**Clostridium difficile** binary toxin CDT

Mechanism, epidemiology, and potential clinical importance

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**Introduction**

*Clostridium difficile* is a human and animal pathogen causing intestinal infection following disturbance of the gut microbiota, usually as a result of prior antibiotic treatment. Since the discovery of *C. difficile* as the major cause of pseudomembranous colitis (PMC), pathogenesis has been linked to production of two large, single unit, glucosylating toxins, toxin A (TcdA) and toxin B (TcdB), are considered the main virulence factors. During the initial studies on *C. difficile* cytotoxicity two large protein toxins were purified and named toxin A (TcdA) and toxin B (TcdB). Clinical studies had confirmed that symptomatic patients were infected with *C. difficile* strains producing both TcdA and TcdB. Initial focus was therefore on the purification of the toxins and production of antibodies that were subsequently used for development of rapid tests for *C. difficile* diagnostics. Other studies addressed the effects of both toxins in animal models and in epithelial cells and increase bacterial adherence. Multiple clinical studies indicate an association between binary toxin in stool, analyses of CDI mortality caused by CDT-producing strains, and the relationship between the expression of CDT toxin and increased mortality caused by CDT-producing strains.
models (hamsters) and the molecular mechanism of action within the cell. Several groups have shown that the cell cytoskeleton was primarily affected in toxin treated cells. At that time other clostridial toxins which directly or indirectly modified actin in the cytoskeleton were already known. They belonged to two groups: clostridial iota toxin-like binary toxins and C2-like toxins, and both were ADP-ribosyltransferases.

To test whether the actin modifying activity of C. difficile TcdA and TcdB is also a result of ADP-ribosyltransferase activity, M. Popoff tested several C. difficile strains. ADP-ribosyltransferase activity was discovered in a single strain in addition to cytotoxicity (TcdB) and enterotoxocity (TcdA) and this strain and toxin properties were described in the first reports of C. difficile binary toxin. Interestingly, these reports described strain CD 196, which was a historical PCR ribotype 027 strain. The next report on C. difficile binary toxin was not published until 1997 and described the sequence of binary toxin encoding genes, the similarity with iota toxin clostridial binary toxins, and the small proportion of tested clinical strains that had binary toxin genes and/or ADP-ribosylating activity (5 of 24 strains). Three years later binary toxin positive strains were reported in C. difficile strains isolated from horses, but not from cats and dogs. In the same year it was reported that binary toxin genes were present in some PCR ribotypes recovered from clinical (human) specimens. According to the number of strains included in different PCR ribotypes in the large Cardiff PCR ribotype (human) specimens, the small proportion of tested clinical strains that had binary toxin genes, the similarity with iota toxin clostridial binary toxins, and the cytoskeleton were already known. They belonged to two groups: clostridial iota toxin-like binary toxins and C2-like toxins. Other members of this toxin family are

**CDT belongs to the family of binary ADP-ribosylating toxins. Other members of this toxin family are C. botulinum C2 toxin, C. perfringens iota toxin, C. sporofere toxin and the B. cereus/thuringiensis vegetative insecticidal proteins (VIP).** All these toxins consist of two separate toxin components. One component is the biologically active component and possesses ADP-ribosyltransferase activity to modify actin and the second component is involved in binding of the toxin to host cells and is responsible for the translocation of the enzyme component into the cytosol.

**Structure of CDTa**

The enzyme component of CDT (strain CD196, O3278, or Q9KH42,UniProtKB) consists of 463 amino acids with a mass of ~53 kDa.11,12 (Fig. 2). The first 45 amino acids are probably a signal sequence and are cleaved by proteolysis. Therefore, the mature toxin component CDTa has a mass of ~49 kDa. This part exhibits about 84 and 82% sequence identity with the enzyme components of C. perfringens iota toxin (Ib) and C. sporofere toxin (CSTb), respectively. Other members of the family of binary actin binding ADP-ribosylating toxins are less related to the enzyme component of CDT with 34 and 29% sequence identity of C. perfringens iota toxin (Ib) and C. sporofere toxin (CSTb), respectively. Nevertheless, in the overall structures of these toxins, CDTa has been crystallized recently. As shown for other toxins, CDTa has a 2 domain structure. Both domains exhibit similar folding most likely resulting from duplication of an ancient ADP-ribosyltransferase gene.2 The N-terminal part is covered by residues 1–215 (note, numbering refers to mature toxin) and consists of 5 a-helices and 8 b-strands, which are probably involved in the interaction with the binding component CDTb, whereas the C-terminal part with residues 224–420 carries ADP-ribosyltransferase activity. As in the N-terminus, the C-terminal part consists of 5 a-helices and 8 b-strands. This part contains the catalytic site. Many bacterial ADP-ribosyltransferases, which in turn exhibit only minimal sequence similarity, belong to the so-called R-S-E family of ADP-ribosyltransferases, which share a typical arginine (R) residue, an S-T-S motif and an EXE motif. Exchange of this residue blocks the ADP-ribosyltransferase activity as well as the NAD-hydrolase activity, which is observed in the presence of H+O as a substrate.2 Recently, the structure of iota toxin, which is highly related to CDT, has been solved in complex with its substrate activator, leading to important insights into the molecular mechanisms of toxin-induced ADP-ribosylation of actin.23

**Structure of CDTb**

The binding component of CDTb consists of 876 amino acids with a molecular mass of 98.8 kDa (strain CD196, O32739 or A8DS70, UniProtKB) (Fig. 2). CDTb is about 80, 79, 44, and
36% identical with the binding components of iota toxin (Ib), C. spiroforme toxin (CSTb), C. botulinum C2 toxin (C2II) and B. cereus/thuringiensis VIP1, respectively. CDTb shares significant sequence similarity (36% identity) with the protective antigen (PA) of B. anthracis toxin.25 This finding is of major importance, because our knowledge about the structure-function relationship of PA was more advanced25 and much was learned from this toxin about activation, membrane interaction, pore formation and translocation of the binding components of binary actin ADP-ribosylating toxins (see below).

