neutralizers of toxin produced by strains of ribotype 027 and 078 and are incomplete neu-

tralizers at higher toxin concentrations (52, 58). CDA1 was also found to be unable to protect against TcdA-induced transepithelial electrical resistance (TEER) loss in Caco-2 cell monolayers (52). Ribotypes 027 and 078 are clinically important due to their prevalence and association with "severe" disease, and hence, it is important that new drugs are effective against these strains. High toxin concentrations may be experienced during the early days of an infection, so drugs capable of modulating toxin activity and hence symptoms could offer clinical advantage. TEER loss is caused by loss of tight junctions between monolayer cells and hence may be considered a surrogate for diarrhea. Diarrhea is intimately linked with patient outcome, definition of "cure," and hence, the length of the hospital stay. Increased length of the hospital stay is the major cause of increased health care costs, so new drugs capable of reducing the duration and severity of diarrhea could offer substantial clinical advantage. Retrospective analysis of CDA1 and MDX-1388 (52, 58) highlighted some of their potential shortcomings, which might potentially relate to their performance in phase I and II clinical trials (34, 56). The somewhat limited neutralization potential of CDA1 may also be of interest in the light of comparisons of the relative effects of purified TcdA and TcdB administered directly into the ceca of mice (64). In general, TcdA was found to effect stronger and more numerous changes (histopathology score and transcriptional changes) sooner than TcdB. Notwithstanding this, mechanisms of toxin neutralization *in vivo* can be different than those *in vitro* (65), with toxin clearance being of importance as well as toxin neutralization.

The results of a phase 2 clinical trial, which included 101 and 99 patients with CDI in the antibody and placebo groups, respectively (all subjects also received standard antibiotic therapy, most often with metronidazole), were reported in 2010 (34). The mean time of receipt of antibodies was day 3 after the start of standard antibiotic treatment (90% of subjects received antibodies by day 5). CDI recurrence occurred significantly less frequently in antibody recipients: 7% versus 25% (18% difference; 95% confidence interval [95% CI], 7% to 29%, $P < 0.001$), representing a 73% overall reduction. Interestingly, this difference in recurrence was driven primarily by the rates seen in outpatients (0% versus 26% in outpatients; $P < 0.001$) compared with inpatients (14% versus 25% inpatients; $P = 0.21$). Recurrence rates in cases caused by the NAP1/027 strain were lower and approached significance in antibody recipients (8% versus 32%; $P = 0.06$). Antibody receipt was protective in patients who had had >1 previous episode of CDI (7% versus 38%; $P = 0.006$).

There were no significant differences observed between the two treatment arms in terms of the severity of diarrhea during the initial CDI episode, the median or mean number of days to resolution of symptoms during the initial episode, or the proportion of subjects who failed therapy. The phase 2 clinical trial results established the proof of principle that MAbs may reduce CDI recurrence and justified the initiation of an ongoing phase 3 clinical trial (registered at ClinicalTrials.gov under registration nos. NCT01241552 and NCT01512239).

Larger trials will also provide more insight into possible differences between 027 and other strains which might relate to toxin sequence differences.

Marozsan et al. (58) also used the "blind" approach to MAb discovery. They immunized mice first with toxoids A and B in order to elicit some level of protection before rounds of challenge with increasing levels of TcdA and TcdB. Survival of the immunized animals ensured that some neutralizing MAbs must have been generated. They identified a number of neutralizing MAbs against TcdA and TcdB (66) and chose to use an equimolar combination of PA-50 and PA-41 for their preclinical evaluation. PA-50 had an affinity of 160 pM for TcdA and an EC_{50} (50% effective concentration) of neutralization against purified toxin of 90 pM (13.5 ng/ml), while PA-41 had an affinity of 590 pM for TcdB and an EC_{50} of neutralization of 4 pM (0.6 ng/ml). These MAbs were shown to be potent neutralizers of toxins derived from nine different ribotypes, by using *C. difficile* culture supernatants and target cells unresponsive to one of the toxins (T-84 and CHO-K1), typically with median EC_{50}s of 32 pM (4.8 ng/ml) against TcdA and 23 pM (3.45 ng/ml) against TcdB. The combination was 90 to 100% protective in a hamster infection model when administered using the same total MAb dose but a somewhat different dosing regimen than that used by Babcock et al. (55). Babcock et al. administered all MAbs prophylactically, while Marozsan et al. gave MAbs on days −1, 1, 3, and 5, a mix of prophylactic and therapeutic dosing that would be expected to produce a different level and duration of coverage during the acute infection phase. Neither MAb offered any protection when administered alone. These MAbs have recently been licensed by Medimmune for clinical development. It is interesting to note that PA-50 was found to bind to the C-terminal region of TcdA, while PA-41 was found to bind to the N-terminal enzymatic region of TcdB. Three other groups (62, 63, 67) independently also opted to use the N-terminal enzymatic portion of TcdB along with the C-terminal cell binding domain of TcdA as the most effective immunogen(s). In vaccine development studies, these researchers showed that TcdA is generally more immunogenic and generates higher neutralizing titers than TcdB. The C-terminal region of TcdB also has more sequence diversity (both intra- and interstrain) than TcdA (68). Further, TcdB variants encode activity/functionality differences (68, 69) with the C-terminal region of R027 TcdB being even less immunogenic than that of R003 (70). These observations may partially explain why the enzymatic domain of TcdB represents a good therapeutic target for both vaccines and the generation of therapeutic MAbs. Davies et al. (52) immunized rats and rabbits with various combinations of toxoid and toxin C-terminal domains and screened MAbs for binding to C-terminal domains before assaying for neutralizing activity. As such, this work is an example of "directed" MAb discovery. Multiple MAbs that completely neutralized TcdA *in vitro* were discovered, and the one chosen for *in vivo* evaluation (CA997) had an affinity for TcdA of ~66 pM and an EC_{50} of neutralization of ~1 ng/ml (~6.6 pM). No single MAb was identified that neutralized TcdB completely. This is perhaps consistent with the work of Lanis et al. (71) and the use of the R027 sequence as an immunogen. Hence, pairs of MAbs were evaluated, settling on the combination of CA1125 and CA1151 that bound TcdB with affinities of 170 pM and 1.8 nM, respectively. The combination of three MAbs conferred 80 to 100% protection to hamsters using the same total dose and dosing regimen as described by Babcock et al. (55). Of note for these MAbs was their high "valency of binding"; CA997 bound to TcdA domains an estimated 12 times and the CA1125/CA1151 combination bound to TcdB an estimated 3 times. High valency of binding may be important in multiple respects for Tcd neutralization. First, antigens decorated with multiple antibody Fc domains are cleared faster and more effectively from circulation than those

