New Perspectives in *Clostridium difficile* Disease Pathogenesis

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**KEYWORDS**

- *Clostridium difficile* • Virulence factors • Spore • Host response • Microbiota

**KEY POINTS**

- *Clostridium difficile* infection pathogenesis is a multifactorial process involving a complex interplay between bacterial virulence factors, the intestinal microbiota and host immune factors.
- *C difficile* spores are the principal vehicle of transmission, infection, and persistence.
- Deficiencies in innate and adaptive immune defense mechanisms affect disease outcomes.
- Inhibiting the function of the toxins, targeting specific host inflammatory pathways, and/or manipulating the intestinal microbiota may offer adjunctive treatments to current antimicrobials.

**INTRODUCTION**

*Clostridium difficile* is a gram-positive, endospore-forming, anaerobic, gastrointestinal pathogen that is the leading worldwide cause of hospital-acquired infective diarrhea.¹ *C difficile* exerts its major pathologic effects through the action of its 2 principal virulence factors, toxin A (TcdA) and toxin B (TcdB). The importance of these homologous exotoxins to *C difficile* pathogenesis is extensively supported by *in vitro* studies using epithelial cell lines derived from human colon cancer and small animal models,²,³ as well as reports showing that *C difficile* clinical isolates lacking both toxin genes are nonpathogenic in humans and animals.⁴⁻⁶ In addition to pathogenic toxin production, the composition and function of the intestinal microbiome and host immune factors have direct impacts on *C difficile* pathogenesis. This article highlights recent developments in the understanding of *C difficile* infection (CDI) pathogenesis.

**Pathogenicity Locus**

The genes encoding TcdA (tcdA) and TcdB (tcdB) are found on the pathogenicity locus (PaLoc), a 19.6-kb chromosomal region that also contains 3 further genes: tcdR,
encoding an RNA polymerase sigma factor that positively regulates toxin expression\(^7\); \(tcdC\), considered a corresponding negative regulator, although still a matter of current debate\(^8\)–\(^11\); and \(tcdE\), which is related to the bacteriophage holins.\(^12\) In addition to \(tcdR\) and \(tcdC\), factors outside the PaLoc, including CodY,\(^13\) a common transcriptional regulator in gram-positive organisms, and the carbon catabolite repression system, involving the catabolite control protein, CcpA,\(^14\) also participate in the regulation of toxin synthesis. Recent phylogenetic analyses reveal that the PaLoc resembles a mobile genetic element that has a complex evolutionary history with distinct PaLoc variants acquiring clade specificity after divergence.\(^15\) These PaLoc variants, referred to as toxinotypes, include variants with intact genes but sequence changes, forms with truncated \(tcdA\), variants of \(tcdB\), and forms with \(tcdC\) encoding mutations and deletions.\(^16\)

**Mechanism of Action and Functional Domains of Toxin A and Toxin B**

Similar to other members of the large clostridial family of toxins, TcdA and TcdB target the Rho/Ras superfamily of GTPases by irreversible modification through glucosylation at Thr-35/Thr-37.\(^6\) Because GTPases are key cellular regulatory proteins, their permanent inactivation within intoxicated epithelial cells leads to dysregulation of actin cytoskeleton and tight junction integrity, intestinal epithelial cell damage, and apoptosis by caspase activation.\(^6\)

Both toxins are single-polypeptide chain, high-molecular-weight exotoxins arranged into large multidomain and functionally distinct structures represented schematically in Fig. 1. The molecular mode of action of the toxins is not completely understood. Based on current data, toxins seem to bind to an as-yet unidentified receptor and enter cells through receptor-mediated endocytosis.\(^17\) Once inside the acidic endosomal compartment, a decrease in pH causes conformational changes within the toxin, allowing pore formation and subsequent translocation of the catalytic glucosyltransferase domain across the endosomal membrane. Knowledge of exactly how this process occurs and which regions of the translocation domain are critical for this process is starting to emerge. Recent findings have uncovered the pore-forming hotspot of the TcdB translocation domain, clustered between amino acid residues 1035 and 1107, which, when individually mutated, reduces cellular toxicity by greater than 1000-fold.\(^18\) Release of the glucosyltransferase enzymatic moiety into the cytosol occurs by an autoproteolytic cleavage event, which is thought to involve exposure to the cysteine protease domain and requires inositol hexakisphosphate (InsP6).\(^19,20\)

