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Review Article

Clostridium Difficile Infection: Virulence Factors, Adaptive Immunity and Vaccine Development

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Abstract

Clostridium difficile (*C. difficile*) is the most common cause of nosocomial bacterial diarrhea in the developed world. Adaptive immune responses to the toxin A (TcdA) and B (TcdB), the two major virulent factors of *C. difficile* determine the outcomes of *C. difficile* Infection (CDI). Active vaccination represents a logical and a cost-effective strategy for the prevention of primary and recurrent CDI, and extensive research in recent years has led to the development of experimental vaccines. In this review, we summarize virulence factors of *C. difficile*, host adaptive immunity and advancement and challenges in the development of vaccines against CDI.

Keywords: *Clostridium difficile* infection; Vaccine; Bacterial toxin; Immunotherapy

Abbreviations

CDI: *Clostridium Difficile* Infection; SLP: Surface Layer Protein; RBD: Receptor Binding Domain; FMT: Fecal Microbiota Transplantation

Introduction

C. difficile is a gram-positive, anaerobic and spore-forming bacterium, and is the leading causes of nosocomial infections in industrialized countries [1,2]. *C. difficile* was first isolated in the stool of neonates in 1935, and was incorrectly assumed to be part of the normal gut flora [3,4]. Subsequently, *C. difficile* was identified in patients with antibiotics treatment as the etiologic pathogen of antibiotic-associated diarrhea and pseudomembranous colitis in humans [5].

C. difficile transmission and colonization in the gastrointestinal tract

C. difficile exists in two forms: an inactive spore form and a disease-causing vegetative form. *C. difficile* spores have been found in the environment -in the soil, water and on surfaces in clinical settings [6-8]. *C. difficile* is able to infect both humans and animals and is transmitted by a fecal-oral route through the ingestion of spores [9,10]. Antibiotic therapies disrupt the intestinal microflora and diminish colonization resistance[11], thereby providing a niche for colonization by intestinal pathogens including *C. difficile* [12,13]. Antibiotic treatments also change the proportion of bile salts and cholalic acid in the large intestine, thus allowing *C. difficile* spores to germinate [14,15].

The spores germinate into the vegetative cells in an anaerobic environment in the presence of bile salts [16]. To adapt to the new environment, the bacterium modifies its surface by expressing several adherence factors. Putative adherence factors include Surface Layer Proteins (SLPs), flagellar proteins FliC and FliD, Cwp66 adhesin, Fbp68 fibronectin binding protein, GroEL heat-shock protein, and certain hydrolytic enzymes such as Cwp84 [17-21].

Major virulent factors - *C. difficile* toxins

Symptoms of CDI are mainly caused by two exotoxins: TcdA and TcdB. these large toxins function as glucosyltransferases that inactivate Rho, Rac and Cdc42 within eukaryotic target cells, leading to actin polymerization, opening of tight junctions, and ultimately cell death. TcdA and TcdB are both holotoxins composed of four functionally distinct domains. The C-terminal Receptor Binding Domain (RBD) is responsible for toxin binding to the cell surface possibly via multi-valent interactions, leading to endocytosis [22]. The middle Transmembrane Domain (TMD) facilitates the insertion of the N-terminus into and through the endosomal membrane [23]. Through autocatalytic cleavage, the Cysteine Protease Domain (CPD) releases the N-terminal Glucosyltransferase (GT) domain into the cytosol of host cell [24]. The GT domain is capable of transferring glucose residues from UDP-glucose to small GTPases including RhoA, Rac1 and Cdc42 [25-27].

The receptors for these toxins have not been clearly identified, although glycoprotein 96 (gp96) was reported as human colonocyte plasma membrane binding protein for TcdA [28]. Following receptor binding and endocytosis, the toxins translocate through the early endosomal compartments into the cytosol. The toxins are auto-cleaved by their own CPD domains in the endosomal compartments, such that only the N-terminal enzymatic GT domain is released into the cytosol. In the cytosol, Rho GTPases are glucosylated, which in turn result in the blocking of downstream signal transduction pathways.