bearing one MAb/Fc (72, 73), and such clearance can contribute significantly to neutralization potential *in vivo* (65). Since circulating toxin has been suspected in patients (74, 75) and has been demonstrated in a piglet model of infection (76), improved toxin clearance may offer clinical advantage to some patients. Neutralized antibody-antigen complexes have also been shown both to educate (16, 17) and vaccinate (18) host immune systems. Consequently, there is an interesting possibility that MAbs with higher valencies of binding might be able to "boost" immunocompetent patients, resulting in more durable protection after the initial "cure." It is noteworthy that multiple binding events are a result of the multiple domain and sequence repeats found in the toxin C-terminal domains (77) and have also been observed in other MAbs. MDX-1388 was found to bind to TcdB at two sites (55), PA-50 bound to TcdA at least twice (58), PCG-4 bound TcdA twice (78), and TTC8 was supposed to bind TcdA more than once (79). Multiple binding events have been observed in nonneutralizing MAbs (80). It is also noteworthy that multiple binding sites are improbable in MAbs such as PA-41 (58) that target catalytic or other domains.

CA997 prevented TcdA-induced TEER loss in Caco-2 monolayers and conferred complete neutralization *in vitro* at higher toxin concentrations. As discussed above, such properties might confer clinical advantage during primary infections. Davies et al. (52) also demonstrated the presence of humanized IgG (hIgG) in the gut mucosa of noninfected animals, further cementing the mechanistic link between serum IgG and protection against a gut pathogen/pathology. The fact that the CDA1/MDX-1388 combination (MK-3415A) showed efficacy for prevention of recurrence but not reduction in the severity or duration of diarrhea is of clinical significance if supported by ongoing phase III studies. Biological therapies such as MAbs offer significant differences and potential for treatment advantage over existing (oral) antibiotics. A single administration by infusion or injection is convenient for those already in a health care facility, ensures patient compliance, and offers the potential for substantial levels of protection equal to or in excess of 3 to 6 weeks. In addition, MAbs can likely be offered to a wider number of patients than other nascent new treatments such as fecal transplant. Although fecal transplant shows great promise, there are a number of technical/manufacturing, ethical, and regulatory questions that need to be settled. Therapeutic MAbs have a proven history in all of these areas. However, MAbs are more expensive to produce than chemical entities, and this is reflected in treatment costs, which are typically in many multiples of those for antibiotics. Hence, the incremental therapeutic advantage compared with antibiotics and the pricing/reimbursement strategy will be critical for successful uptake of MAb therapies in the clinic. It remains to be seen whether improvement in recurrence alone or additional effectiveness against primary infections is needed to support the widespread use of MAb therapies.

The analysis of ongoing and future trials is awaited with interest to fully understand the clinical potential of MAbs in the management of CDI and for information they may yield on disease pathogenesis.

The understanding of the properties of MAbs that might offer meaningful clinical advantage has necessarily had very substantial overlap with research into vaccine development, including immunogen, adjuvant, routes of administration, and the development of diagnostic reagents and animal models. Table 1 contains a comprehensive collection of instructive research in these areas aimed at both substantiating discussion in this minireview and broadly informing readers. Nonpublished communications also make clear that MAb approaches to the treatment of CDIs are or have been in research phase or early clinical development in a number of biotechnology and pharmaceutical institutions. We expect therefore that information offering additional clarity on desirable properties of and clinical potential of therapeutic MAbs will also transpire in the coming years.

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