The relative importance of each toxin in disease pathogenesis is still a matter of debate. Both toxins seem to be lethal in animal challenge models, supported further by \(C\) difficile genetic manipulation studies reporting that TcdA-TcdB\(^+\) and TcdA\(^+\)TcdB\(^-\) mutants of \(C\) difficile caused disease in hamsters.\(^21,22\) Nevertheless, an earlier report generating equivalent mutants in the same \(C\) difficile strain found that only TcdB was essential for virulence, whereas TcdA was dispensable.\(^23\) In support of the dominant role of TcdB, all naturally occurring pathogenic strains produce TcdB (but not necessarily TcdA), suggesting that TcdB may play the dominant role in human infection.\(^23\)–\(^25\)

**Other Clostridium difficile Virulence Components**

Some \(C\) difficile strains (eg, 027 and 078 ribotypes) also produce an adenosine diphosphate ribosyltransferase toxin, commonly referred to as \(C\) difficile binary toxin (CDT). This toxin is composed of an enzymatically active A component (CDTa), which causes ADP-ribosylation of G-actin, and a cell-binding and translocation B
The CDTb component is activated by serine proteases and binds to a lipolysis-stimulated lipoprotein receptor. Although the biological significance of CDT during infection remains unclear, in vitro studies show that purified CDT is toxic to Vero cells and may increase adherence of *Clostridium difficile* to intestinal epithelial cells.

**Fig. 1.** (A) Structure of TcdA and TcdB. TcdA and TcdB are large multidomain proteins consisting of an N-terminal catalytic domain with glucosyltransferase activity, a delivery or pore-forming domain, a cysteine protease region involved in toxin entry into target epithelial cells, and a C-terminal host cell–binding region consisting of combined repetitive oligopeptide repeats (CROPs). The P zone represents the hydrophobic region of the delivery domain that has been proposed to form the *C difficile* TcdB translocation pore, clustered between amino acids 1035 and 1107. (B) Mechanism of action of TcdA and TcdB. Toxin binds to the surface of epithelial cells using the C-terminal receptor-binding domain. Binding triggers toxin internalization via clathrin-mediated endocytosis. Acidification of the endosome creates a pore that is thought to enable translocation of the glucosyltransferase domain into the cytosol. Exposure of the cysteine protease domain to inositol hexakisphosphate (InsP6) activates an autoproteolytic cleavage event, resulting in the release of the glucosyltransferase domain into the cytosol. The glucosyltransferase domain transfers a glucose moiety from the donor substrate uridine diphosphate (UDP)-glucose to a threonine residue (Thr-37 in RhoA), thereby inactivating intracellular Rho and Ras family GTPases.

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epithelial cells by the formation of netlike microtubule protrusions. CDT-induced protrusions contain trafficking vesicles and endoplasmic reticulum, connected to microtubules via the calcium sensor Stim1. Recent data indicate that CDT alters the secretory machinery of host cells and reroutes fibronectin from basolateral to the apical side of intestinal epithelial cells, where protrusions are formed. Released fibronectin and the microtubule-based protrusion meshwork increase adherence of clostridia. Beyond C difficile toxins, other factors seem to be involved in pathogenesis of C difficile. The surface layer proteins (SLPs) of C difficile form a paracrystalline regular array that coats the outer layer of the bacterial cell. These SLPs mediate adherence of C difficile to host cells, modulate inflammatory and antibody responses, display a high degree of variability between classic and epidemic strains and may influence the pathogen’s ability to attach to the mucosa or unstirred mucus layer.