CDTb is expressed with a signal sequence of 42 amino acids. The binding component is divided into 4 domains. So far the binding domain has been crystalized from C2II26 and PA 27 but not from CDTb, Ib or CSTb. Mainly deduced from the similar structure of PA,25 the functions of the domains of CDTb are as follows: The N-terminal 257 residues form the activation domain I, which is followed by domain II until residue 480. Domain II is involved in membrane insertion and pore formation. Domain III is responsible for oligomerization and the C-terminal domain IV (from amino acid 592 to 876) is involved in receptor binding. The latter domain is very similar between CDTb, Ib or CSTb but does not share significant sequence similarity with C2II, VIP2 or PA. Moreover, the binding components of CDT, iota toxin and C. spiroforme toxin can be exchanged among each other, whereas C2II is not able to deliver the enzyme component of these toxins into target cells. This finding classifies CDT, iota toxin and CST into the subfamily of iota-like binary toxins.19

As known for all the binding components, CDTb has to be activated by serine-type proteases.11 Activating cleavage of the full length binding component occurs most likely between Lys209 and Leu210 to release a 20 kDa peptide and a 75 kDa peptide. The large peptide is the activated binding component and forms most likely heptamers. Heptamer formation has been shown for C2II,28 PA (although octamer formation was also suggested29) and for iota toxin.30,31 Whether the activation occurs before or after receptor binding is not clear. Both monomeric and heptameric CDTb bind to its cell surface receptor LSR (lipolysis-stimulated lipoprotein receptor).

Using a gene trapping approach and haploid cells (HAP1 cells), the host cell receptor for CDT was identified as the lipolysis-stimulated lipoprotein receptor (LSR).32 Knockout of the LSR gene in HAP1 cells resulted in specific resistance toward CDT, whereas the related C2 toxin still killed the cells. Expression of recombinant LSR in LSR−/− HAP1 cells reconstituted the toxicity. Moreover, it was observed that HeLa cells do not express LSR and are insensitive to CDT. Accordingly, expression of LSR in HeLa caused CDT-induced cytotoxicity. Moreover, a direct interaction of the binding component of CDT to LSR or its extracellular part could be shown.32 LSR was found to be a type I

Figure 1. Schematic representation of the CDT region and flanking genes. The regions from the nontoxigenic isolate CD37 (A), the binary toxin-negative isolate strain 630 (B), and the binary toxin-positive isolate QCD-32 g58 (C) are shown. The positions of the 5′ flanking genes CD2601 and CD2602, the 3′ flanking gene trpS, the response regulator gene cdtR, and the CDT binary toxin-encoding genes cdtAB, or their pseudogenes, are shown. For each variant of the CDT region the positions of the 5′ and 3′ conserved boundaries are shown, and the size of the entire CdtLoc is indicated. The unique 68-bp sequence that is present in C2DII and other nontoxigenic isolates in place of the CdtLoc is shown in bold. Adapted from J Bacteriol 2007; 189: 7290–7291, with permission from American Society for Microbiology.14

www.landesbioscience.com Gut Microbes 17
single-pass transmembrane protein of 581 amino acids and a mass of ~65 kDa (Fig. 2). It has an extracellular immunoglobulin-like domain and a long intracellular part. Three splice variants have been described but their specific functions were not clear. LSR was found highly expressed in liver and also in many other tissues including gut (both small intestine and colon), lung, kidney, adrenal glands, testes and ovaries. Importantly, LSR was also discovered to be the receptor for Clostridium perfringens iota toxin and C. difficile toxin A.40 It was reported that fatty acids, which are released by lipolysis of low density lipoproteins (LDL) by an LDL-receptor independent pathway, were suggested to be involved in uptake and removal of apoB, CD44 knockout mice are at least partially resistant to iota toxin toxicity, suggesting a function in uptake of iota-like toxins.13,41 In addition to the 75kd LDL-receptor (LR) which is structurally similar to tight junction protein occludin,40 LSR belongs to a family of immunoglobulin-like domain-containing receptor (ILDR), which has two other members ILDR2 and 3. Because ILDR2 and 3 are only ~30% identical with LSR, it is questionable that they are receptors for CDT.41

Recently, it has been suggested that in addition to LSR, CD44 may be involved in binding and/or endocytosis of iota-like toxins.42 CD44 is a multifunctional glycoprotein on the surface of mammalian cells, which is associated with lipid rafts and forms cell-surface clusters. Although its role in toxin binding is not clear, CD44 knockout mice are at least partially resistant to iota toxin toxicity, suggesting a function in up-take of binary iota-like toxins.

CDT uptake

Studies on the uptake of CDT into target cells are scarce (see model Fig. 3). However, high sequence similarity of CDT with iota toxin and usage of the same cell surface receptor (LSR) by both toxins allow drawing conclusions from uptake studies with iota toxins. Studies with iota toxin suggest that the monomeric activated binding component interacts with the cell surface receptor (LSR) followed by accumulation in lipid rafts, oligomerization and binding of the enzyme component.41,43 Thereafter, the toxin-receptor complex is internalized to reach endosomal compartments. The low pH of endosomes is probably a trigger for membrane insertion of the binding component and pore formation to allow translocation of the enzyme component into the cytosol. This is deduced from the finding that bafilomycin A, which blocks proton ATPase and thereby inhibits acidification of endosomes, prevents translocation of the enzyme component.42,44 However, pore formation by oligomerized binding component, resulting in potassium release,43 also occurs without acidification. In some cells (e.g., human epithelial carcinoma cells [A431]), the binding component of iota toxin causes necrosis without the enzyme component.45 From early endosomes, the binding component traffic to late endosomes and is probably then degraded in lysosomes.46

The mechanism of pore formation by CDTb-like proteins is best understood from PA and C2II. PA forms cell-surface clusters. Although its role in toxin binding is not clear, CD44 may be involved in binding and/or endocytosis of iota-like toxins.41 CD44 is a multifunctional glycoprotein on the surface of mammalian cells, which is associated with lipid rafts and forms cell-surface clusters. Although its role in toxin binding is not clear, CD44 knockout mice are at least partially resistant to iota toxin toxicity, suggesting a function in up-take of binary iota-like toxins.

