**Clostridium difficile** Toxins TcdA and TcdB Cause Colonic Tissue Damage by Distinct Mechanisms

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As the major cause of antibiotic-associated diarrhea, *Clostridium difficile* is a serious problem in health care facilities worldwide. *C. difficile* produces two large toxins, TcdA and TcdB, which are the primary virulence factors in disease. The respective functions of these toxins have been difficult to discern, in part because the cytotoxicity profiles for these toxins differ with concentration and cell type. The goal of this study was to develop a cell culture model that would allow a side-by-side mechanistic comparison of the toxins. Conditionally immortalized, young adult mouse colonic (YAMC) epithelial cells demonstrate an exquisite sensitivity to both toxins with phenotypes that agree with observations in tissue explants. TcdA intoxication results in an apoptotic cell death that is dependent on the glucosyltransferase activity of the toxin. In contrast, TcdB has a bimodal mechanism; it induces apoptosis in a glucosyltransferase-dependent manner at lower concentrations and glucosyltransferase-independent necrotic death at higher concentrations. The direct comparison of the responses to TcdA and TcdB in cells and colonic explants provides the opportunity to unify a large body of observations made by many independent investigators.

*Clostridium difficile* is the most common cause of antibiotic-associated diarrhea in the United States, and *C. difficile* infection (CDI) has been steadily increasing in prevalence and severity over the last 15 years (1–3). Symptoms of CDI can range from mild diarrhea to pseudomembranous colitis, and hallmarks of the disease include neutrophil infiltration, fluid release, and necrotic lesions in the colonic epithelium (4, 5). The bacteria produce two main virulence factors, large toxins called TcdA and TcdB (6, 7).

The respective function and relative importance of each toxin in pathogenesis have been active topics of investigation. Genetic knockout experiments in *C. difficile* have shown that both toxins are important for disease pathology, although TcdB alone is sufficient to cause death in both the hamster and mouse models (6–8). For many years, TcdA and TcdB have been thought to act synergistically, with TcdA acting as an enterotoxin and TcdB acting as a cytoxin (9, 10). The general term enterotoxin refers to the capacity of TcdA to induce inflammation, cytokine release, and fluid secretion in animal intoxication models (11–13). While TcdB does not always induce these same phenotypes in models, such as the ileal loop model, it has been shown to disrupt the integrity of the epithelial structure in human explant and xenograft models (14, 15). TcdB is also notably more potent as a cytoxin in cell culture models (9, 10, 16).

The toxins have an N-terminal glucosyltransferase domain (GTD) that is delivered into the host cytosol by the C-terminal portion of the protein (17, 18). The GTD has been shown to target and inactivate a number of Rho-family GTPases (19, 20). This inactivation has been linked to a cell rounding or cytopathic effect (CPE) (21–24) and to an apoptotic cytotoxic effect (25–33). In tissue culture models, the apoptotic effects of TcdA and TcdB occur at toxin concentrations of picomolar or lower and are evident at 24 to 48 h postintoxication (26, 28, 29, 34–36). TcdB also induces a glucosyltransferase-independent necrosis that is mediated by the assembly and activation of the NADPH oxidase (NOX) complex, subsequently producing high levels of reactive oxygen species (ROS) (34, 37–40). Indicators of necrosis are apparent within 2 to 4 h using nanomolar concentrations of TcdB in both tissue culture and colonic explant models.

Most published reports discuss the effects of a single toxin; it is rare to see the effects of the toxins compared side-by-side in colonic cell and tissue models. In this study, we wanted to investigate the mechanisms and pathological outcomes associated with TcdA and TcdB intoxication under comparable conditions. We reasoned that the antiapoptotic mutations associated with transformed cell lines were preventing TcdA-induced cell death pathways. Young adult mouse colonic (YAMC) epithelial cells are derived from the Immortomouse, which expresses a temperature-sensitive simian virus 40 (SV40) T antigen that suppresses p53 (41). The cells can be carried as an antiapoptotic cell line at the permissive temperature of 33°C, and then, when they are shifted to the nonpermissive temperature of 37°C, YAMC cells behave as primary cells with an intact p53 pathway able to undergo normal apoptosis. Using this tool, we were able to investigate the effects of TcdA and TcdB side-by-side using the same time points and assay readouts. Our observations provide an opportunity to unify the many, seemingly conflicting reports describing the mechanisms by which TcdA and TcdB cause cell death in epithelial cells.
MATERIALS AND METHODS

Recombinant protein expression and purification. The glucosyltransferase domain double point mutation (TcdA D285/287N and TcdB D286/288N) plasmids (pBL764 and pBL765, respectively) were made using the TcdA and TcdB parent plasmids (42) according to the QuickChange protocol (Stratagene). Recombinant TcdA, TcdA D285/287N, TcdB, and TcdB D286/288N proteins were expressed in Bacillus megaterium and purified as previously described (42).