Previously, it was believed that TcdA initiated intestinal epithelial damage and mucosal disruption that allowed TcdB to gain access to underlying cells. However, more recent studies indicate that

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TcdB is more potent than TcdA in inducing epithelial injury and electrophysiological changes in human colonic strips in vitro [29]. Recent studies using TcdA-negative *C. difficile* mutants demonstrate the importance of TcdB in a CDI hamster model [30]. There have also been numerous reports on TcdA-negative/TcdB-positive strains of *C. difficile* isolated from CDI patients. In summary, both toxins are central to disease pathogenesis.

Up to 35% of *C. difficile* strains also express a third toxin, binary toxin. This toxin has been shown to enhance virulence of *C. difficile* through the formation of host cell microtubule protrusions that facilitate bacterial attachment [31,32]. Binary toxin comprises two subunits (CDTa and CDTb) and catalyzes the ADP-ribosylation of G-actin, which leads to the depolymerization of F-actin filaments [33,34]. Working synergistically with TcdA and TcdB, binary toxin has been associated with more severe disease and increased recurrence of CDI in recent outbreaks [35-37].

Epidemiology

There is an increased incidence and severity of CDI in the general population of North America and Europe [38]. In the U.S., CDI remains the most common cause of hospital acquired diarrhea with the number of hospitalized patients with any CDI discharge diagnosis doubling from 139,000 in 2000 to 336,600 in 2009 at a cost of $1 billion annually [39]. The possible cause of this outbreak is a hypervirulent strain now known as NAP1/B1/027, which has also caused outbreaks in the rest of the world [40-43].

The emergence of *C. difficile* strain NAP1/BI/027 has been associated with community-based outbreaks and in individuals not on antibiotics, increased severity and infections less responsive to treatment [44]. First noted in 2001, *C. difficile* NAP1/B1/027 strains were shown to produce 16-fold more TcdA and 23-fold more TcdB than historic *C. difficile* strains [45,46]. These strains also exhibit high-level of fluoroquinolone resistance, possibly a higher rate of sporulation and also produce binary toxin [47,48].

Recurrent CDI is one of the major challenges in managing CDI, afflicting more than 20% of CDI patients following the resolution of the initial infection [49]. Recurrent CDI occurs either due to relapse (i.e., endogenous persistence of the same strain of *C. difficile*) or reinfection (i.e., acquisition of a new strain of *C. difficile* from an exogenous source) [50,51].

Adaptive immune response to CDI

The newborns and infants lack intestinal colonization resistance. As a result, 60% to 70% of healthy infants are asymptomatic carriers of *C. difficile* during the first 12 months of life [52]. The mechanisms by which infants are resistant to developing CDI-associated symptoms are still mysterious. Many healthy children and adults (~60%) have detectable serum IgG and IgA antibodies to TcdA and TcdB even in the absence of *C. difficile* colonization or active infection [53-56]. It is likely that antibody production is stimulated in infancy and perpetuated through adult life by environmental exposure to *C. difficile* and to other clostridial species. Serum IgG antibodies directed against TcdA and TcdB are associated with protection against CDI. Clinical studies have shown that asymptomatic patients have increased serum anti-toxin IgG compared to patients who develop symptomatic disease [57,58]. Clinical studies have also shown that acquired immunity after an initial episode, manifested as increased serum anti-toxin IgG, protects against recurrent CDI [59]. Advanced age [60,61], malnutrition, female gender, and medical comorbidities tend to diminish host protective response to *C. difficile* in adults [62], and may be associated with more severe infection [63,64].

Generally, patients colonized with *C. difficile* who can boost an anamnestic systemic immune response to *C. difficile* toxins are less likely to develop symptoms [55]. Likewise, symptomatic patients who can mount an immune response early in the course of their illness are less likely to have recurrent CDI. The immune responses to TcdA, TcdB and non-toxin antigens are correlated with protection against symptomatic disease [65]. This observation is important as effective protection against initial symptoms or recurrence is likely to involve vaccines aimed at inducing immunity to both toxins and to non-toxin antigens.

