Overlapping Roles for Toxins in *Clostridium difficile* Infection

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(See the brief report by Kuehne et al on pages 83–6.)

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All bacterial infections are of great concern to society. Some bacterial infections, however, seem to have periods of high drama or wider public health import. The 100 or so known species of *Clostridium* (from the Greek *kloster* or “spindle”; phylum Firmicutes) belong to a group of obligately anaerobic, gram-positive, spore-forming, rod-shaped bacteria. Of these, only a handful are pathogenic to humans. Yet this handful is known to cause many types of serious bacterial diseases, including some of the most common (eg, *Clostridium perfringens* food poisoning), to some of the most dramatic: Gas gangrene (clostridial myonecrosis), tetanus, and botulism are the most commonly recognized names (etiologic agents: *C. perfringens*, *Clostridium tetani*, and *Clostridium botulinum*, respectively) [1]. Over the years researchers have learned that the pathogenesis, indeed many of the acute observed pathologies, of these diseases are mediated predominately by secreted bacterial toxins, or protein exotoxins, of which the Clostridia are prolific producers. Some of these (the botulinum neurotoxins) represent the most poisonous agents known [2]. Bacterial toxins’ gene expression, processing, secretion, protein structures, molecular targets, biochemical activities, and especially the varied, unique biologic consequences of these toxins to our cells and to ourselves have served as mainstays of bacterial disease research for decades. Each advance in toxin research has honed understanding of the cognate infectious cycles, and many derivative medical countermeasures—especially the toxoid vaccines—are proven and marvelous success stories.

Today we are up against a relatively new and potent clostridial challenger, *Clostridium difficile*, a cause of serious gastrointestinal tract infections and a troublesome public health risk [3, 4]. Cases of *C. difficile* infection (CDI) continue to increase, afflicting mostly older people receiving antibiotics for reasons unrelated to CDI. The clinical presentation of CDI can range from asymptomatic colonization to a fulminant colitis with the development of toxic megacolon [5]. Although CDI was initially recognized as a nosocomial infection, the recent rise of community-acquired CDI has been cause for alarm [6, 7]. The details of the *C. difficile* infectious cycle and its pathogenesis appear to be significantly more complex than, say, tetanus, botulism, or even gas gangrene. CDI arises after antibiotic administration disrupts the ecology of the healthy, normal gastrointestinal bacterial community, compromising colonization resistance and allowing for *C. difficile* to find an environmental “toehold” for setting up shop after spore ingestion and germination [8]. Inflammation and activities of our innate immune responses are implicated as either helping to contain the organism, assisting the organism in persistence, contributing to acute pathologies, or all of the above [9]. Any roles for acquired immunity, either in clearing the pathogen or in long-term immunity, are in the early stages of investigation. Recurrence is common after treatment, and spread to new victims via fecal-oral transmission is a torment to healthcare facilities [10]. High levels of recurrence and transmission are believed to be due to, at least in part, the robust resistance properties of its spore to both antibiotics and surface decontamination agents.

So where do *C. difficile* toxins fit into this complicated picture? Three *C. difficile* exotoxins have been discovered, intensely researched, and highly suspected as potential factors contributing to CDI, perhaps key factors to virulence. However, correlates...
Clostridium difficile strains can carry the genes (tcdA and tcdB) for 2 A/B-type cytotoxins named TcdA and TcdB, both similar in terms of their primary structures and known activities [11, 12]. The 2 toxins act by binding to and entering host target cells and then, via enzymatic glycosylation, inactivating intestinal cells’ actin-regulating GTPases (Rho, Rac, and Cdc42). The vast majority of isolates from CDI patients produce TcdB but many of these also produce TcdA. In addition to these 2 cytotoxins, certain strains of C. difficile produce a third toxin, commonly referred to as “binary toxin,” which is the product of 2 genes, cdtA and cdtB [13]. Like TcdA and TcdB, binary toxin is a secreted A/B-type cytotoxin that also acts by binding to and entering intestinal cells, and disrupting host actin, albeit by a different enzymatic means, an ADP-ribosyltransferase activity. Although commonly produced by the recently epidemic BI/NAP1/027 strains of C. difficile, a strain some studies have scored as causing more severe forms of CDI, the role of binary toxin in the pathogenesis of CDI remains unclear.