YAMC cell culture and viability assays. YAMC cells were maintained in RPMI 1640 supplemented with 5% fetal bovine serum, 1 mg/ml insulin, 10 μM alpha-thioglycolate, 1 μM hydrocortisone, and 5 U/ml mouse interferon gamma. Cells were maintained at 33°C with 5% CO2. For assays performed at 37°C, cells were plated and incubated at 37°C with 5% CO2 overnight prior to intoxication. Viability was measured at the concentrations and time points indicated below using the CellTiter Glo luminescent cell viability assay (catalog number G7573; Promega). Lactate dehydrogenase (LDH) release was quantified using the CytoTox Glo assay (catalog number G9290; Promega). Apoptosis was assessed by measuring active caspase-3 and -7 levels using the Apo-ONE homogeneous caspase-3/7 assay (catalog number Promega, G7792). ROS production was assayed with carboxy-2′,7′-dichlorodihydrofluorescein diacetate (catalog number C400; Life Technologies) as previously described (37).

Colonic explants. Animal husbandry and experimental procedures related to the porcine colonic explants were performed in accordance with the Vanderbilt University Institutional Animal Care and Use Committee (IACUC) policy. Discarded colon tissues were obtained from pigs following euthanization at the end of IACUC-approved animal use protocols and prepared for intoxication as previously described (34). Tissue was challenged with 10 nM TcdA, TcdB, TcdA D285/287N, or TcdB D286/288N for 5 h at 37°C. The sections were cut by the Vanderbilt University Translational Pathology Shared Resource Core. Staining of tissues for detection of caspase-3 and ROS was done as previously described (34, 37). All slides stained with fluorescent markers were analyzed with an LSM 510 confocal microscope.

Statistical analysis. Statistical analysis was performed using a two-way analysis of variance (ANOVA) and post hoc test in GraphPad Prism software. Two-tailed, paired Student’s t tests were performed using Excel software.

RESULTS

TcdA induces a robust cell death in conditionally immortalized cells. We hypothesized that the lack of rapid TcdA-induced cell death in typical tissue culture models was due to mutations in the apoptotic pathways of many transformed cell lines. To test our hypothesis, we obtained YAMC cells, a conditionally immortalized cell line with temperature-sensitive large T antigen (ts58) that can affect p53 function and, more broadly, drive cells into S phase to promote cell death (43). YAMC cells were challenged with the TcdA and TcdB toxins at both the permissive (33°C) and nonpermissive (37°C) temperatures, and cell death was quantified using CellTiter Glo, an ATP-sensitive viability indicator. Consistent with what is observed in transformed cell lines, TcdA does not induce appreciable cell death at 33°C, where p53 is inactivated (Fig. 1A). Consistent with our previous observations in transformed HeLa and Caco2 cell lines (34), TcdB kills cells efficiently at concentrations of ≥100 pM but does not cause cell death at concentrations of ≤10 pM (Fig. 1B). These changes were also significant relative to the responses at 33°C (P < 0.0001).

Both TcdA and TcdB bind YAMC cells and glucosylate Rac1 with similar efficiencies at 33°C and 37°C (see Fig. S1 in the supplemental material), suggesting that the increased cytotoxicity of the toxins at 37°C is not the result of different binding, entry, or glucosylating activities. Similar results were observed in HeLa cells, which are p53 null (although we were unable to detect the
TcdA binding by Western blotting in these cells) (see Fig. S2 in the supplemental material). As another control experiment, we used a live/dead indicator to assess the toxin-induced CPE and cell death in HeLa cells at the two different temperatures. The cells rounded in response to 100 pM or 10 nM concentrations of TcdA at both temperatures, but as has been observed in previous studies (34, 37), there was very little cell death (see Fig. S3 in the supplemental material). With TcdB applied at a 10 pM or 10 nM concentration, the cells were either round at both temperatures or dead, respectively. These experiments support the hypothesis that the different effects of the toxins at different temperatures are not a result of different binding and glucosylating activities but, rather, are a result of a cell death mechanism that depends on the expression of ts58.

