Chapter 5

Assays for Measuring *C. difficile* Toxin Activity and Inhibition in Mammalian Cells

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http://dx.doi.org/10.5772/68127

Abstract

Clostridium difficile infections (CDIs) are the leading cause of hospital-acquired infectious diarrhea. The symptoms of CDI are caused by two exotoxins, TcdA and TcdB, which are structurally and functionally highly homologous. Both toxins bind to specific receptors on mammalian cells, are internalized through endocytosis, translocate to the cytoplasm, and inactivate Rho-type GTPases via covalent glucosylation. This leads to downstream events that include morphological changes and disruption of epithelial tight junctions, release of pro-inflammatory mediators, and cell death. Assays used to assess the effects of toxins on cells have historically relied on evaluation of cell rounding or quantitation of ATP levels to estimate cell death—assays which can be qualitative and variable. In this chapter, several assays are described that robustly and quantitatively measure early and late toxin-dependent events in cells, including (i) toxin binding, (ii) Rac1 glucosylation, (iii) changes in cellular morphology (measured as dynamic mass redistribution), (iv) loss of epithelial integrity (measured as transepithelial electrical resistance), and (v) cell death (measured as total cellular protein using a colorimetric assay). The assays were validated using the highly specific monoclonal antitoxin antibodies, actoxumab and bezlotoxumab, which neutralize TcdA and TcdB, respectively.

Keywords: *C. difficile*, toxins, cell-based assays, epithelial cells, antitoxins

1. Introduction

Clostridium difficile (*C. difficile*) is an anaerobic, gram-positive, spore-forming bacterium that colonizes the lower intestinal tract of patients whose normal gut microflora has been disrupted by treatment with broad-spectrum antibiotics [1]. The symptoms of *C. difficile*

infection (CDI)—which include diarrhea and, in severe cases, pseudomembranous colitis, colonic rupture, and death [1, 2]—are caused by two exotoxins, toxin A (TcdA) and toxin B (TcdB) [3]. Both toxins have similar structural and functional characteristics. After binding to specific receptors on the surface of gut epithelial cells, they are internalized through endocytosis, translocate to the cytoplasm, and inactivate Rho-type GTPases via covalent glucosylation [4–7]. This leads to a variety of downstream events, including morphological changes associated with disruption of epithelial tight junctions, release of pro-inflammatory mediators (including interleukin-1β, tumor necrosis factor alpha, and interleukin-8), and eventually cell death [3, 8]. The damaging effects on the gut epithelium and initiation of a host inflammatory response are thought to underlie the clinical manifestation of CDI.

Current treatment for *C. difficile* infections includes discontinuing the offending broad-spectrum antibiotic and initiating therapy with narrower spectrum agents such as vancomycin, metronidazole, or fidaxomicin [9, 10]. Unfortunately, these treatments do not directly address the damaging effects of the toxins on the gut and perpetuate the gut dysbiosis that caused CDI in the first place. As a result, up to 25% or more patients successfully cured of an initial episode of CDI with these antibiotics suffer a recurrent episode within days to weeks. To address this, recent approaches to CDI treatment, including vaccines and monoclonal antibodies, have focused on neutralizing the effects of TcdA and TcdB, specifically, rather than the organisms itself [11–13]. Foremost among these novel therapies is bezlotoxumab, the anti-TcdB antibody recently approved by the Food and Drug Administration for reducing recurrence of CDI in patients 18 years of age or older who are receiving antibacterial drug treatment of CDI and are at a high risk for CDI recurrence.

The renewed interest in toxin-directed therapies underscores the importance of having robust quantitative assays in place to assess the activity of the *C. difficile* toxins. Historically, studying the effects of TcdA and TcdB on mammalian cells has been hampered by time-consuming and subjective assays that rely, for example, on visualization of cells to assess cell rounding or on the variable quantitation of ATP levels to measure cell death [13]. Thus, there is a scarcity of robust quantitative assays that measure the various cellular events associated with the intoxication cascade, making it difficult to evaluate new toxin-directed agents. In this chapter, we describe multiple quantitative cell-based assays that were newly developed, or adapted and optimized from previous reports, and used to interrogate the effect of the *C. difficile* toxins on epithelial cells. The assays are validated using the highly specific and potent antitoxin antibodies, actoxumab and bezlotoxumab, which bind to and neutralize TcdA and TcdB, respectively [13–15].

2. Materials and methods

2.1. TcdA- and TcdB-binding assay (Western blot)

TcdA (1 μg/ml) or TcdB (0.1 μg/ml) (The Native Antigen Company, Upper Heyford, the UK and tgcBIOMICS, Bingen, Germany) was incubated with or without 200 μg/ml actoxumab or bezlotoxumab in Vero cell culture medium (Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin)

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for 30 min at 37°C; these mixtures were then chilled on ice and added to plates of pre-chilled Vero cells (ATCC, Rockville, MD). Plates were incubated for 30 min on ice to allow binding of toxins. Following incubation, plates were washed three times with cold phosphate buffered saline (PBS) and cells were harvested by scraping. Cell membranes were isolated at 4°C with the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Scientific, Grand Island, NY), according to the manufacturer's instructions, and solubilized in a total volume of 100 μL solubilization buffer with HALT protease/phosphatase inhibitors (Thermo Scientific). Following addition of Laemmli sample buffer, samples were incubated for 5 min at 95°C and resolved by SDS PAGE in 4–12% polyacrylamide gels and transferred to a nitrocellulose membrane. The nitrocellulose membrane containing transferred protein was blocked in Odyssey blocking buffer (Li-Cor) followed by incubation with actoxumab, bezlotoxumab, or an anti-cadherin antibody (Cell Signaling Technology, Beverly, MA) as the primary antibody for 1 h at room temperature (RT). After washing, the nitrocellulose membrane was incubated with a goat anti-human IgG antibody coupled to IRDye® 800CW (Li-Cor) for 30 min at RT. After additional washing, bands were visualized using the Odyssey imaging system (Li-Cor).

2.2. TcdA-binding assay (flow cytometry)

TcdA, from ribotype 087 (The Native Antigen Company, Upper Heyford, the UK), was fluorescently labeled using the Lightning Link Atto488 Antibody Labeling kit (Novus Biosciences, Littleton, CO) as directed by the manufacturer. About 50 μg of lyophilized TcdA was reconstituted for a minimum of 30 min in sterile ddH2O at RT before adding the LL-modifier buffer. The toxin/LL-modifier buffer solution was transferred to a vial containing the lyophilized Lightning Link mix. The mixture was pipetted up and down and incubated at RT in the dark. After 5 h, LL-quencher buffer was added and incubated at RT in the dark for 30 min and then stored at 4°C until use the following day. Several concentrations of TcdA-Atto488 were incubated with or without 200 μg/ml actoxumab at RT for 60 min, protected from light. Samples were then chilled on ice. Adherent HT29 cells (ATCC, Rockville, MD) were resuspended in the cell medium (McCoy's 5A Modified medium supplemented with 10% FBS, 2 mM glutamine, 0.75% sodium bicarbonate, 100 U/ml penicillin, and 100 U/ml streptomycin), following treatment with Accutase (Innovative Cell Technologies), washed once with cold Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS++) containing 1% bovine serum albumin (BSA), and then chilled on ice. 100 μL of each toxin/antibody sample was added to separate vials containing 3×10^5 cells. After mixing, samples were incubated on ice in the dark. After 30 min, 1 ml of ice cold DPBS++/1% BSA was added to each sample. To remove unbound toxin, cell suspensions were washed twice with ice cold DPBS++/1% BSA by centrifuging for 5 min at 4°C at 200 × g and removing the supernatant. Washed cells were resuspended in 500 μl cold DPBS++/1% BSA and analyzed by flow cytometry using an LSRII instrument (BD Biosciences) with excitation and emission wavelengths of 488 and 530 nm, respectively. 10,000 events were measured for each sample.