Figure 2. Scheme of the structure of CDT. (A) The binary toxin consists of a binding component (CDTb) and an enzymatic component (CDTa). Both are expressed with leader sequences, which are not shown here. CDTb is activated by proteolytic cleavage to release ~20 and 75 kDa fragments. The 75 kDa fragment is the active binding component, which oligomerizes to form heptamers. (B) The lipolysis-stimulated lipoprotein receptor (LSR) is the target cell receptor of CDT. The protein possesses an extracellular part with an immunoglobulin-like structure, a transmembrane region and a large intracellular part.
Figure 3. Uptake and mode of action of CDT. The proteolytically activated binding component of CDTb forms heptamers and binds to its cell surface receptor LSR. Alternatively, monomeric CDT binds to the receptor and, thereafter, polymerizes to form heptamers. Then, the enzymatic component CDTa interacts with CDTb. The LSR–CDT complex is endocytosed. At low pH of endosomes, the binding component inserts into the endosomal membrane and forms a pore. Through the pore, the enzymatic component is translocated into the cytosol. This process depends on cytosolic chaperon system, including heat shock proteins (HSP, cyclophilin A and FK506-binding protein 51). In the cytosol, CDTa ADP-ribosylates actin. ADP-ribosylated actin is not able to polymerize and is trapped in its monomeric form. Moreover, ADP-ribosylated actin acts like a capping protein to block polymerization at the barbed (plus) ends of F-actin. This causes enhanced depolymerization of the actin cytoskeleton. The depolymerization of cortical actin, which is located beneath the cell membrane, results in formation of long protrusions, which are microtubule-based. The protrusions form a network on the surface of epithelial cells, which increases the interaction interface, and enhances adherence and colonization of clostridia.
Actin, the substrate of CDT

After translocation into the cytosol, CDTa ADP-ribosylates actin. Actin is a 45 kDa cytoskeletal protein, which is abundant in all cells and tissues and highly conserved (e.g., ~90% sequence identity between human and yeast actin). Six mammalian actin isoforms are known, which differ maximally in 25 amino acid residues. Actin is involved in a large array of cellular functions. It participates in establishment of cell morphology, is crucial for cell adhesion, motility and cytokinesis. It plays crucial roles in a large array of cellular functions. C. difficile studies have shown that CDT increases adherence of bacteria. Importantly, while CDT-induced protrusions are best studied in cell culture, they also occur in intact tissue and in vivo studies have shown that CDT increases adherence of C. difficile in a mouse model of infection. All these data suggest that destruction of the actin cytoskeleton induces increased adherence and colonization of the toxin-producing bacteria (Fig. 4).

Evolving Epidemiology of Binary Toxin Positive C. difficile Strains

Toxinotyping and PCR ribotyping have been widely used to characterize C. difficile strains and the presence of binary toxin genes has been correlated with different toxinotypes and ribotypes. Toxinotyping categorized strains on the basis of genetic variations within the pathogenicity locus (PaLoc) encoding genes for the glucosylating toxins A and B and 3 accessory genes and was not obviously linked to the binary toxin genes which reside at a chromosomal site distant from the PaLoc. However, virtually all strains that contained complete aat and cdtA genes belonged to one of several variant toxinotypes (non-toxigenic 0) or “non-toxogenic” strains that did not have a PaLoc. The reason for this correlation is unknown, however a large proportion of toxigenic 0 strains contained truncated forms or pseudogenes (Fig. 3) of the binary toxin genes (79% of all tested toxigenic 0 strains). Not all variant toxinotypes have been shown to contain binary toxin genes, including the wide-spread...
toxinotype VIII which does not produce toxin A yet caused clinical syndromes similar to toxin A and B-producing strains (Table 1).13,15

PCR ribotyping, a more generalizable typing method is based on amplification of the 16S-23S rRNA gene intergenic spacer region and therefore, is not obviously linked to binary toxin genes similar to toxinotyping. Certain ribotypes are consistently associated with the presence of binary toxin, including the epidemic PCR ribotype 027/toxinotype III strains (also known as BI/NAP1)13 (Table 1).

Prior to the emergence of the 027/BI/NAP1 strain in the early 2000s most surveys of C. difficile strains documented a prevalence of binary toxin genes of <10%.79-84 Only 5.5% of strains in isolates from the Anaerobe Reference Unit at Cardiff were positive for binary toxin genes when reported in 2000.13 Likewise, a 5-y survey of consecutive clinical isolates in one hospital in Chicago between 1996 and 2001 found a binary toxin gene prevalence of 5.8%.85 The epidemiology of binary toxin positive C. difficile strains in the US changed dramatically over the next decade, largely as the result of the emergence of the epidemic 027/BI/NAP1 strain. In the US, the 027/BI/NAP1 strain was reported as the predominant strain in 8 hospital CDI outbreaks in 7 states between 2000 and 2003.1 In one affected hospital in Pittsburgh, 17 colectomies were performed for fulminant CDI in 2000 compared with an average of 2.7 per year over the previous decade.86 By 2009, this strain was endemic throughout the US, accounting for 61% of all clinical isolates from 25 acute healthcare facilities in Chicago.87 Shortly after the reports in the US a multi-hospital regional CDI outbreak occurred in Canada involving over 12 hospitals in the Montreal area between 2003 and 2004.2 Increased rates and severity of CDI were reported with an estimated 2,000 deaths directly attributable to CDI. The 027/BI/NAP1 strain accounted for 82% of the clinical isolates during this outbreak.2 This strain was subsequently documented in several European countries where outbreaks of severe CDI were also noted.88

The proportion of binary toxin positive strains has also increased in human CDI cases independent from the spread of type 027/BI/NAP1. In Italy strains isolated in different time intervals were compared and binary toxin positive strains represented 0% (before 1990), 24% (1991 to 1999), and 45% (2000 to 2001).89 The same trend was also observed in two large European studies in which the majority of European Union countries have participated. In the first study strains were collected during a 2 mo period in 2005 from 14 countries and 17.2% of all