TcdB causes a loss of membrane integrity faster than TcdA. The extent of the temperature effect was less pronounced for the cells treated with TcdB at concentrations of ≥100 pM (Fig. 1). This led us to hypothesize that TcdA and TcdB kill cells through distinct mechanisms at concentrations of ≥100 pM. To evaluate this further, we assessed the impact of TcdA and TcdB on cell membrane integrity over time using an LDH indicator (Fig. 2). We detected LDH release as early as 2 h after cells were challenged with ≥0.1 nM TcdB (Fig. 2A), consistent with a necrotic mechanism of cytotoxicity. The LDH signal was high in cells treated with high TcdB concentrations (100 pM to 100 nM) for up to 8 h postintoxication (Fig. 2A to C). Concentrations below 100 pM did not demonstrate an appreciable rise in LDH levels at the 2-, 4-, or 8-h time points, and the LDH signal was not significantly different from the signal produced from cells intoxicated with TcdA (Fig. 2A to C). However, an increase in the LDH signal at lower concentrations was detected at 24 h postintoxication with TcdB (Fig. 2D). LDH release was detected in response to all concentrations of TcdA but only after 24 h of intoxication (Fig. 2D). At the 24-h time point, there was no significant difference in the LDH signals from cells treated with either TcdA or TcdB.

TcdA induces cell death by a mechanism distinct from that for TcdB at higher toxin concentrations. The difference in the rate at which TcdA and TcdB induce a loss of membrane integrity further supported our hypothesis that the toxins kill cells by different mechanisms at higher concentrations. We expected TcdB to induce ROS-driven necrosis at these higher concentrations based on previous work in HeLa and Caco-2 cells (37) and therefore measured ROS production in response to each toxin in YAMC cells (Fig. 3A). TcdB induced the production of high levels of ROS at higher concentrations (≥100 pM), where we have previously observed necrosis, but not at toxin concentrations below...
FIG 3  TcdA and TcdB induce cell death by different mechanisms. (A) YAMC cells were tested for ROS production in response to TcdA (24 h) or TcdB (6 h) using a fluorescent ROS reporter. Statistical analysis by two-way ANOVA revealed that the results for TcdA and TcdB were significantly different from each other at concentrations of ≥100 pM (*, P < 0.0001). (B) YAMC cells were also tested for activated caspase-3/7 using Apo-ONE in response to TcdA and TcdA D285/287N. A two-way ANOVA revealed a significant difference between TcdA and TcdA D285/287N at all concentrations (*, P < 0.0001). (C) Activated caspase was measured in response to TcdB or TcdB D286/288N, as described in the legend to panel B. A two-way ANOVA showed no significant difference between TcdB and TcdB D286/288N at concentrations of ≥100 pM. At concentrations of ≥10 pM, there was a significant difference between TcdB and TcdB D286/288N (*, P < 0.01). In all panels, data represent the averages from three experiments performed in triplicate, and error bars represent the standard deviations of the means.
TcdA induces glucosyltransferase-dependent caspase-3 activation, while TcdB induces glucosyltransferase-independent ROS production in colonic tissue. We next wanted to test whether our observations that TcdA and TcdB induced cell death by different mechanisms at higher concentrations in YAMC cells could also be observed in colonic explants. We intoxicated tissue with 10 nM TcdA, TcdB, or their corresponding glucosyltransferase-deficient mutants and assessed ROS production using a fluorescent indicator and apoptosis using an antibody specific for active caspase-3. While the ROS signal in TcdA-treated tissue was minor (Fig. 4A), we detected a robust signal for active caspase-3 that was attenuated with the TcdA D285/287N mutant (Fig. 4B). Consistent with previous observations (37), we detected significant ROS in colonic explants treated with both TcdB and TcdB D286/288N (Fig. 4A). There was very little active caspase-3 signal in these samples (Fig. 4B). Together, these data support our observations in YAMC cells and suggest that TcdA and TcdB induce cell death by different mechanisms at concentrations of ≥100 pM.

**DISCUSSION**

To more fully elucidate the mechanisms of TcdA- and TcdB-induced cytotoxicity, we designed a study that allowed the analysis of comparable concentrations of toxins at identical time points in the same tissue or cell type. The comparison permits an understanding of the different cellular processes engaged by each toxin, the concentration at which the toxin is capable of injuring the cell or tissue, and the relative contribution of the glucosyltransferase activity of the toxins.