2.3. Rac1 glucosylation assay

Vero cells were seeded at a cell density of 5×10^3 cells/well in a 384-well collagen-coated plate and grown overnight at 37°C in 5% CO_2. TcdA and TcdB (The Native Antigen Company,

Upper Heyford, the UK and tgcBIOMICS, Bingen, Germany) were serially diluted in Vero cell culture medium, and 50 μl was added to each well. For assays determining neutralization effects of actoxumab and bezlotoxumab, TcdA and TcdB were pre-incubated at 90% effective concentrations (EC_{90}) with actoxumab and bezlotoxumab, respectively (various concentrations), for 1 h at RT in Vero cell culture medium, prior to addition of cells as above. Following incubation at 37°C in 5% CO_2 for 3 h, medium containing toxin alone or toxin+antibody was removed by aspiration. Cells were immediately fixed with 50 μl/well fixing solution (4% paraformaldehyde in modified Dulbecco's phosphate-buffered saline (DPBS/modified)) for 1 h at RT. Following fixation, cells were washed four times for 5 min with 50 μl/well permeabilization solution (0.1% Triton-X-100 in DPBS/modified) at RT with gentle shaking. Cells were then blocked with 50 μl/well Odyssey blocking buffer (Li-Cor) overnight at 4°C. After removing blocking buffer, cells were incubated with 25 μl/well mouse anti-Rac1 (BD Biosciences #610651, recognizing non-glucosylated Rac1), or anti-Rac1 clone 23A8 (Millipore #05-389, recognizing total Rac1), diluted at 1:75 and 1:200, respectively, in Odyssey blocking buffer and incubated for 2 h at RT with gentle shaking. Cells were washed four times for 5 min with 50 μl/well wash solution (0.1% tween 20 in DPBS/modified) at RT with gentle shaking. Cells were then incubated with 25 μl/well secondary antibodies (IRDye 800 CW goat anti-mouse and CellTag 700 stain, diluted at 1:800 and 1:1000, respectively, in Odyssey blocking buffer) at RT for 1 h with gentle shaking protected from light. Cells were again washed four times for 5 min with 50 μl/well wash solution at room temperature with gentle shaking. After the final wash, any remaining solution was removed from the wells, and the plates were scanned on the Li-Cor Odyssey classic (Li-Cor) with detection in both 700 and 800 nm channels (A700 and A800). Cell number normalization/well was calculated using the ratio of A800/A700, and remaining percent of non-glucosylated Rac1 was determined using the ratio of normalized A800 of treated cells/normalized A800 of untreated cells multiplied by 100. Analysis was performed with GraphPad Prism (version 6.04) using the 4-parameter nonlinear regression formula.

2.4. Dynamic mass redistribution (Epic) assay

Vero cells were seeded at a cell density of 5×10^3 cells/well in a 384-well fibronectin-coated Epic plate (Corning #5042) and grown overnight at 37°C in 5% CO_2. On the day of assay, medium was aspirated and replaced with 40 μl/well assay buffer (HBSS in 20 mM HEPES) and equilibrated at RT for 1 h. TcdA and TcdB (The Native Antigen Company, Upper Heyford, UK and tgcBIOMICS, Bingen, Germany) were serially diluted in assay buffer and equilibrated at RT for approximately 10 min. For assays determining neutralization effects of actoxumab and bezlotoxumab, TcdA and TcdB were pre-incubated at EC_{90} concentrations for 1 h at RT with actoxumab and bezlotoxumab, respectively (various concentrations). Following pre-incubations, 10 μl/well of the toxins alone or toxin/antibody solutions were added to Vero cells using a Matrix Platemate (Thermo Scientific) and gently mixed. The plate was read every 12 s for 200 min using the Epic BT-157900 (Corning). As a baseline, wells containing assay buffer alone were used. The dynamic mass redistribution (DMR) values were recorded at 180 min at which point the signal had plateaued (not shown). The recorded DMR values (corrected for assay buffer alone) were collected with EpicAnalyzer software and analyzed with GraphPad Prism (version 6.04) using the four-parameter nonlinear regression formula.

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2.5. Transepithelial electrical resistance (TER) assay

To initiate the 2-dimensional culture system, 0.5–1 × 10^5 Caco-2 cells (ATCC, Rockville, MD) were seeded into each well of the 24-well insert plates (Falcon #351181 HTS Multiwell Insert System—1.0 um pore size/PET membrane), with 250 μl Caco-2 cell culture medium (EMEM supplemented with 10% FBS, 1× non-essential amino acid, 0.075% sodium bicarbonate, 100 U/ml penicillin, and 100 U/ml streptomycin) in the apical chamber and 800 μl in the basolateral chamber. Caco-2 cells were cultured for at least 14 days at 37°C with 5% CO_2 to ensure full differentiation and confluency, which were confirmed by plateauing of the TER reading at ≥ 600 Ω cm^2. TER was measured using the Epithelial Volt-Ohm Meter Millicell ERS-2 (EMD Millipore, Billerica, MA, USA). To assess the effect of toxins on the cell monolayer, TcdA and TcdB (The Native Antigen Company, Upper Heyford, UK and tgcBIOMICS, Bingen, Germany) were added to the apical chamber. To evaluate the ability of the antibodies to neutralize toxin effects, actoxumab or bezlotoxumab was added to the apical chamber immediately before addition of TcdA or TcdB to the apical chamber. For neutralization studies, 10 ng/ml TcdA was combined with various concentrations (from 0 to 50 μg/ml) of actoxumab, and 100 ng/ml TcdB was combined with various concentrations (from 0 to 100 μg/ml) of bezlotoxumab. TER measurements were obtained immediately before and, at 6, 24, and 48 h, after addition of toxins/antibodies to the apical chamber. TER values were normalized to values obtained in the absence of toxin at each time point to account for minor time-dependent variability.