Table 1. Correlation of Toxinotypes and PCR ribotypes among binary toxin-positive C. difficile strains (CDT)+

| Toxin production type | Toxinotypes | Ribotypes | Molecular background of PaLoc | Epidemiologic associations |
|-----------------------|-------------|-----------|-------------------------------|---------------------------|
| Minor types           |             |           |                               |                           |
| Major types           |             |           |                               |                           |
| A+B+CDT+              |             |           |                               |                           |
| A-B+CDT+              |             |           |                               |                           |
| A-B-CDT+              |             |           |                               |                           |
| X                     | 016         |           | TcdA: rearrangement in PaLoc and large deletion probably causing changes in regulation and low or no transcription of truncated TcdA |
| XVI, XVIII, some Viking strains | ND | ND | TcdA mechanism unknown | |
| XXX                   | 280, 281    |           | TcdA not present | |
| A-B+CDT+              |             |           |                               |                           |
| A-B-CDT+              |             |           |                               |                           |
| A-B-CDT+              |             |           |                               |                           |
| Xax, Xib              | 033         | Yes      | A-B: only small non-functional part of PaLoc present | Uncommon strains found mainly in asymptomatic patients |
| some strains without PaLoc | ND |           |                               |                           |
| A-B-CDT+              |             |           |                               |                           |
| A-B-CDT+              |             |           |                               |                           |

*CDT+: Presence of full length CDT locus, implying the potential for expression of binary toxin. Some A+B+ strains contain portions of the CDT locus, but are predicted as non-binary toxin producing strains (CDT), ND, not done.
toxinogenic isolates were binary toxin positive (determined as presence of non-truncated binary toxin genes). In the second study performed in a 1 mo period in year 2008, strains were obtained from 35 countries and the proportion of binary positive strains among all toxinogenic strains was 23%. Only 5% of the isolates were ribotype 027.

Binary toxin-positive (CDT+) strains were also frequently recovered from animals. Interestingly, while CDT+ strains have represented less than 10% of all human isolates prior to the outbreak of 027/BI/NAP1, they were typically associated with animals and have represented from 20 to 100% of all animal isolates. One particular CDT+ group predominated among pigs and calves and has been characterized as PCR ribotype 078 and toxinoype V.79,80 Toxinoype V strains recovered from these animals raised for food production appeared to be clonally related to type V strains recovered from humans with CDI.81

Although some investigators have reported the recovery of C. difficile, including CDT+ strains, in meat destined for human consumption,82–84 foodborne transmission of C. difficile has not been confirmed. Goorhuis et al. reported an increase in the incidence of ribotype 078 strains among patients in the Netherlands from 3% to 13% between 2005 and 2008.85 Compared with CDT cases associated with 027 strains, 078 cases were younger and more frequently had community-associated disease but had similar rates of severe diarrhea and attributable mortality.86 Additionally, binary toxin positive strains other than ribotype 078 have been reported to often be associated with community onset C. difficile.87,88 Further characterization of CDT+ strains would be helpful to clarify the epidemiology of binary toxin in C. difficile.

Importance of Binary Toxin as a Possible Pathogenesis Factor in C. difficile

The pathogenic potential of binary toxin was somewhat masked by studies of CDI severity that focused on specific PCR ribotypes, PFGE types or REA group strains of C. difficile that possess binary toxin, but also have other genetic characteristics that might account for enhanced virulence. Among these potential virulence factors were possible increased sporulation compared with other strains, better adhesion properties, and deletions and mutations in the tcdC gene that could result in increased production of TcdA or TcdB toxins. For example, a recent study comparing the association of CDT severity with PCR ribotype 027 failed to find an association between severe infection and ribotype 027 as the cause of CDI using multivariate analysis, even though ribotype 027 had a higher proportion of severe disease than any other ribotype.109 Previous similar studies without as large CDI patient populations have also failed to show an association of the PCR ribotype 027 strain with severe CDI.110,111 In contrast, Petrella et al.112 reported a significantly lower treatment response rate and higher recurrence rate for 027/NAP1/BI strains than for other strains in two large clinical trials of vancomycin and fidaxomicin for CDI treatment. Miller et al. reported a Canada-wide survey in 2005 by the Canadian Nosocomial Infection Surveillance Program (CNISP) of 1008 patients with clinical data and infecting strain analysis in which 31% were infected with the 027/NAP1 strain.113 This study confirmed a strong age association with infection due to this epidemic strain: 12.5% of NAP1-associated infections compared with 5.9% of non-NAP1-associated infections resulted in a severe outcome (p < 0.001). Patients 60–80 y of age infected with the NAP1 strain were approximately twice as likely to die or have a severe outcome compared with same aged patients with other strains. An important deficiency of strain-specific studies is that they may unknowingly harbor other binary toxin positive strains in the control groups, since the number of ribotypes known to carry binary toxin is quite extensive (Table 1). If this were to occur the severity of disease in the control group could be increased if large numbers of binary toxin-positive strains were included within the comparator group.

One study has attempted to differentiate the possible clinical effects of binary toxin from other genetic properties such as tcdC mutations in clinical isolates obtained over a 2-y period at one NHS Foundation Trust in London.114 The prevalence of tcdC mutations and binary toxin genes was analyzed in 207 C. difficile isolates and compared with measured risk factors of patient age and laboratory findings as well as patient outcomes (disease severity, ICU admission, mortality, recurrence and length of stay). The prevalence of tcdC truncating mutations was 15% whereas binary toxin genes were present in nearly twice as many isolates, 28%. Patients infected with isolates containing tcdC truncating mutations had significantly elevated C-reactive protein and peripheral white blood cell (WBC) counts compared with patients not infected with these strains, but there was no difference in patient outcome. In contrast, patients infected with C. difficile strains containing binary toxin not only had significantly higher peripheral WBC, but also had a significantly higher 30-d all-cause mortality (31% vs 14%, P = 0.02). In this study only 8% of isolates were ribotype 027 whereas 28% of isolates contained binary toxin genes.

A similar analysis was done on 212 C. difficile isolates containing genes for toxins A and B compared with 265 isolates containing genes for toxins A, B, and binary toxin and PCR ribotyped. Of the isolates possessing toxin A, B, and binary toxin genes that were not ribotype 027, 24 (33%) were PCR ribotype 078, 26 (36%) were PCR ribotype 066, and 22 (31%) were PCR ribotype 023 and 9 other PCR ribotypes. The case mortality rate at 30 d after diagnosis was 54/193 (28%) for PCR ribotype 027 strains, 20/72 (27.8%) for binary toxin-positive non-027 isolates, and 36/212 (17%) for patients infected with toxin A and B positive isolates. Among binary toxin-positive non-027 isolates, the 30-d mortality was 29.2% for ribotype 078, 30.8% for ribotype 066, and 22.7% for ribotype 023 and others. Based on the similar...
case-fatality rates for strains possessing toxins A, B, and binary toxin, wherein strains were compared with the case-fatality rate for strains possessing only toxins A and B. In univariate analysis, the relative risk of death within 30 days was 1.8 (95% CI 1.2–2.7) for binary toxin-positive strains and by multivariate analysis adjusting for age, gender, and geographic region the relative risk was 1.6 (95% CI 1.0–2.4).