**INFLUENCE OF THE GUT MICROBIAL AND METABOLIC ENVIRONMENT ON CLOSTRIDIUM DIFFICILE**

The resident microbiota of the human gastrointestinal tract plays a central role in promoting intestinal homeostasis through diverse mechanisms, including degradation of xenobiotic substances, synthesis of vitamins and other beneficial metabolites, immune system regulation, and colonization resistance against invading pathogenic microorganisms. Multiple studies in mice and humans have shown that antibiotics have profound and, in some cases, long-lasting effects on the community structure of the gut microbiota, and in turn reduce colonization resistance against C difficile. This change in function is reflected in the intestinal metabolome, which includes both host-derived and microbial-derived metabolites. Fecal samples from patients who develop CDI after antibiotic treatment show decreased microbial diversity and other changes in the microbiota, compared with patients who do not develop CDI. Moreover, the intestinal microbiomes of patients with recurrent disease are characterized by markedly decreased microbial diversity compared with control subjects and patients with an initial episode of CDI.

The mechanisms by which antibiotics lead to loss of colonization resistance are beginning to be elucidated. Antimicrobial-induced dysbiosis results in loss of protective toll-like receptor (TLR) signaling, accumulation of proinflammatory T helper 17 T cells, and increased epithelial permeability. Subsequent infection with C difficile leads to additional toxin-mediated epithelial injury, access to lamina propria immune cells, and perpetuation of the proinflammatory response.

In murine infection models, dysbiosis can promote C difficile transmission by creating a supershedder phenotype, allowing C difficile to transmit very effectively. On closer inspection, the microbiota from these supershedders produced fewer short-chain fatty acids compared with naive and clindamycin-treated controls. Short-chain fatty acids, including acetate, butyrate, and propionate, are important nutrients for mucosal and immune homeostasis. Further evidence indicates that the metabolic environment of the murine intestinal tract following antibiotic treatment is enriched with primary bile acids and carbohydrates that support germination and growth of C difficile in vitro and ex vivo. Another study suggests that sialic acids are increasingly released by gut commensals after antibiotic treatment, possibly enhancing C difficile growth.

Underscoring the significant impact of dysbiosis on C difficile–mediated disease, there is now clear compelling evidence to suggest that therapeutic restoration of the diversity of the intestinal microbiota can restore colonization resistance. Patients
with recurrent CDI can eliminate *C. difficile* after receiving a healthy microbiota through strategies such as fecal microbiota transplantation.\textsuperscript{47,48}

**ROLE OF THE HOST IMMUNE RESPONSE IN CLOSTRIDIUM DIFFICILE INFECTION**

Following CDI, both the innate and adaptive arms of the immune system are activated, suggesting that host immune responses are important determinants of disease pathogenesis.\textsuperscript{32,49,50}

**Innate Immune Responses**

The early pathogenesis of CDI is predominantly characterized by acute intestinal inflammation that is mediated by the inducible innate immune response. *C. difficile* is able to subvert the normally protective effects afforded by the mucus layer overlying the epithelium by downregulating mucin exocytosis from mucin-producing human colonic epithelial cells during infection.\textsuperscript{51,52} However, host epithelial-derived antimicrobial peptides, defensins, and cathelicidins can significantly reduce *C. difficile* toxin–induced tissue damage and inflammation.\textsuperscript{53,54}

*In vitro* studies have shown that *C. difficile* TcdA acts rapidly on intestinal epithelial cells, causing cellular rounding, detachment, apoptosis, and the secretion of proinflammatory cytokines, including the potent neutrophil chemoattractant interleukin (IL)-8.\textsuperscript{55} Neutrophils contribute significantly to tissue damage, because they contain a potent arsenal of oxidants and proteases in azurophilic granules.\textsuperscript{56}

Following the loss of epithelial cells, exposure of lamina propria cells to TcdA *in vitro* induces apoptosis in macrophages, eosinophils, and T cells,\textsuperscript{57} which trigger dissemination of the inflammatory cascade via further release of proinflammatory cytokines and chemokines including IL-12, IL-18, interferon gamma, IL-1β, tumor necrosis factor alpha, macrophage inflammatory protein (MIP) 1 alpha, MIP-2, IL-8, and leptin.\textsuperscript{58,59}