Effective non-vaccination based strategies against CDI

Currently, the most common treatment for CDI involves discontinuing the original antibiotic in use at the time of diagnosis followed by administration of vancomycin or metronidazole [66,67]. Resistant strains to both antibiotics have been reported [68,69]. In addition, their protracted administration prevents the re-establishment of natural resistance to *C. difficile* by allowing the host microflora to repopulate the gut, and thereby predisposes the host to recurrent CDI [70]. Several antibiotics appear to be more effective in preventing CDI relapse such as Rifaximin [71], Nitazoxanide [72] and Fidaxomicin [73], some of which are already FDA approved, whereas others are currently undergoing clinical trials [74-76].

Efficacy of Intravenous Injection of Immunoglobulin G (IVIG) as a treatment for recurrent CDI has been studied in patients with severe or recurrent CDI [77-80]. However, the lack of large scale, randomized and controlled studies precludes a definitive evaluation of this approach. Meanwhile, the limited availability of IVIG hinders its general use as a therapy for severe or recurrent CDI.

Passive immunotherapy for the treatment of CDI has been also studied using two fully human monoclonal antibody targeting the RBD of TcdA (CDA1) and TcdB (CDB1) [81]. Although a reduced recurrence of *C. difficile* diarrhea was reported, this antibody therapy did not improve the severity of the diarrheal illness, the duration of hospitalization, or the time to resolution of the diarrhea [82].

Stool transplantation, commonly known as Fecal Microbiota Transplantation (FMT) has shown promise in numerous studied as an effective treatment of recurrent or refractory CDI, although the effectiveness varied by route of instillation, relationship to stool donor, volume of FMT given, and treatments received before infusion [83-85].

Vaccination against toxin antigens

Because of the key roles of TcdA and TcdB in the pathogenesis of CDI, most vaccine developments in the past have focused on the two toxins as major targets for vaccination. Given the success of toxoid-based vaccines against multiple pathogens such as *Clostridium tetani* and *Corynebacterium diphtheriae*, the first candidate vaccine against *C. difficile* in clinical trial is a toxoid-based vaccine containing

formalin-inactivated purified native full-length toxins adjuvanted with alum, developed by Sanofi Pasteur [86,87]. Based on the Phase II results, an adjuvanted high-dose vaccine formulation was selected for further evaluation in a global efficacy program. This ongoing Phase III trial began in August 2013 with plans to include up to 200 sites in 17 countries, involving 15.000 volunteers to evaluate the efficacy of the toxoid vaccine to prevent primary CDI in elderly patients with comorbidities who are at a risk for CDI. However, this vaccine has risk issues related to residue toxicity due to formalin treatment of the toxins and the inherent instability of the large holotoxins.

Holotoxins , by definition, are made up of multiple domains that perform distinct functions of the toxins. The large sized toxins are difficult to purify and produce, and are unstable over time. In addition, they require formalin inactivation and contain some contaminating antigens. To circumvent these problems, many groups have focused on generating recombinant toxin fragments [88-91]. The RBD of TcdA is able to induce neutralizing antibodies against TcdA [92]. The first report demonstrated that antiserum induced by subcutaneous immunization with a nontoxic recombinant peptide comprising 33 of the 38 repeating units of TcdA RBD region neutralized the enterotoxic and cytotoxic activity of TcdA and that hamsters vaccinated with the recombinant peptide were partially protected against *C. difficile* disease [93]. Several groups have studied optimization of epitope repertoire, immunization route, delivery vehicles using partial or intact of TcdA RBD [94-96]. In summary, many subdomains of RBD of TcdA alone are found to contain protective epitopes.

Since an optimal vaccine strategy should target both TcdA and TcdB, the search for an efficacious vaccine targeting both toxins prompted the use of combined recombinant peptides. A single recombinant fusion protein containing portions of the RBD from both TcdA and TcdB induced high levels of serum antibodies capable of neutralizing toxin activity both *in vitro* and *in vivo*. Immunization with the fusion protein reduced disease severity and conferred significant protection against a lethal dose of *C. difficile* spores in hamsters [97].