2.6. Sulforhodamine B assay

To study the effects of *C. difficile* toxins on cytotoxicity and the ability of actoxumab and bezlotoxumab to neutralize those effects, the sulforhodamine B (SRB) assay was employed to measure total cellular protein as a surrogate of cell number [16]. Vero or T-84 (T-84 growth medium—DMEM/F-12K supplemented with 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin) cells were seeded into 96-well plates at 2000 and 3000 cells/well, respectively, and incubated overnight at 37°C with 5% CO_2. Varying concentrations of purified TcdA and TcdB (tgcBIOMICS, Bingen, Germany) were diluted in the appropriate growth media, incubated at 37°C for 2 h, and added to cells. Following a 24-h incubation at 37°C with 5% CO_2, the medium was aspirated and plates were washed twice with PBS. About 200 μl per well of complete medium was added, and plates were incubated for an additional 48 (Vero cells) or 72 h (T-84 cells). After incubation, the medium was removed, and cells were fixed with 100 μl/well of 10% cold trichloroacetic acid (TCA) for 1 h at 4°C. The TCA was then removed and plates were washed four times with distilled water. After washing, 100 μl/well of 100 μg/ml SRB in 10% acetic acid was added, and plates were incubated for 15 min at room temperature (RT). The plates were then washed four times with 10% acetic acid and air-dried. Addition of 150 μl/well of 10 mM tris was followed by a 10-min incubation at RT with shaking. Absorbance was then measured at 570 nm with a SpectraMax plate reader (Molecular Biosystems). Treated and untreated cells were compared, and 90% lethal concentrations (LC_{90}, that is, concentrations of TcdA or TcdB required to cause a 90% reduction in cell number) were calculated. Antibody-mediated toxin neutralization was measured by incubating serially diluted actoxumab or bezlotoxumab (at concentrations ranging from 1 ng/ml to 192 μg/ml) with purified TcdA or TcdB at LC_{90} for 2 h at 37°C. The toxin/antibody

mixtures were then added to Vero or T-84 cells as described above and incubated for 24 h at 37°C with 5% CO_2. The cells were then washed twice with PBS and treated and analyzed as described above.

To assess the cytotoxicity of *C. difficile* toxins derived from bacterial culture supernatants, strain VPI 10463 (ribotype 087) (ATCC) was grown in chopped meat medium (Anaerobe Systems) under anaerobic conditions at 37°C for 72–96 h, and culture supernatants were collected, filtered twice through a 0.22 μm filter, and stored at 4°C. For TcdB immunodepletion, cell culture supernatants were combined and mixed with bezlotoxumab and protein A-agarose beads for 4–6 h at 4°C. After incubation, the beads were removed by centrifugation. Supernatants were then collected, filtered (0.22 μm), and stored at 4°C. Cytotoxicity and antibody-mediated neutralization of the untreated (for determinations on TcdB) or immunodepleted (for determinations on TcdA) supernatants were measured as described above.

3. Results

3.1. Overview of mammalian cell intoxication by TcdA and TcdB

TcdA and TcdB are large, monomeric proteins (300 and 270 kDa, respectively) with similar structures and functions (**Figure 1**) [17, 18]. The functional domains of the toxins are arranged according to the ABCD model [17]: the N-terminal A domain contains the glucosyltransferase enzymatic activity, the B domain is a putative receptor-binding domain composed of a series of long and short repeats known as combined repetitive oligopeptides (CROPs), the cysteine protease (C) domain is responsible for autocatalytic processing, and the D domain is involved in pore formation and toxin translocation. Both toxins bind to receptors on the surface of the epithelial cells that line the wall of the lower intestine (and possibly other cell types). Once bound, they are internalized via receptor-mediated endocytosis [19]. Acidification of the endosome promotes a conformational change that enables translocation of the N-terminal glucosyltransferase domain of the toxin into the cytoplasm. Cellular inositol hexakisphosphate (InsP6) allows cleavage of the toxin by the cysteine protease domain, releasing the glucosyltransferase domain into the cytoplasm where it inactivates Rho-type GTPases through covalent glucosylation (from UDP-glucose) [20]. This in turn causes changes in epithelial cell morphology due to actin depolymerization, loss of tight junction integrity, and eventually, cell death (**Figure 1**) [21]. The assays described in this chapter measure many of the various steps, described above, involved in the intoxication cascade (steps 1–5, as denoted in **Figure 1**).

3.2. Cell surface binding of TcdA and TcdB (step 1 in Figure 1)

Binding of toxins to the cell surface of target cells is the first step in TcdA and TcdB cell entry, leading to the downstream effects of the toxins. We assessed cell surface binding of TcdA and TcdB by Western blotting of cell membranes isolated from Vero cells incubated

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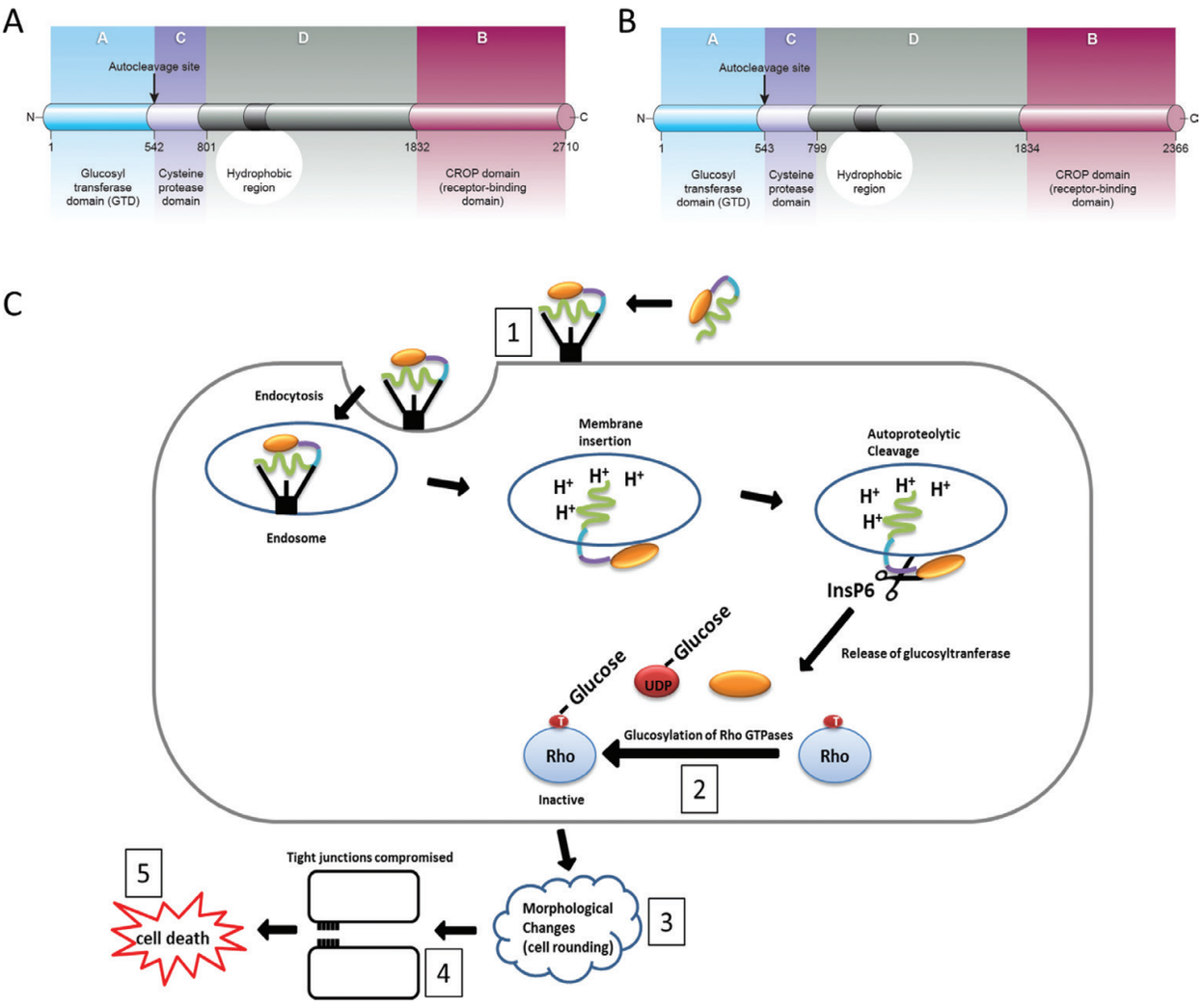