Using YAMC cells, we could readily detect TcdA-induced cell death over a wide range of concentrations (10 fM to 100 nM) in 18 h (Fig. 1B). TcdB-induced cell death was also readily detectable down to 10 fM at the same time point. The sensitivity of YAMC cells to TcdA- and TcdB-induced cell death at low concentrations is dependent on maintenance of the cells at 37°C, a temperature that prevents the expression of ts58. The large T antigen binds several cellular factors, including p53, a tumor suppressor known to regulate cell cycle, mitotic division, and apoptotic cell death pathways. There are conflicting reports regarding the function of p53 in TcdA-induced apoptosis (35, 45). One study using a non-transformed colonic cell line concluded that TcdA-induced apoptosis is dependent upon p53 function (45). Another study used two cell lines with different p53 expression profiles and directly compared the lines in the context of p53 presence or absence to conclude that TcdA-induced apoptosis is independent of p53 (35). In the second study, the cell line that expressed p53 was mutated in various other components of the apoptotic pathway, however. The data in this study suggest that TcdA-induced apoptosis is dependent upon p53 function (45).
study are consistent with a view where p53 function is important for TcdA- and TcdB-induced apoptosis, although we acknowledge that p53 is just one of many tS8 targets.

The temperature-dependent TcdB-induced cytotoxicity profiles observed in YAMC cells (Fig. 1) are consistent with the disparity in cell death events reported by many different groups over the years. Studies where TcdB was used at high picomolar to nanomolar concentrations report necrosis (34, 37–39), while subpicomolar intoxication results in an apoptotic event (30–33, 46). Notably, TcdB does not require p53 function at concentrations of ≥100 pM that result in necrotic cell death (Fig. 1A). We have previously shown that TcdB induces a glucosyltransferase-independent necrotic cell death as a result of aberrant ROS production through the NADPH oxidase (NOX) complex (34, 37). To further investigate the possibility that TcdB induces a concentration-dependent mechanism that switches from apoptosis to necrosis, we used cellular indicators to determine the death pathway activated at a given concentration. We observed that TcdA induces an apoptotic cell death at all concentrations (Fig. 3B) and the induction of apoptosis by TcdA is completely dependent on a fully active glucosyltransferase (Fig. 3B), and we interpret the LDH release at 24 h (Fig. 2D) to be the result of necrosis secondary to apoptosis. Most interestingly, we could observe two distinct cell death mechanisms occurring in response to TcdB. At higher concentrations (≥100 pM), we saw clear indications of a necrotic cell death, including ROS production (Fig. 3A), rapid LDH release (Fig. 2A), and minimal caspase-3/7 activation (Fig. 3C). At lower concentrations (≤10 pM), there were no indications of necrosis, we saw a rise in caspase-3/7 activation (Fig. 3C), indicating apoptotic cell death. Also as clearly demonstrated in Fig. 3C, the activation of the apoptotic cell death pathway at lower concentrations of TcdB mirrors that seen at lower concentrations of TcdA, in that it requires glucosyltransferase activity. This is the first report of TcdB inducing a bimodal cell death mechanism, dependent upon the concentration of toxin, allowing the unification of the observations described by groups with seemingly opposing data.

We were able to extrapolate and confirm our findings in the colonic explant model. At a concentration of 10 nM toxin, TcdA induces glucosyltransferase-dependent apoptotic cell death, while TcdB induces the glucosyltransferase-independent production of ROS (Fig. 4). While our current explant model system do not allow for observation for longer times, we anticipate that at lower concentrations (≤10 pM) both toxins will cause damage through a glucosyltransferase-dependent apoptotic mechanism.

The next question is, how can TcdA and TcdB cause cell death by different mechanisms? While differences in GTPase substrates between TcdA and TcdB have been reported, we would not expect substrate differences to account for differences in apoptosis and necrosis since the mechanism of necrosis is glucosyltransferase independent. Rather, we think that the most likely reason is that the toxins engage different receptors. While the human receptor for TcdA has yet to be identified, two receptors for TcdB have been described: poliovirus receptor-like protein 3 (PVRL3) and chondroitin sulfate proteoglycan 4 (CSPG4) (47, 48). PVRL3 seems to account for the cell death occurring at high concentrations in HLa cells (where CSPG4 is also highly expressed), but it can mediate both necrotic and apoptotic mechanisms in Caco2 cells (47, 48). We hypothesize that TcdB binding to PVRL3 initiates the assembly and activation of the NOX complex and that the lack of PVRL3 binding by TcdA accounts for the differences in the cell death responses of the two toxins. What is unclear, however, is how PVRL3 can be involved in both apoptotic and necrotic mechanisms depending only on the TcdB concentration. This is a topic of ongoing investigation.

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