Another recent study used bacterial spores (*Bacillus subtilis*) as a delivery vehicle to evaluate the C-terminal repeat domains of TcdA and TcdB as protective antigens. Their findings show that oral immunization of the C-terminal repeat domain of TcdA is sufficient to confer protection through serum IgG and fecal IgA in a hamster model against challenge with a *C. difficile* strain producing both TcdA and TcdB [98]. However, the result is contradictory to evidences reported by previous work done by other groups [91, 99, 100], although the difference may lie in the induction of a mucosal anti-TcdA response vs. a parenteral anti-TcdA response.

In contrast to the previous assumption that only RBD regions induce protective antibodies, a recent study demonstrated that alternative neutralizing epitopes within toxins are promising vaccine candidates [101]. While the C-terminal repeat regions played the principal role in generating neutralizing antibodies to TcdA, in the case of TcdB, the central region domains dominated the neutralizing immune response. For both TcdA and TcdB, fragments which comprised domains from both the central and C-terminal repeat region of the toxins were found to induce the most potent immune responses [90]. Using a systemically delivered vaccine, researchers found that while neutralizing antibodies to the binding domains of both TcdA and TcdB are moderately protective, enhanced survival is observed when fragments from the GT region of toxin B replacing those from the binding domain of this toxin [102].

Chimeric vaccines expressed using a *Bacillus megaterium* expression system, comprising of the full-length TcdB with the original RBD domain replaced by the corresponding portion of TcdA was able to confer complete and long-lasting protection and prevented spore-induced disease relapse [100]. These novel results indicate the potential of GT domain of TcdB to induce neutralizing antibodies. Importantly, this chimeric vaccine, generated with toxin sequences of VPI10463 strain, protects experimental animals from challenge with hypervirulent BI/NAP1/027 strains. This provides evidence for the potential of the GT domain to confer broad protection across diverse strains.

Various groups have studied multiple recombinant formulations and adjuvants in recent years, such as repeating units of RBD with fragment C of tetanus toxin, flagellin of *Salmonella typhimurium* etc. In one study, following intraperitoneal injection with a vaccine targeting the RBD of the toxins, adjuvanted with alum and *S. typhimurium* FliC mice were able to mount a protective immune response against *C.difficile* challenge [103].

Besides TcdA and TcdB, binary toxin could become a potent candidate for the immunization therapy of CDI [104]. All in all, a wide variety of recombinant fusion protein vaccines are booming in present years, and DNA vaccine technology, known to provide humoral and cell-mediated immunity, has also been evaluated as proof of concept for a safe and easily-manufactured vaccine against CDI [94].

Vaccination against non-toxin antigens

Colonization and adherence to gut mucosa by *C. difficile* is an important step in disease pathogenesis [105]. The factors involved in this process represent intriguing targets for vaccine development [106,107]. This approach could complement antitoxin strategies, because colonization of the gut and adhesion to mucosal surfaces precedes toxin production. *C. difficile* surface proteins have been identified which may function as adhesions such as the flagellar proteins FliD and FliC or as proteases such as the Cwp 84 protein [13,108].

It was reported that infected patients developed antibodies to FliC, FliD, Cwp 84, and the Cwp 66 C-terminal domain, but not to the Cwp 66 N-terminal domain. An early study confirms the expression of these surface proteins of *C. difficile* during the course of the disease [109]. In addition, the FliC, FliD, and Cwp 84 proteins appeared to be good potential vaccine candidates [109,110]. Flagellar cap protein FliD of *C. difficile* has been tested for its use as a vaccine candidate via several immunization routes: intranasal, rectal, and intragastric. FliC-FliD immunized mice showed reduced intestinal colonization by *C. difficile* [111]. The endospores of *B. subtilis* can serve as a tool for surface presentation of heterologous proteins. The unique properties of the spore protective layers make them perfect vehicles for orally administered vaccines. In a recent study, researchers successfully displayed a fragment of *C. difficile* FliD protein on the surface of *B. subtilis* spores [112]. The recombinant spores may be good candidates

for *C. difficile* oral vaccines. Additional antigens present on the spore surface such as BclA1 has also been tested as a vaccine candidate using this approach [113,114]. Preliminary results show partial protection in hamster challenge experiments.