In an earlier study with fewer patients Barbut et al. identified 26 patients in 1999 and 2000 who had CDI caused by toxinotype II isolates and did a case control study of 42 patients with CDI caused by isolates that possessed only toxin A and B genes (not binary toxin genes) who were hospitalized at the same time on the same ward. Patients infected with binary toxin-positive strains were more likely to be community-associated ($P = 0.017$) and were more likely to have CDI as the cause of hospitalization ($P = 0.003$) by univariate analysis. In a later study conducted at one large teaching hospital in Paris from 2000 to 2004 Barbut et al. analyzed 131 C. difficile strains causing CDI. Binary toxin-positive isolates were found in 11% and were responsible for more severe diarrhea ($P = 0.01$) and higher case-fatality rate ($P = 0.03$) than isolates that did not contain binary toxin. Binary toxin-positive isolates were found in toxinotypes I, III, IV, V, VI, IX, XII, XIV, and XXIV, and only one of the toxinotype III isolates was related to the epidemic ribotype 027 strain. These observations suggest a possible link of the presence of binary toxin in a wide variety of C. difficile toxin strain variants to increased CDI mortality.

An additional study analyzed potential virulence factors, including tcdC deletions and binary toxin genes, in C. difficile isolates obtained from 69 consecutive patients who were followed for CDI recurrence. In this study, 41% of patients had at least one CDI recurrence and 38% of the isolates from the initial episode were PCR ribotype 027. The presence of binary toxin gene was significantly associated with recurrence ($P = 0.02$) and need for hospital admission with recurrence ($P = 0.02$). tcdC deletion and ribotype 027 were not associated with recurrence.

Attributable mortality and severe diarrhea were similar in PCR ribotype 078 ($n = 54$) and 027 ($n = 124$) in one study from the Netherlands and both were greater than in 501 non-027/078 cases. More recently, PCR ribotype 078 strains of C. difficile that produce binary toxin were found to be associated with the highest 14-d mortality (36/63, 25%) in a very large patient study from the United Kingdom, exceeding the next highest 14-d mortality of 20% (111/560) for ribotype 027 strains that also produce binary toxin. The effect of binary toxin in animal studies of CDI has been difficult to assess. Geric et al. were able to identify a prevalence of at least 2% binary toxin-positive genes in isolates that did not possess toxin A and B genes. These isolates provided an opportunity to test the virulence in animal models of strains possessing only binary toxin genes. Supernatants from four A-/B- CDT+ strains were found to cause marked fluid accumulation in the rabbit ileal loop model. However, when hamsters were infected with these strains following clindamycin, they colonized the gastrointestinal tract but did not cause diarrhea or death. It was speculated that binary toxin by itself may not be sufficient to cause disease. More recently, Kuene et al. published data on the use of CDT+ toxin technology to inactivate tcdA, tcdB, and binary toxin (tcdC) genes. Isolates lacking toxin A or toxin B remained fully virulent in the hamster model, but when both toxin A and toxin B were inactivated and only the binary toxin genes remained active, 3 of 8 animals died. The symptoms in these animals were not typical of hamster CDI. The animals had signs of wet tail but had no cecal lesions, however, they did demonstrate hemorrhage and inflammation in their small intestines which was not seen in any other animals. In addition, when toxin B was inactivated, the presence of binary toxin with toxin A caused significantly more rapid hamster death ($p < 0.05$) than when only toxin A was present. These observations suggest that there may be an important role for binary toxin in C. difficile pathogenesis.

Future Implications for Binary Toxin Research

It is clear that the pathogenic potential of C. difficile organisms containing binary toxin has not been fully explored. It is also apparent that there is not a direct correlation between presence of the tcdA and tcdB genes and production of CDTa and CDTb polypeptides and binary toxin CDT. Detection of the binary toxin genes may not be sufficient to indicate the presence of the toxin in stool as measured by CDTb in stool. Only 19 of 36 (53%) of stools containing CDT+ isolates had CDTb detected in the stool specimen. Levels of CDTb detected in broth culture also correlated positively with levels in stool specimens with generally higher levels in stool than in broth supernatants. Given these limitations of genetic detection for binary toxin CDT, there is a clinical need for detection of binary toxin in the stool of patients with CDI. At present there are no binary toxin clinical assays available, however, a prototype novel enzyme immunoassay for CDTb has been developed for research purposes by TechLab, Inc.

In an oral presentation at the 4th International Clostridium difficile Symposium Heinrichs et al. (Heinrichs JH, Wang S, Mieziewski M, Seizer X, Xie A, Zorman J, et al. Design, production and pre-clinical evaluation of a novel toxin-based vaccine for the prevention of Clostridium difficile disease. 4th International Clostridium difficile Symposium September 2012; Bled, Slovenia, Abstract 01, www.ccds.si/abstracts.php) showed preliminary pre-clinical data on the benefit of including binary toxin antigens in a C. difficile candidate vaccine. Their group first developed a recombinant vaccine targeting toxin A and toxin B that was highly effective in preventing disease in the hamster model when challenged with C. difficile strain VPI10463 that produces very high levels of toxin A and toxin B but lacks binary toxin. However, when hamsters were challenged with C. difficile strain BI17, a NAP1/BI/027 strain that produces toxins A, B, and binary toxin, the hamsters were not protected by this vaccine. They then developed CDTa and CDTb antigens recombinantly and tested them independently and together in combination with the TcdA and TcdB antigens in trivalent and tetravalent antigen combinations. The combination of all 4 antigens provided significantly superior protection of hamsters.
compared with TcdA and TcdB divalent vaccine (p < 0.0001) and was superior to TcdA, TcdB and either CDTa or CDTb monovalent CDTs or CDTs antigens in trivalent combinations. This protective effect was shown for an additional NAP1/BI/027 strain, strain BI6, and for challenge with strain 630 (binary toxin negative strain) as well. The authors also showed that neutralizing antibody to binary toxin was associated with protection in the hamsters challenged with strain BI17. In contrast to the results of Kuehne et al.,113 they found a lack of fatal illness in hamsters challenged with a C. difficile strain that produced only binary toxin and not toxin A or B, although some of the animals developed soft stools and wet-tail consistent with the hamster observations of Kuehne et al. using their model.114