Figure 1. *Clostridium difficile* toxin structure and mechanism of action. (A) Domain organization of TcdA. (B) Domain organization of TcdB. (C) Mechanism of intoxication of mammalian cells by TcdA and TcdB. Toxins A and B bind to receptors on the surface of target cells (1) and are endocytosed. Endosomal toxins are acidified causing exposure of hydrophobic regions of the protein that allow their insertion into the membrane, forming pore(s). The N-terminal catalytic domain is then translocated from the endosomal compartment into the cytoplasm, where the glucosyltransferase domain is released by inositol hexakisphosphate (InsP6)-dependent auto-cleavage. The toxins then glucosylate Rho-type GTPases (2) from UDP-glucose, causing actin depolymerization, changes in cell morphology (3), disruption of tight junctions (4), and cell death (5). Cellular events numbered 1–5 correspond to the steps assessed by the various assays described in this chapter. Figure adapted from Jank and Aktories [17].

with TcdA or TcdB at 4°C. As shown in **Figure 2**, membrane fractions isolated from cells incubated with TcdA (see **Figure 2A**, top panel) or TcdB (**Figure 2B**, top panel) contain toxins, indicating cell surface binding of the toxins. Actoxumab and bezlotoxumab bind to and neutralize purified TcdA and TcdB, respectively, from a variety of *C. difficile* strains [15]. Pre-incubation of TcdA with actoxumab but not bezlotoxumab efficiently blocked binding of TcdA to cells (**Figure 2A**), while pre-incubation of TcdB with bezlotoxumab but not actoxumab efficiently blocked binding of TcdB to cells (**Figure 2B**), confirming the specificity of toxins binding to cells.

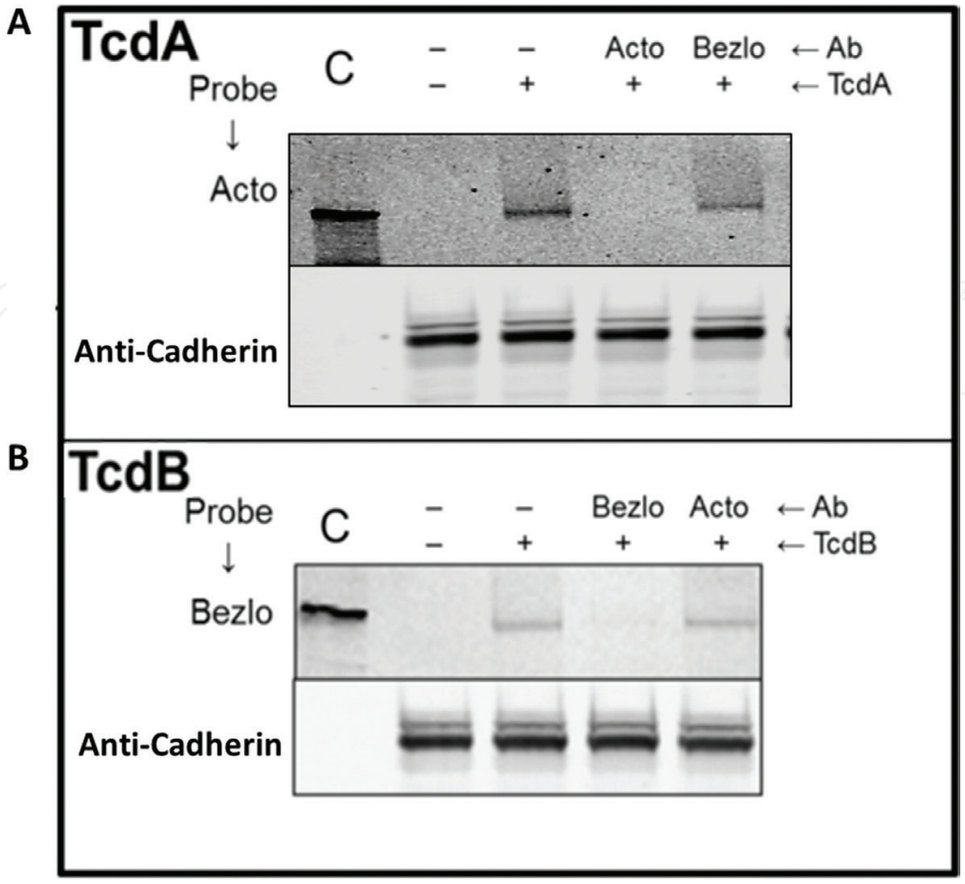

Figure 2. Cell surface binding of TcdA and TcdB as measured by Western blot. Western blots of cell membranes isolated from Vero cells following incubation with (A) TcdA or (B) TcdB, in the presence of vehicle, actoxumab, or bezlotoxumab (200 μg/ml), as indicated. The top blots in each panel show TcdA and TcdB, while the bottom blots show cadherin, used as a loading control.

Binding of TcdA to cells and the prevention, thereof, by actoxumab were also assessed by flow cytometry (**Figure 3**). Incubation of HT29 cells with increasing levels of fluorescently labeled TcdA (TcdA-Atto488) led to an elevated mean fluorescence intensity (MFI), indicating binding of TcdA to the cell surface in a concentration-dependent manner. In the presence of actoxumab, however, the MFI for each toxin concentration was reduced to background levels showing that actoxumab blocked binding of TcdA to the cell surface. No significant changes in MFI were measured in the presence of bezlotoxumab, indicating that the effect of actoxumab is specific (data not shown).

3.3. Glucosylation of Rac1 by TcdA and TcdB (step 2 in Figure 1)

Inactivation of Rho-type GTPases is a key step in the intoxication of host cells, leading to the downstream cytopathic and cytotoxic effects of the *C. difficile* toxins. Historically, the glucosylation of Rho GTPases was assessed by polyacrylamide gel-based assays that use either radioactively labeled glucose or antibodies to detect the glucosylated and non-glucosylated protein on a gel [22]. These assays are laborious, low throughput, qualitative, and do not detect glucosylation directly in the cell. A novel assay was therefore developed to measure

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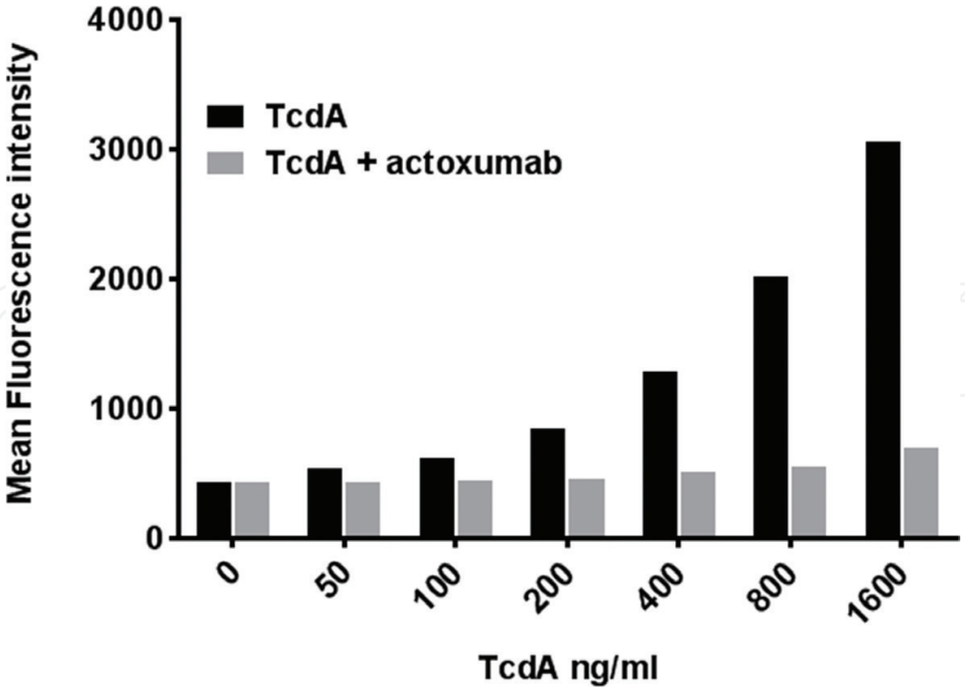