Structural explorations have shown that *C. difficile* may express three phosphorylated polysaccharides, named PSI, PSII and PSIII. Anti-PSII antibodies can be raised in farm animals, mice and hamster models; humans and horses carry anti-PSII IgA and IgG antibodies from natural exposure to *C. difficile*, respectively; phosphate is an indispensable immunogenic epitope and vaccine-induced PSII antibodies recognize PSII on *C. difficile* [115]. It has now been established that PSII is a conserved antigen abundantly present on the cell-surface and biofilm of *C. difficile* [116]. The presence of PSII hexasaccharide hapten-specific antibodies in the stool supernatants of CDI patients further highlights the suggestion that PSII is expressed during infection in the gut, is immunogenic in CDI patients and thus could be a potential vaccines target. [117].

A Lipoteichoic Acid (LTA) has recently been shown to be conserved in the majority of strains from *C. difficile* and as such is being considered as a possible vaccine antigen. A study has illustrated that the LTA polymer is a highly conserved surface polymer of *C. difficile* that is easily accessible to the immune system and as such merits consideration as a vaccine antigen to combat *C. difficile* infection [118].

Multicomponent vaccines that prevent colonization and neutralize toxin activity are also being developed. Romano and colleagues evaluated the efficacy of PSII glycoconjugates where recombinant toxins A and B fragments (TcdA_B2 and TcdB_GT respectively) have been used as carriers in a naive mice model [119]. Both glycoconjugates elicited anti-PSII IgG titers although only the TcdB_GT conjugate induced a response comparable to that obtained with CRM197 (non-toxic mutant of diphtheria toxin). Moreover, TcdA_B2 and TcdB_GT conjugated to PSII retained the ability to elicit IgG with neutralizing activity against the respective toxins. These results are a crucial proof of concept for the development of glycoconjugate vaccines against CDI that combine different *C. difficile* antigens to potentially prevent bacterial colonization of the gut and neutralize toxin activity.

Conclusions –future vaccine development

In 1974, pseudomembranous colitis was identified in patients who had received clindamycin [120]. Four years later, Bartlett et al identified toxigenic *C difficile* as a cause of the disease [68]. Adaptive immune responses to *C. difficile* influence the outcomes of CDI; therefore immune-based therapies have a high chance of success in stopping the spread of this epidemic. A large number of studies mentioned above have confirmed the efficiency of protective immunity induced by *C difficile* vaccine, in animal models of CDI as well as in humans enrolled in clinical trials.

Both toxoid-based and recombinant vaccines have proven to be highly immunogenic in healthy and at-risk volunteers. Three experimental vaccines against *C. difficile* are currently under clinical evaluation, all of them aim to prevent of CDI in adults and elderly [86, 121, 122]. The challenge for the vaccine will reside in its ability of inducing in elderly and immuno-compromised individuals a rapid, long lasting, and protective immunity.

As key players in colonization, *C. difficile* surface proteins were evaluated in animal models of CDI [109, 111]. PSII is as a surface antigen conserved among the most common strains and can represent a relevant target for the development of a carbohydrate-based vaccine [115]. Conjugation of *C. difficile* carbohydrate antigens to toxin fragments is a promising approach for the design of a conjugate vaccine which targets both surface exposed carbohydrate as well as secreted toxins. Further evaluation is needed to fully understand the capacity of such constructs to prevent colonization and neutralize toxin activity.

In recent years, the emergence of a "new" hypervirulent strain has led to more severe complications and an associated increase in mortality. The target population is also broadening to include a younger, community-based population. Therefore, there is a clear need for novel non-antimicrobial approaches against *C. difficile* which can potentially stop the growth of the CDI epidemic. Vaccination targeting both toxins and *C. difficile* colonization represents a logic and cost-effective means to end this epidemic.

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