Although the results of the above study are still preliminary, if confirmed, they suggest significant pathogenic capability of binary toxin in combination with toxins A and B in the hamster model as also shown by Kuehne et al.,113 which could account for the increased mortality and disease severity associated with binary toxin-containing C. difficile strains infecting humans. Whether binary toxin targeting for vaccine development is required has not been determined, and existing clinical data for monoclonal antibodies directed against only toxin A and toxin B indicated that prevention of CDR recurrence following CDI treatment was achieved for strains that make binary toxin.114 Lowy et al. showed that the recurrence rate among patients with the epidemic BI/NAP1/027 strain was 8% for the antibody group and 32% for the placebo group (P = 0.06) suggesting that at least for recurrent CDI, antibody directed at binary toxin was apparently not necessary.114 However, protection against primary challenge with C. difficile by monoclonal antibodies or vaccines is more difficult than prevention of CDI recurrence in the hamster model115 and prevention of primary CDI may be an important distinction requiring the addition of binary toxin antigen to the vaccine mix.

One additional area of experimental interest is the use of binary toxin as an intracellular toxin delivery system to dissect aspects of cell function.116 The AB binary toxins are particularly well suited for this purpose as 1) their toxins are particularly potent due to the enzymatic A-domain, 2) they possess highly sophisticated mechanisms to translocate their A-domain across cell membranes, and 3) the toxins are highly specific with regard to their host cell substrate.117 Most work has been done with the C. botulinum C2 binary toxin. The receptor for C2 toxin seems to be universally present on vertebrate cells making it a useful system for study of multiple cell lines. The ability of the enzymatic (ADP-ribosylating) portion of C2 toxin to depolymerize F-actin has been used to study the function of the actin skeleton in cellular processes such as exocytosis, cell migration, leukocyte activation and endothelium permeability.117 Construction of chimeric fusion proteins by mixing the A and B domains of various binary toxins to achieve more selective binding of toxin to specific cell types such as tumor cells can be achieved. In addition, the A domain may be substituted with proteins or nucleic acids that can be introduced into the cytosol of specific cell types to correct deficiencies. Specific antibody domains can also be fused to the A-domain to create targeted immunotoxins.118 There are significant challenges as the pore size of the B-domain requires unfolding of proteins in order to translocate followed by refolding of the protein which requires a host cell chaperone. Increased knowledge of these mechanisms and their structure-function relationships should allow binary toxins to be useful carriers of nucleic acids and foreign proteins into mammalian cells.

Conclusions

Binary toxin CDT may be an important virulence factor of C. difficile for which the significance remains to be determined. Several clinical studies suggest an association between the presence of binary toxin in infecting C. difficile strains and increased mortality of the patients. Further studies of the effects of binary toxin-producing strains are needed including measures of binary toxin in stool, analyses of strains carrying binary toxin across multiple PCR ribotypes and C. difficile variants to the presence of binary toxin in C. difficile strains, and further study of the epidemiology, mortality and risk factors for CDI caused by binary toxin-positive C. difficile strains.

Disclosure of Potential Conflicts of Interest

Gerding DN holds patents for prevention and treatment of CDI licensed to ViroPharma and is a consultant for ViroPharma, Merck, Roche, Novartis, Opipter, Cibaax, Cangene, Sanofi Pasteur, Summit PLC and Actelion. Johnson S has served as a consultant for Optimer, Pfizer, and Bio-K+. Rupnik M has served as a consultant for Astellas, Cibaax and Summit PLC. Akkers K reports no potential conflicts.

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References

1. McDonnell LG, Kepler CE, Thompson A, Omura BC Jr, Kuehne SV, Sambl SP, Johnson S, Gerding DN. An epidemic toxin-gene-variant strain of Clostridium difficile. N Engl J Med 2005; 353:2431-41. PMID:16120265, http://dx.doi.org/10.1056/NEJMoa051590

2. Lee NG, Festler J, Miller MA, Ogihara M, Libmann MJ, Michael S, Boustead AM, Nguyen T, Ferretto S, Kelly M, et al. A predomnantly cloned multi-ribotype epidemic outbreak of Clostridium difficile-associated diarrhea with high morbidity and mortality. N Engl J Med 2005; 353:2442-8. PMID:16120262, http://dx.doi.org/10.1056/NEJMoa051639

3. Govorkov A, Bakker D, Carter J, Dohant SB, Stobierski C, Nataro J, Dewar D, Baggett JS, Dohant PW, Kepler CE. Emergence of Clostridium difficile infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin Infect Dis. 2008; 47:1162-70; PMID:18843578, http://dx.doi.org/10.1086/592297

4. Rupnik M. Heterogeneity of large clostridial toxins: importance of Clostridium difficile/clostridial toxin PE/EMS Microbiol. Rev. 2008; 72:74-83; PMID:18397287; http://dx.doi.org/10.1128/978978-1. Johnson S, Gerding DN, Mcfarlane C, Kuijper EJ, Baggett JS. Clostridium difficile toxinotypes. FEMS Microbiol. Lett. 2008; 278:101-10a, http://dx.doi.org/10.1111/j.1574-6976.2008.00110.x

5. Dohant SB, van Loo GJ, Stobierski C, Nataro J, Dewar D, Baggett JS, Dohant PW, Kepler CE. Emergence of Clostridium difficile infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin Infect Dis. 2008; 47:1162-70; PMID:18843578, http://dx.doi.org/10.1086/592297

6. Rupnik M. Heterogeneity of large clostridial toxins: importance of Clostridium difficile/clostridial toxin PE/EMS Microbiol. Rev. 2008; 72:74-83; PMID:18397287; http://dx.doi.org/10.1128/978978-1. Johnson S, Gerding DN, Mcfarlane C, Kuijper EJ, Baggett JS. Clostridium difficile toxinotypes. FEMS Microbiol. Lett. 2008; 278:101-10a, http://dx.doi.org/10.1111/j.1574-6976.2008.00110.x
20. Nagaoka Y, Nakanishi S, Kadowaki K, Yoneda T, Tomoeda M, Fukushima H, Oda Y, Furuse M. Analysis of the ‘angulin’ protein: a novel mucin-like integron product of the Clostridium difficile binary toxin family. Infect Immun. 2008 Apr; 76(4):1339-48.