Figure 3. Cell surface binding of TcdA as measured by flow cytometry. A representative experiment showing flow cytometry analysis of HT29 cells pre-incubated with a titration of TcdA-Atto488 in the presence or absence of actoxumab. Following incubation, mean fluorescence intensity (MFI) was measured with excitation and emission wavelengths of 488 and 530 nm, respectively.

TcdA- and TcdB-mediated Rac1 glucosylation in a high throughput and quantitative 384-well in-cell Western assay, using antibodies that detect non-glucosylated and total Rac1. A dose-dependent decrease in non-glucosylated Rac1 was observed in the presence of TcdA and TcdB from various ribotypes (027, 078, and the control 087 (strain VPI 10463)) (**Figure 4A** and **B**), while total Rac1 was minimally affected (not shown). Vero cells were found to be more sensitive to TcdB than TcdA, consistent with previous observation by Torres et al. [23]. In addition, differences in sensitivity of Vero cells to toxins of the different *C. difficile* ribotypes were noted. For instance, Vero cells were found to be more sensitive to TcdA of ribotype 087 (VPI 10463) than of ribotypes 027 and 078, while TcdB showed the opposite effect, with cells being more sensitive to TcdB of ribotypes 027 and 078 compared to ribotype 087.

Actoxumab and bezlotoxumab neutralized the effects of TcdA and TcdB (at EC_{90} concentrations), respectively (**Figure 4C** and **D**). Notably, the potency of actoxumab and bezlotoxumab on their respective toxins was lower for toxins of ribotype 027 and 078 compared to ribotype 087. This is consistent with the lower affinities of the antibodies against toxins of these ribotypes, as previously described by Hernandez et al. [15].

3.4. Changes in cell morphology induced by TcdA and TcdB (step 3 in Figure 1)

The cytopathic effects of TcdA and TcdB on gut epithelium are visualized as profound morphological changes, typically cell rounding, due to the glucosylation and inactivation of Rho-type GTPases and subsequent disruption of actin polymerization. Historically, these cytopathic

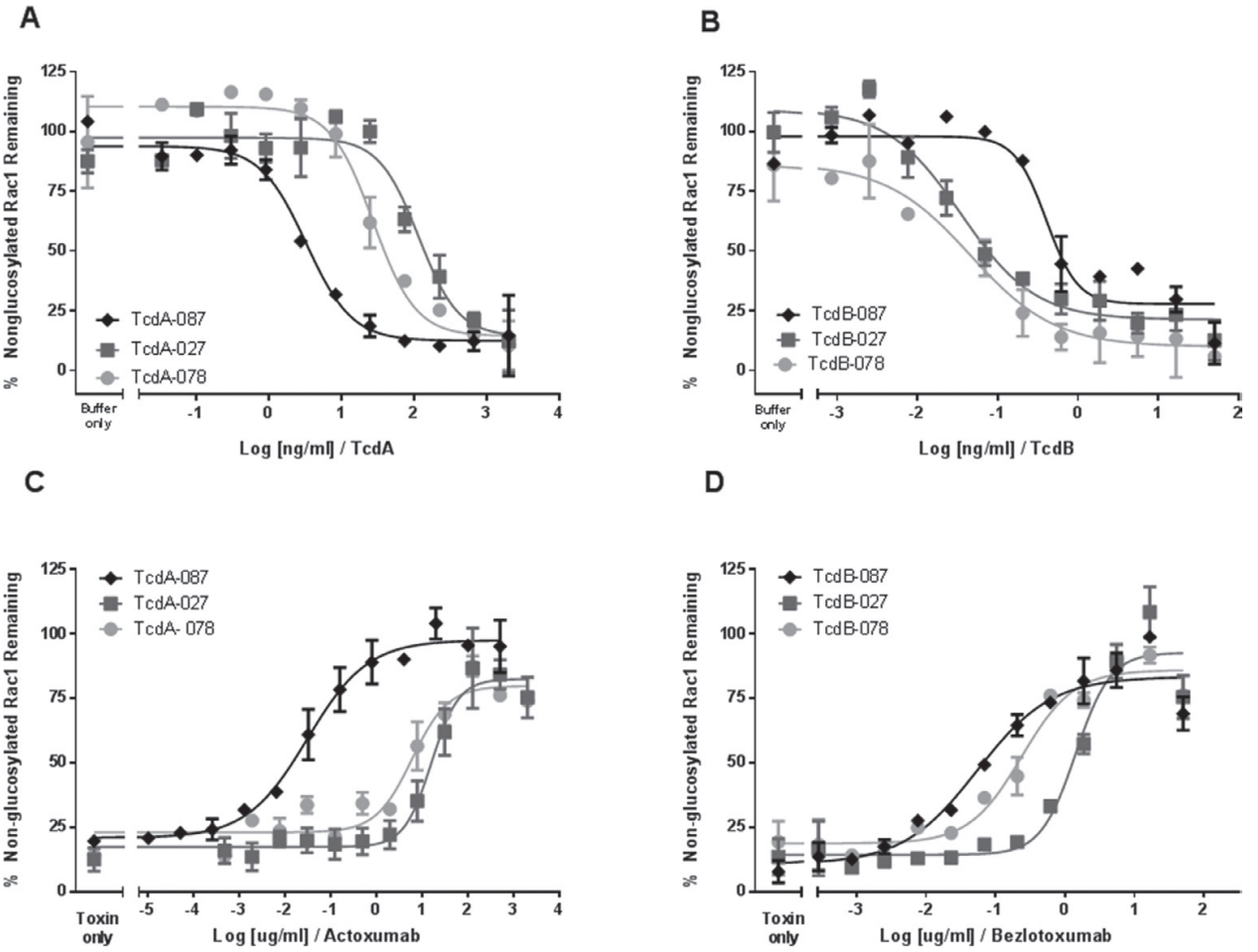

Figure 4. TcdA- and TcdB-mediated Rac1 glucosylation and neutralization thereof by actoxumab and bezlotoxumab. Effect of TcdA (A) and TcdB (B) of ribotypes 027, 078, and 087 on glucosylation of Rac1. Neutralization of TcdA-mediated Rac1 glucosylation by actoxumab (C) and of TcdB-mediated Rac1 glucosylation by bezlotoxumab (D).

effects have been assessed qualitatively through visual determination of cell rounding [23]. Improved phenotypic assays used to investigate changes in cell morphology involve the quantification of length-to-width ratios of fluorescently labeled cells [23, 24]. This latter technique is quantitative and has an improved throughput, although it requires consistent staining and substantial data analysis. To better understand and quantify toxin-induced morphological changes in unlabeled cells, an assay was developed to examine dynamic mass distribution (DMR) in Vero cells using the Epic instrument. In this assay, plates containing optical sensors are used to capture translocation of cellular mass of unlabeled cells in response to ligand binding, allowing changes in cell shape to be quantified. The concentration-dependent effects of TcdA and TcdB on mass redistribution were determined at 180 min (at which time the effects have plateaued, not shown) (**Figure 5A**). As with the Rac1 glucosylation assay, Vero cells are much more sensitive to TcdB than TcdA in the DMR assay. The neutralizing effects of actoxumab and bezlotoxumab on toxin-induced morphological changes were assessed at EC_{90} concentrations of TcdA and TcdB, respectively. Actoxumab and bezlotoxumab fully neutralized the effects of TcdA and TcdB, respectively, on DMR (**Figure 5B**).