In addition, *C. difficile* can activate both surface and intracellular innate immune sensors, including the IL-1β/inflammasome, TLR4, TLR5, and nucleotide-binding oligomerization domain 1 signaling pathways.\textsuperscript{60–63} Nitric oxide has also emerged as an important innate immune defense mechanism against *C. difficile*, ameliorating disease in murine models by inhibiting neutrophil migration, hypoxia-inducible factor 1-alpha, and inflammasome activity, as well as directly inhibiting the potency of the toxins.\textsuperscript{64}

The protective effects of nitric oxide signal transduction seem in large part to be mediated by pleiotropic S-nitrosylation signals that result from increased nitric oxide synthase 2A activity.\textsuperscript{65}

Both toxins may also lead to activation, degranulation, and the release of inflammatory mediators from mast cells\textsuperscript{66} and can stimulate a strong neuroinflammatory response via secretion of various neuro peptides and neuroimmune signals.\textsuperscript{62} Intestinal dendritic cells also respond to *C. difficile* antigens, including SLPS and *C. difficile* toxins, by promoting the release of regulatory and antiinflammatory cytokines such as IL-10, IL-23, and IL-4. These cytokines initiate cellular repair processes, dampen the inflammatory response, and activate regulatory T cells and B lymphocytes to promote the protective adaptive antibody response.\textsuperscript{50,67,68}

**Adaptive Immune Responses**

Reports from multiple animal models and human studies clearly indicate that humoral immune responses to TcdA and TcdB influence the outcomes of CDI.\textsuperscript{69} Most notably, symptomless carriers of toxigenic *C. difficile* and those with a single episode of CDI without recurrence show more robust anti-TcdA immunoglobulin (Ig) G immune
responses than patients with symptomatic and recurrent disease. More recently, several reports have observed an association between higher levels of anti-TcdB antibodies and lower levels of disease. It is not known whether this is caused by a central role of anti-TcdB in preventing recurrence or whether TcdB is more immunogenic than TcdA in humans. There is also evidence suggesting that sera responses may be nondurable or weaker in the elderly, observations that are consistent with the phenomenon known as immune senescence.

The importance of the adaptive immune response in modulating CDI outcomes is perhaps best highlighted by the number of experimental vaccines currently under development. Furthermore, a phase II human trial showed a large (72%) reduction in *C. difficile* recurrence rate in subjects given a mixture of 2 neutralizing human IgG1 anti-TcdA and TcdB antibodies.

**Clostridium difficile** Sporulation and Germination

*C. difficile* spores are the main vehicle of persistence (CDI recurrence) and transmission of strains. This finding has been supported by recent studies in which a mutant strain of *C. difficile*, unable to produce the Spo0A protein (a transcriptional regulatory protein essential for the initiation of sporulation), did not persist or transmit disease in a mouse model. *C. difficile* spores are metabolically dormant and therefore intrinsically resistant to antibiotics and attacks from the host’s immune system, and once shed into the environment are resistant to disinfectants.

In order to initiate infection in the host, ingested *C. difficile* spores must germinate into toxin-expressing vegetative cells in the intestinal tract. *C. difficile* spores germinate in response to specific bile salts (cholate, taurocholate, glycocholate, and deoxycholate) and L-glycine acts as a cogerminant. These salts bind to CspC, a catalytically dead serine protease that acts as a germinant receptor. New evidence points to strain-to-strain variability in the germination response to bile salts, with variation also observed in the germination efficiency of *C. difficile* spores.

**Summary**

Although knowledge of the mechanisms underlying *C. difficile* pathogenesis has increased markedly in recent years, many important outstanding questions remain. For example, what are the human receptors for TcdA and TcdB; which cells do they target in *vivo*; how significant are the roles of binary toxin and nontoxin components in determining bacterial virulence; how do innate immune responses influence disease progression and outcome in *C. difficile*; and which factors are involved in modulating *C. difficile* spore resistance, germination, and sporulation? Further advances in murine models of infection, microbial culturing, DNA sequencing technologies, gene-editing tools, mucosal immunology, and *in vivo*-like three-dimensional intestinal organoid platforms should help facilitate the development of novel and more targeted therapies for this difficult pathogen.

**References**