19. Senda Y, Inoue H, Umezawa S, Inoue Y, Kuboki T, Yamanaka H, Akiyama Y, Sudo T. Antigenic structure of CDT from Clostridium difficile. Infect Immun. 2005 May; 73(5):3199-205.

18. Kuroki AE, Thomas KE, Stringer D, Hong DQ, Feld AD, Yang TH, Toh CH, Williams ER, Briggs JM, Krain KA. The protective antigen component of Clostridium difficile toxin A forms a functional complex with the enzymatic component. J Biol Chem. 2006 May 19; 281(21):13430-4.

17. Strack RB, Deutscher MJ. RNA polymerase II: a complex enzyme of many subunits. Annu Rev Biochem. 1996; 65:525-61.

16. Lebherz TH, Wengel J. Mutations in the major subunit of RNA polymerase II of the frog Xenopus laevis do not affect the ability to be reconstituted into a complete enzyme. Nucleic Acids Res. 1989 Oct 1; 17(20):9377-85.

15. Marvaud JC, Popoff MR, Stiles BG. Evidence for a role as chylomicron remnant receptor. J Cell Sci. 2003 Sep; 116(Pt 18):3441-6.

14. Theodorou P, Wilcoxon C, Nölke T, Dömling A, Bihain BE, Darmon P. Molecular cloning of a lipolysis-stimulated remnant receptor isoenzyme with different substrate specificities and implications for triglyceride metabolism. J Biol Chem. 1999 Jul 23; 274(29):20044-52.

13. Marvaud JC, Krone R, Bihain BE. Lipolysis-stimulated remnant receptor and its relationship to the LDL receptor. Curr Drug Targets. 2004 Dec; 5(6):691-702.

12. Nagaoka Y, Nakanishi S, Kadowaki K, Yoneda T, Tomoeda M, Fukushima H, Oda Y, Furuse M. Analysis of the ‘angulin’ protein: a novel mucin-like integron product of the Clostridium difficile binary toxin family. Infect Immun. 2008 Apr; 76(4):1339-48.

11. Senda Y, Inoue H, Umezawa S, Inoue Y, Kuboki T, Yamanaka H, Akiyama Y, Sudo T. Antigenic structure of CDT from Clostridium difficile. Infect Immun. 2005 May; 73(5):3199-205.

10. Kuroki AE, Thomas KE, Stringer D, Hong DQ, Feld AD, Yang TH, Toh CH, Williams ER, Briggs JM, Krain KA. The protective antigen component of Clostridium difficile toxin A forms a functional complex with the enzymatic component. J Biol Chem. 2006 May 19; 281(21):13430-4.

9. Theodorou P, Wilcoxon C, Nölke T, Dömling A, Bihain BE, Darmon P. Molecular cloning of a lipolysis-stimulated remnant receptor isoenzyme with different substrate specificities and implications for triglyceride metabolism. J Biol Chem. 1999 Jul 23; 274(29):20044-52.

8. Marvaud JC, Krone R, Bihain BE. Lipolysis-stimulated remnant receptor and its relationship to the LDL receptor. Curr Drug Targets. 2004 Dec; 5(6):691-702.

7. Nagaoka Y, Nakanishi S, Kadowaki K, Yoneda T, Tomoeda M, Fukushima H, Oda Y, Furuse M. Analysis of the ‘angulin’ protein: a novel mucin-like integron product of the Clostridium difficile binary toxin family. Infect Immun. 2008 Apr; 76(4):1339-48.

6. Theodorou P, Wilcoxon C, Nölke T, Dömling A, Bihain BE, Darmon P. Molecular cloning of a lipolysis-stimulated remnant receptor isoenzyme with different substrate specificities and implications for triglyceride metabolism. J Biol Chem. 1999 Jul 23; 274(29):20044-52.

5. Marvaud JC, Krone R, Bihain BE. Lipolysis-stimulated remnant receptor and its relationship to the LDL receptor. Curr Drug Targets. 2004 Dec; 5(6):691-702.
A phenylalanine Hsp90 is necessary for cytotoxic action of the bi0354278

48. Kaiser K, Scharf C, Ernst R, Schwab C, Popp M, Fischer G, Bucher J, Aktories K, Barth H. Membrane translocations of binary actin-ADP-ribosylating toxins from Clostridium difficile and Clostridium perfringens are facilitated by cofilin A and Hsp90. Infect Immun. 2011; 79:393-21, PMID:21786481.
49. Kaiser K, Scharf C, Ernst R, Schwab C, Popp M, Fischer G, Bucher J, Aktories K, Barth H. Membrane translocation of Clostridium difficile C2 toxin and FXP-related membrane translocation of the toxin in mammalian cells. Cell Microbiol. 2011; 13:805-16, PMID:21732708.
50. Pillard TD, Cooper JS. Activity of a bacterial poly(A)-binding protein 51 interacts with FLAG epitope-tagged Clostridium difficile 0231 toxin. Biochemistry 2001; 40:4316-23, PMID:11263802.
51. Dominguez R, Holcombe KC. Actin structures and their function. Annu Rev Biophys. 2011; 40:169-86, PMID:21274225.
52. Willows WD, Rupnik M, Obuch-Woszczatynski P, Wieczorek S, Suda T, Ishidoh K, Sakurai J. Novel actin-targeting mono-ADP-ribosyltransferase from Photorhabdus luminescens. J Biol Chem 2010; 285:37818-26, PMID:20771674.
53. Aktories K, Popoff M, Fischer G, Barth H. The host cell chaperone Hsp90 is essential for translocation of the binary Clostridium botulinum C2 toxin into the cytosol. J Biol Chem. 2010; 285:37818-26, PMID:20771674.
54. Kaiser K, Scharf C, Ernst R, Schwab C, Popp M, Fischer G, Bucher J, Aktories K, Barth H. Membrane translocations of binary actin-ADP-ribosylating toxins from Clostridium difficile and Clostridium perfringens are facilitated by cofilin A and Hsp90. Infect Immun. 2011; 79:393-21, PMID:21786481.
55. Kaiser K, Scharf C, Ernst R, Schwab C, Popp M, Fischer G, Bucher J, Aktories K, Barth H. Membrane translocation of Clostridium difficile C2 toxin and FXP-related membrane translocation of the toxin in mammalian cells. Cell Microbiol. 2011; 13:805-16, PMID:21732708.
56. Pillard TD, Cooper JS. Activity of a bacterial poly(A)-binding protein 51 interacts with FLAG epitope-tagged Clostridium difficile 0231 toxin. Biochemistry 2001; 40:4316-23, PMID:11263802.
57. Dominguez R, Holcombe KC. Actin structures and their function. Annu Rev Biophys. 2011; 40:169-86, PMID:21274225.
58. Willows WD, Rupnik M, Obuch-Woszczatynski P, Wieczorek S, Suda T, Ishidoh K, Sakurai J. Novel actin-targeting mono-ADP-ribosyltransferase from Photorhabdus luminescens. J Biol Chem 2010; 285:37818-26, PMID:20771674.
59. Aktories K, Popoff M, Fischer G, Barth H. The host cell chaperone Hsp90 is essential for translocation of the binary Clostridium botulinum C2 toxin into the cytosol. J Biol Chem. 2010; 285:37818-26, PMID:20771674.
Goul