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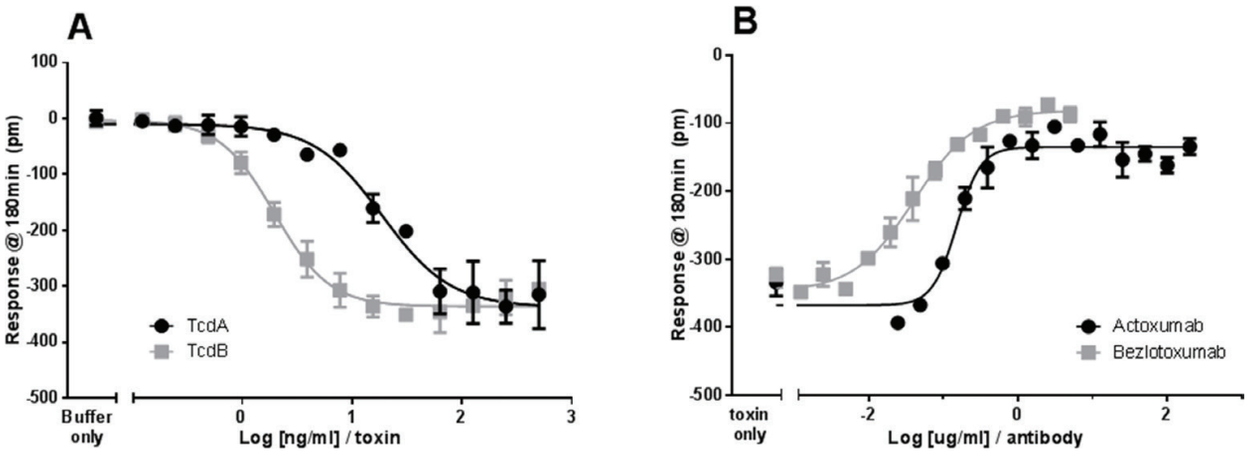

Figure 5. Effects of TcdA and TcdB on dynamic mass redistribution and neutralization by actoxumab and bezlotoxumab. (A) Concentration-dependent effects of TcdA and TcdB on DMR. (B) Neutralizing effects of actoxumab and bezlotoxumab on toxin-induced effects on DMR.

3.5. Toxin-induced disruption of epithelial tight junctions (step 4 in Figure 1)

To gain an understanding of the effect of *C. difficile* toxins on the integrity of the gut wall epithelium, a two-dimensional cell culture system was utilized wherein a single monolayer of colonic epithelial cells (Caco-2) is grown on a permeable membrane, separating distinct apical and basolateral compartments [25–28]. The system simulates the polarized nature of the intact intestinal mucosal epithelium, which separates the gut lumen (apical side) from the subepithelial/systemic space (basolateral side). The integrity of the epithelial layer is monitored by measuring the transepithelial electrical resistance (TER), with a decrease in TER suggesting that the integrity of the epithelial monolayer has been compromised [26]. In this system, TcdA and TcdB added to the apical side of the cell monolayer (mimicking the presence of toxin on the lumenal side of the gut) caused significant time- and concentration-dependent decreases in TER (**Figure 6A** and **B**). Neutralization of the toxin-induced effects by actoxumab and bezlotoxumab was assessed at EC_{90} concentrations of TcdA and TcdB, respectively. Both antibodies dose-dependently neutralized the effects of their respective toxins (**Figure 6C** and **D**).

3.6. Toxin-induced cytotoxicity (step 5 in Figure 1)

The traditional way of assessing the cytoxic effects of *C. difficile* on host cells involves measuring cellular ATP levels of intoxicated cells. This method is plagued with low signal to noise ratios and variability due to substantial ATP levels remaining in cells that are not yet dead and still undergoing morphological changes due to intoxication [13]. Additionally, normal metabolism-related fluctuations in ATP levels that are unrelated to cell viability can further affect the assay readout. We developed a more robust colorimetric assay that measures cellular protein content as a surrogate of cell growth and survival [14]. The sulforhodamine B (SRB) assay was used to determine the cytotoxic effects of purified *C. difficile* toxins of the reference strain VPI 10463 (ribotype 087) and from strains of ribotypes from the so-called hyper-virulent ribotypes 027 and 078. All toxins tested caused a robust concentration-dependent decrease in cell viability