Although it appears that all 3 toxins can disrupt actin, potentially leading to breaches in the integrity of the intestinal barrier and contributing to pathologies, formally proving the roles or even the requirements for each these 3 toxins in the C. difficile infectious cycle has been challenging, often piecemeal in nature, and controversial among investigators. A major technical challenge to direct inquiry has been the lack of amenable molecular genetic tools with which to generate the requisite number of isogenic toxin mutants to perform the type of internally controlled, head-to-head animal challenge studies most commonly accepted as “proof of principle.” In this issue of the Journal of Infectious Diseases, Kuehne and colleagues have done just that. Using their genetic system called “ClostTron,” these investigators generated a series of isogenic mutants from an R20291 epidemic strain of C. difficile, covering all possible single, double, and triple (null) combinations of the 3 toxins, TcdA, TcdB, and binary toxin. The parental wild-type and each mutant strain were used for cell toxicity assays in vitro, and direct challenge of Golden Syrian hamsters. All animals receiving the toxin null (triple mutant strain) strain survived, with no indication of illness. In contrast, those receiving fully toxigenic wild-type parental or double mutants expressing only TcdB or only TcdA died at nearly the same levels and with only slightly different kinetics, although animals receiving the TcdA-only strain trended toward fewer deaths at slightly slower rates.

New insights into roles for binary toxin were also garnered in this study. Naturally occurring strains of C. difficile that do not produce either TcdA or TcdB but do produce binary toxin have been described. In a rabbit ileal loop model, these strains can trigger marked fluid accumulation [14]. However, in this same study, no disease was observed when these strains were used to challenge hamsters. As these were naturally occurring strains and in most cases did not arise from patients who had clinical disease, it is possible that these differing results are due to strain differences outside of these toxin loci. In the current study by Kuehne et al, isogenic mutant strains expressing only binary toxin were attenuated. As expected, strains that expressed binary toxin with either TcdA or TcdB were fully virulent. What was notable to the investigators was that the presence of binary toxin in concert with TcdA restored full virulence, when compared to strains producing only TcdA. Collectively, the study results can be summarized as follows: (1) Mutants making TcdB only (or those with TcdB plus binary toxin) are as virulent as wild type; (2) mutants making TcdA plus binary toxin also are as virulent as wild type; (3) mutants making TcdA alone are slightly less virulent than wild type; (4) mutants making binary toxin alone or no toxins (null) are severely attenuated. Although only a single clinical strain and 1 animal model of C. difficile infection were used here, this study provides additional solid and internally controlled evidence for central roles of the 3 toxins in CDI. The use of a representative of the recent epidemic strain adds to previous work creating isogenic tcdA and tcdB mutants in the laboratory strain 630 [15, 16]. Furthermore, these results appear to provide solid support in favor of the group’s long-held position that, when planning to build new C. difficile countermeasures targeting its toxins, it would be prudent to consider both TcdB and TcdA and, potentially, that the binary toxin needs to be considered in these respects as well.

Whereas these results studying toxin virulence factors are important, it should also be stressed that future therapies for CDI will likely target aspects of the pathogenesis that extend beyond toxins. For example, novel therapies that interrupt the process of spore germination have been proposed and tested [17]. Modulation of the gut microbiota through interventions such as probiotic administration and fecal transplantation is being investigated [18, 19]. It is likely that a combination of approaches targeting different stages of the C. difficile infection cycle, informed through research on all aspects of pathogenesis, will be required to make significant inroads into handling this important infection.

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