Clostridium difficile

infection in the
in Europe. J Clin Gastroenterol Hepatol 2009; 7:868-73; PMID:19605755; http://dx.doi.org/10.1038/jdg.2009.108.

Bacrer J, Moth C, Atkins D, Johnson S, Gerding DN. Clinical severity of Clostridium difficile infection caused by the epidemic C. difficile H1 strain. Clin Infect Dis 2012; 55:351-3; PMID:22684204; http://dx.doi.org/10.1093/jac/dks273.

Keel K, Barbier JT, Kuijper EJ; ECDIS Study Group. Pathogenesis of C. difficile-associated infections: results of a retrospective study of 128 strain-typed cases from a teaching hospital in the United Kingdom. Clin Infect Dis 2010; 50:71-81; PMID:20384512.

Morgan OWE, Rodriguez B, Biscarini V, Vellard NR, Brown DS, Braun J, Braucher M. Clinical severity of Clostridium difficile PCR ribotype 027: a case-case study. Mol Cell 2008; 3:1821; PMID:18335498; http://dx.doi.org/10.1016/j.molcel.2008.03.047.

Petrella LA, Sandil SD, Ciszkis A, Nagesh K, Kuri Y, Sears PV, Bahrami H, Johnson S, Gerding DN. Discordant case and increased recurrence rates for Clostridium difficile infection among the elderly: a case-control study. J Med Microbiol 2013; 62:355-62; PMID:21396957; http://dx.doi.org/10.1099/jmm.0.05804-0.

Goldenberg SD, Bresch G. Lack of association of binary toxin-positive strains with severe disease in a monocentric setting. Clin Gastroenterol Hepatol 2011; 9:194-201; PMID:21425265; http://dx.doi.org/10.1016/j.cgh.2010.10.018.

Goldenberg SD, Bresch G. Lack of association of binary toxin-positive strains with severe disease in a monocentric setting. Clin Gastroenterol Hepatol 2011; 9:194-201; PMID:21425265; http://dx.doi.org/10.1016/j.cgh.2010.10.018.

Rupnik M, Artzi B, Carman RJ, Aguiliete PM, Reilly PJ, Gerding DN. Lack of association of binary toxin-positive strains with severe disease in a monocentric setting. Clin Gastroenterol Hepatol 2011; 9:194-201; PMID:21425265; http://dx.doi.org/10.1016/j.cgh.2010.10.018.

Rupnik M, Artzi B, Carman RJ, Aguiliete PM, Reilly PJ, Gerding DN. Lack of association of binary toxin-positive strains with severe disease in a monocentric setting. Clin Gastroenterol Hepatol 2011; 9:194-201; PMID:21425265; http://dx.doi.org/10.1016/j.cgh.2010.10.018.

Rupnik M, Artzi B, Carman RJ, Aguiliete PM, Reilly PJ, Gerding DN. Lack of association of binary toxin-positive strains with severe disease in a monocentric setting. Clin Gastroenterol Hepatol 2011; 9:194-201; PMID:21425265; http://dx.doi.org/10.1016/j.cgh.2010.10.018.

Rupnik M, Artzi B, Carman RJ, Aguiliete PM, Reilly PJ, Gerding DN. Lack of association of binary toxin-positive strains with severe disease in a monocentric setting. Clin Gastroenterol Hepatol 2011; 9:194-201; PMID:21425265; http://dx.doi.org/10.1016/j.cgh.2010.10.018.

Rupnik M, Artzi B, Carman RJ, Aguiliete PM, Reilly PJ, Gerding DN. Lack of association of binary toxin-positive strains with severe disease in a monocentric setting. Clin Gastroenterol Hepatol 2011; 9:194-201; PMID:21425265; http://dx.doi.org/10.1016/j.cgh.2010.10.018.

Rupnik M, Artzi B, Carman RJ, Aguiliete PM, Reilly PJ, Gerding DN. Lack of association of binary toxin-positive strains with severe disease in a monocentric setting. Clin Gastroenterol Hepatol 2011; 9:194-201; PMID:21425265; http://dx.doi.org/10.1016/j.cgh.2010.10.018.

Rupnik M, Artzi B, Carman RJ, Aguiliete PM, Reilly PJ, Gerding DN. Lack of association of binary toxin-positive strains with severe disease in a monocentric setting. Clin Gastroenterol Hepatol 2011; 9:194-201; PMID:21425265; http://dx.doi.org/10.1016/j.cgh.2010.10.018.

Rupnik M, Artzi B, Carman RJ, Aguiliete PM, Reilly PJ, Gerding DN. Lack of association of binary toxin-positive strains with severe disease in a monocentric setting. Clin Gastroenterol Hepatol 2011; 9:194-201; PMID:21425265; http://dx.doi.org/10.1016/j.cgh.2010.10.018.