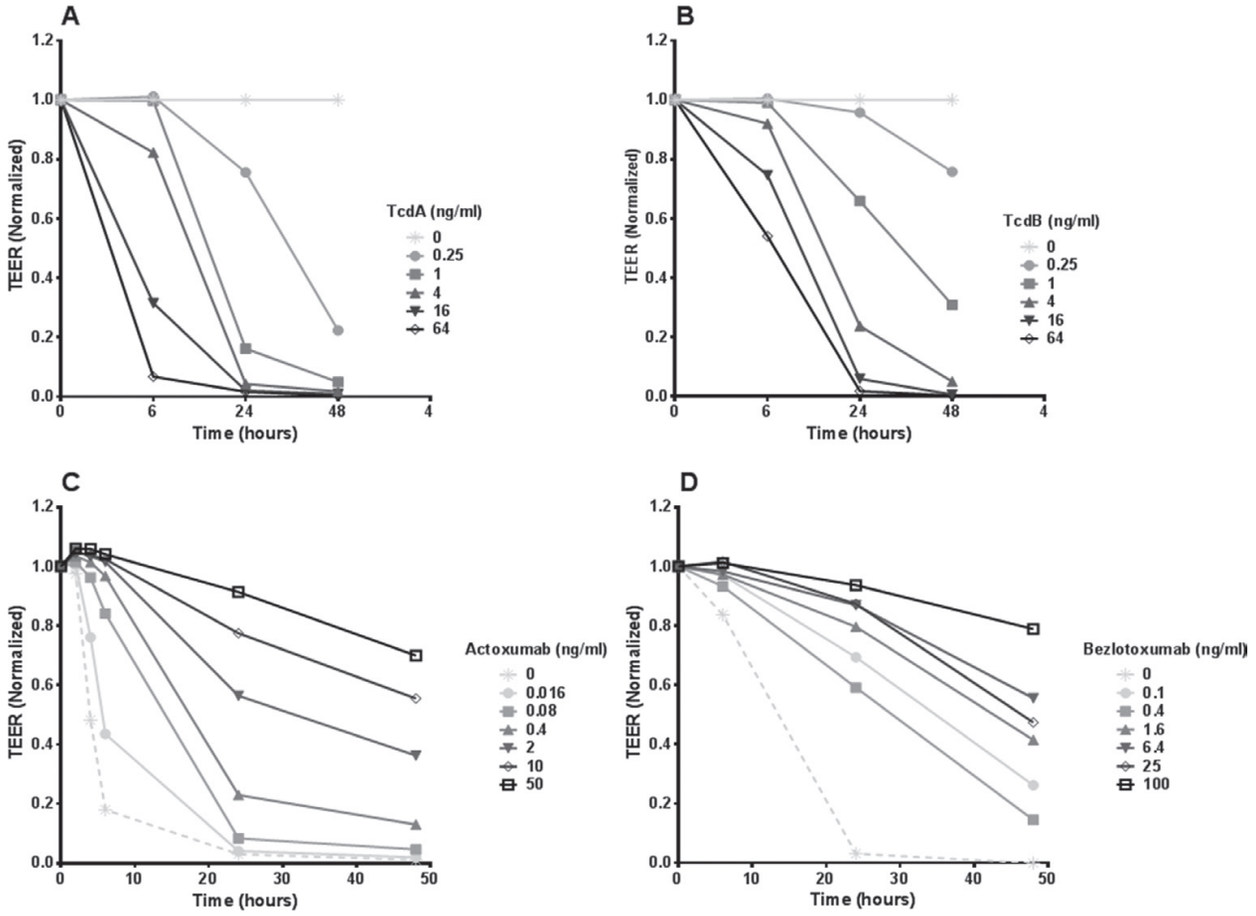

Figure 6. Effects of TcdA and TcdB on integrity of Caco-2 cell monolayers and neutralization by actoxumab and bezlotoxumab. Time- and concentration-dependent effects on TER of TcdA (A) or TcdB (B) added to the apical side of Caco-2 monolayers. Time- and dose-dependent neutralization of TcdA by actoxumab (C) and of TcdB by bezlotoxumab (D), added to the apical side.

(**Figure 7A** and **B**). As with other assays described herein and as previously observed by Torres et al. [23], Vero cells are significantly more sensitive to TcdB than to TcdA. The ability of actoxumab and bezlotoxumab to neutralize TcdA and TcdB, respectively, was assessed at toxin concentrations that are associated with a 90% decrease in cell viability (LC_{90}). Both antibodies fully neutralized the effects of their respective toxins from all ribotypes tested (**Figure 7C** and **D**). However, the neutralization potencies of both antibodies for toxins of ribotypes 027 and 078 were significantly lower than toxins of ribotype 087, similar to data obtained in the Rac1 glucosylation assay above (Section 2.3) and consistent with previous data in the SRB assay [15].

The robust nature of the SRB assay also allows for the study of the cytotoxic effects of unpurified *C. difficile* toxins directly from culture supernatants for clinical strains for which purified toxins are not available. For these studies, Vero cells were treated with serially diluted culture supernatants of the reference strain VPI 10463, containing both toxins (not shown), in the absence or presence of actoxumab, bezlotoxumab, or the combination of both antibodies. In the absence of antibodies, there was a concentration-dependent decrease in cell viability, presumably due to the presence of toxin in the supernatant. Addition of actoxumab had no effect on the cytotoxicity of supernatant, while addition of 10 μg/ml bezlotoxumab either

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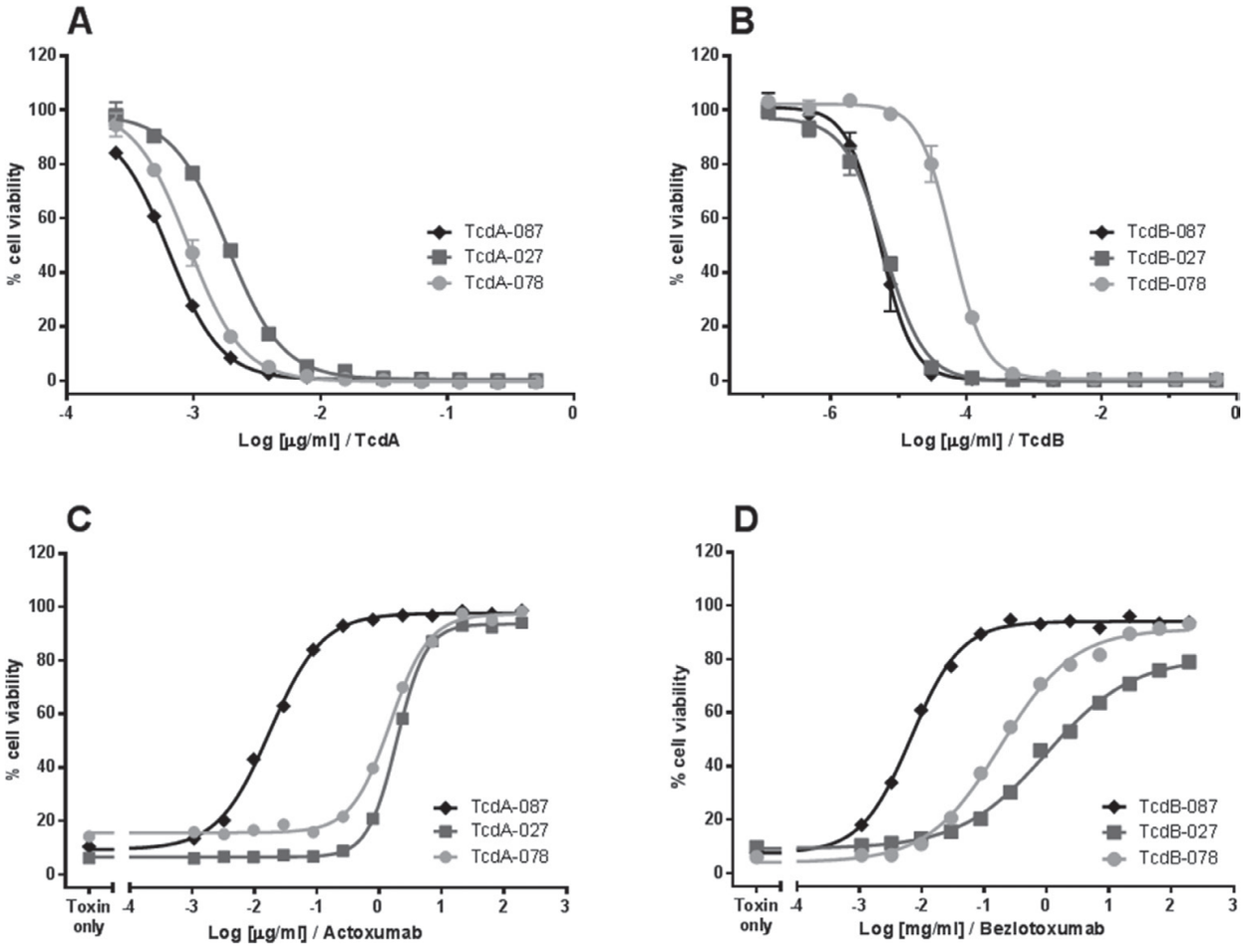

Figure 7. Purified TcdA- and TcdB-mediated effects on cell viability and neutralization by actoxumab and bezlotoxumab. Reduction in Vero cell viability induced by TcdA (A) and TcdB (B) using purified toxins from ribotypes 087, 027, and 078. Neutralization of TcdA by actoxumab (C) and of TcdB by bezlotoxumab (D). Figure reproduced from Hernandez et al. [15] (Copyright © American Society for Microbiology [Antimicrob Agents Chemother. 59, 2015, 1052–1060. DOI:10.1128/AAC.04433-14]).

by itself or in combination with 10 μg/ml actoxumab significantly shifted the concentration-response curve to the right, indicating that most of the cytotoxic activity in the supernatant is due to TcdB (**Figure 8A**). This is not surprising as Vero cells are more sensitive to TcdB than to TcdA. To assess the cytotoxic activity associated with TcdA, TcdB was first removed from the supernatant using an immunodepletion approach (see Section 2). In this case, 10 μg/ml actoxumab, alone or in combination with 10 μg/ml bezlotoxumab, shifted the response curve to the right, whereas bezlotoxumab showed minimal effect, confirming that the cytotoxic activity in immunodepleted supernatants is associated mainly with TcdA (**Figure 8B**). To confirm this finding, full concentration-response curves of actoxumab and bezlotoxumab were generated against dilutions of intact or immunodepleted supernatants associated with ~90% reduction in cell viability (EC_{90}); actoxumab neutralized the cytotoxic activity of immunodepleted supernatants, whereas bezlotoxumab neutralized the cytotoxic activity of intact supernatants, and no cross-neutralization was observed (**Figure 8C** and **D**). This approach has been used successfully to assess the activities of actoxumab and bezlotoxumab on TcdA and TcdB of dozens of clinical isolates of *C. difficile*, covering 18 distinct ribotypes (seven toxinotypes) [15].

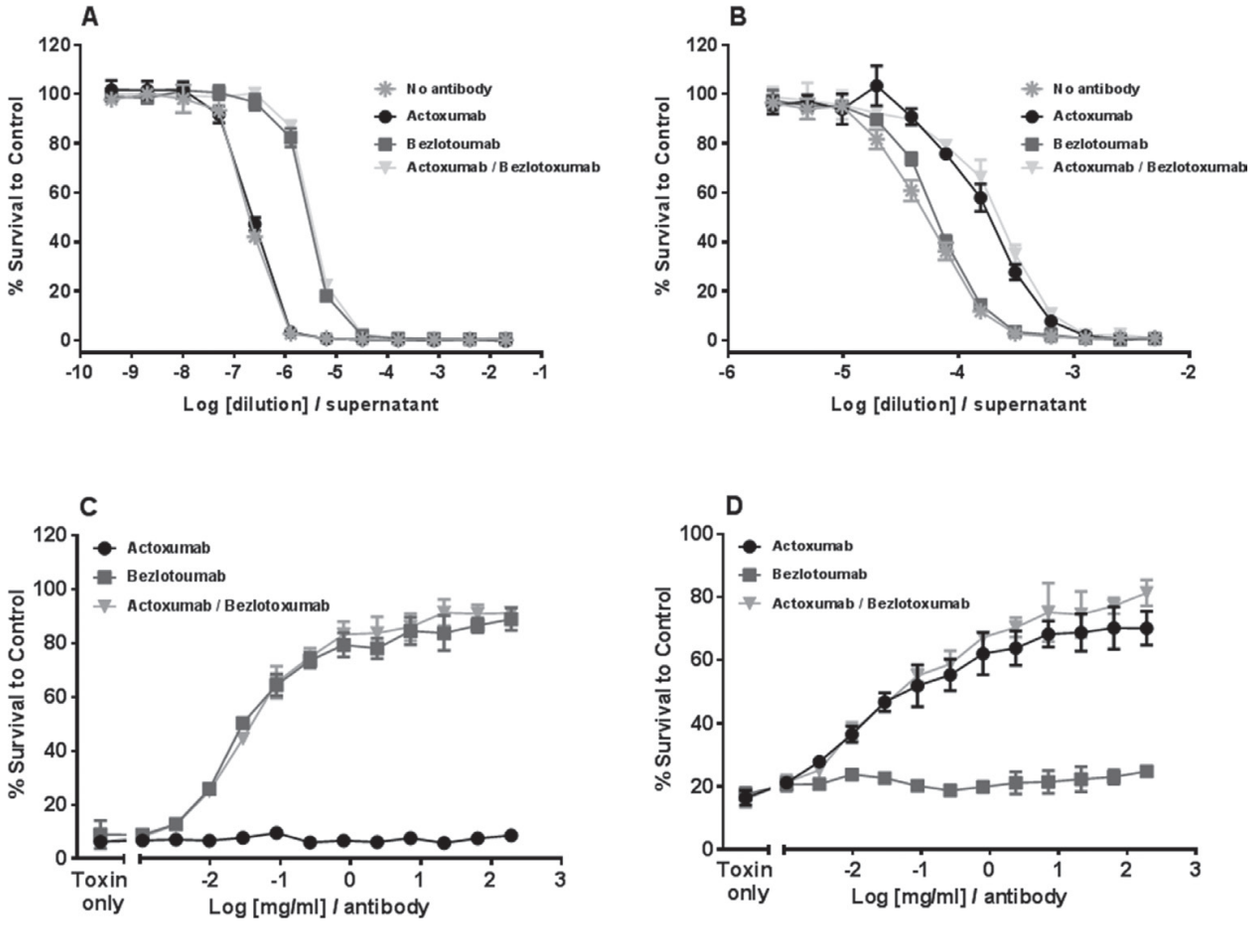

Figure 8. Unpurified TcdA- and TcdB-mediated effects on Vero cell viability and neutralization by actoxumab and bezlotoxumab. Cytotoxic effects of serially diluted intact (A) or immunodepleted (B) supernatants in the presence or absence of actoxumab, bezlotoxumab, or a combination of the two antibodies. Neutralization of cytotoxic activity by bezlotoxumab, but not actoxumab, in intact supernatant at EC_{90} dilution (C) and by actoxumab but not bezlotoxumab in immunodepleted supernatant at EC_{90} dilution (D).

4. Conclusions

In this chapter, we have described novel cell-based assays for analyzing multiple distinct steps in the intoxication cascade associated with TcdA and TcdB. Unlike historical assays that measure toxin effects qualitatively, such as the visual assessment of cell rounding, or are variable and often unreliable, such as quantitation of ATP levels to estimate cell death, the assays presented here can quantitatively and robustly assess the effects of toxins in mammalian cells. We show how the initial event of toxin binding to host cells can be assessed using cell surface binding assays with labeled or unlabeled toxins in flow cytometry and Western blot formats, respectively. The more proximal events that follow internalization of the toxins, namely Rac1 glucosylation and cell rounding, can be studied with novel quantitative assays by in-cell Western and dynamic mass redistribution assays, respectively. Finally, we show how the TER and SRB assays can be utilized to assess the final stages of intoxication, tight junction disruption, and cell death, respectively. We also show how the SRB assay can be used to accurately measure the activities of TcdA and TcdB from unpurified toxins in culture supernatants of

C. difficile strains for which purified toxins are not available. The assays described were validated with the antitoxin antibodies actoxumab (anti-TcdA) and bezlotoxumab (anti-TcdB) to demonstrate their utility in evaluating pharmacological blockade of toxins. These assays may be useful in future studies aimed at better understanding of *C. difficile* toxin function, as well as in characterizing toxin inhibitors as tools or as potential therapeutics.

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