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Development and Optimization of a High-Throughput Assay To Measure Neutralizing Antibodies against *Clostridium difficile* Binary Toxin

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***Clostridium difficile* strains producing binary toxin, in addition to toxin A (TcdA) and toxin B (TcdB), have been associated with more severe disease and increased recurrence of *C. difficile* infection in recent outbreaks. Binary toxin comprises two subunits (CDTa and CDTb) and catalyzes the ADP-ribosylation of globular actin (G-actin), which leads to the depolymerization of filamentous actin (F-actin) filaments. A robust assay is highly desirable for detecting the cytotoxic effect of the toxin and the presence of neutralizing antibodies in animal and human sera to evaluate vaccine efficacy. We describe here the optimization, using design-of-experiment (DOE) methodology, of a high-throughput assay to measure the toxin potency and neutralizing antibodies (NAb) against binary toxin. Vero cells were chosen from a panel of cells screened for sensitivity and specificity. We have successfully optimized the CDTa-to-CDTb molar ratio, toxin concentration, cell-seeding density, and sera-toxin preincubation time in the NAb assay using DOE methodology. This assay is robust, produces linear results across serial dilutions of hyperimmune serum, and can be used to quantify neutralizing antibodies in sera from hamsters and monkeys immunized with *C. difficile* binary toxin-containing vaccines. The assay will be useful for *C. difficile* diagnosis, for epidemiology studies, and for selecting and optimizing vaccine candidates.**

Clostridium difficile is a Gram-positive bacterium and the primary cause of hospital-acquired diarrhea. *C. difficile* infection (CDI) occurs when antibiotic treatment disrupts the normal bacterial flora of the intestine, which allows for the colonization of *C. difficile* bacteria. CDI is characterized by symptoms that range from mild diarrhea to severe and often life-threatening colitis. The incidence of more severe CDI has been increasing in recent years due to the emergence of epidemic hypervirulent strains (1, 2).

Two glucosylating toxins, TcdA and TcdB, are considered the main virulence factors of *C. difficile*. These two toxins catalyze the glucosylation of proteins RhoA, Rac, and Cdc42, which leads to the disruption of actin filaments and collapse of the cell cytoskeleton, disruption of other signaling pathways, and eventually, cell death (3–5). In addition to TcdA and TcdB, hypervirulent strains have been found to express a binary toxin referred to as *C. difficile* transferase (CDT). Much of what we know of CDT was learned through a comparison to related binary toxins found in other clostridial species, such as *Clostridium botulinum* C2 toxin and *Clostridium perfringens* iota toxin (6–8). Binary toxins are characterized as having an enzymatically active "A" component that causes ADP-ribosylation of globular actin (G-actin) and a cell binding and translocation "B" component. For *C. difficile*, CDTa and CDTb are transcribed monocistronically but translated as two separate proteins. The proposed mechanism of action for the *C. difficile* binary toxin has been described in the literature (7). Briefly, the precursor CDTb is cleaved by trypsin, and the subsequently activated CDTb protein binds to the cell surface receptor as either a preformed heptamer or as monomers that subsequently oligomerize to form heptamers. CDTa binds to the CDTb heptamer on the cell surface. The receptor-binary-toxin complex is then internalized via receptor-mediated endocytosis into the endosomes. In the acidic environment in the endosome, the CDTb heptamer then forms a pore, which allows the CDTa enzymatic component to enter the cell. The CDTa component catalyzes the ADP-ribosylation of G-actin at Arg_{177} and prevents actin polymerization, causing disruption of the cytoskeleton and fluid loss, rounding of the cells, and eventually, cell death (8, 9). The cell surface receptor for *C. difficile* binary toxin has been identified as lipolysis-stimulated lipoprotein receptor (LSR), which is also the receptor for two other binary toxins, *C. perfringens* iota toxin and *Clostridium spiroforme* toxin (CST) (10).

Although the exact role of binary toxin in CDI is not clear, there is increasing evidence of a correlation between CDI severity and *C. difficile* strains expressing binary toxin. In studies using a binary toxin-containing TcdA-negative TcdB-negative strain, researchers have shown that binary toxin alone can cause fluid accumulation in rabbit ileal loop assays and that this strain can col-

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TABLE 1 Description of recombinant binary toxin components used in this study

Toxin name	Expression system	Mutation(s)	Expression tag	Use in this study
CDTa	*E. coli*	None	His tag at C terminus	NAb assay, hamster immunization
1mCDTa	*E. coli*	C_2A	His tag at C terminus	NAb assay
ProCDTb	*E. coli*	None	GST tag at N terminus[a]	NAb assay
CDTb	*E. coli*	None	GST tag at N terminus[b]	Hamster immunization
3mCDTa	Baculovirus	S_{345}F, E_{385}Q, E_{387}Q	No	Monkey immunization
ProCDTb	Baculovirus	None	No	Monkey immunization

[a] GST_Pro domain was removed by chymotrypsin activation before use in the NAb assay. The activated protein is referred to as a_CDTb.
[b] GST domain was removed during the protein purification process.

onize hamsters; however, binary toxin alone is not sufficient to cause disease (11). However, it is postulated that binary toxin enhances the pathogenesis of *C. difficile* by inducing the redistribution and protrusion of microtubules at intestinal epithelial cell surfaces, thereby increasing bacterial adherence and colonization (12). This increase in pathogenesis is supported by epidemiological studies showing higher fatality rates in patients infected with binary toxin-containing strains versus patients infected with strains that do not produce binary toxin (13, 14). Binary toxin has also been found to be associated with hypervirulent strains of *C. difficile* that cause increased CDI severity, higher death rates, longer hospital stays, and increased recurrence of CDI (1, 2, 15). However, epidemiological studies also have been performed that suggest that binary toxin is not a significant predictor of severe CDI (16, 17).

The typical treatment regimen for CDI is discontinuation of the antibiotic treatment, which alters the microbiota of the gut, followed by treatment with a narrow-spectrum antibiotic that specifically targets the *C. difficile* bacterium (18, 19). More recently, monoclonal antibodies to *C. difficile* toxins A and B have been shown to prevent CDI onset and reduce the rate of recurrence of disease (20). In addition, immunization with TcdA and TcdB toxins that have been either inactivated recombinantly or by chemical methods have shown efficacy in preventing disease (21, 22). The efficacy of these vaccines is presumably due to the production of antibodies that can neutralize the activities of these toxins. We have developed a vaccine containing binary toxin in addition to TcdA and TcdB, which may increase the protective efficacy of this vaccine for hypervirulent strains expressing binary toxin (23). Vaccine development requires a functional assay that can measure neutralizing antibody responses against binary toxin in animal models and clinical trials. We describe here the development and optimization of an assay to measure the cytotoxicity of *C. difficile* binary toxin and an assay to test the ability of serum to neutralize this toxin.

MATERIALS AND METHODS

Binary toxin constructs (*C. difficile* NAP1 strain [GenBank accession no. R20291] sequences) CDTa, 1mCDTa (C_2A), glutathione *S*-transferase (GST)_ProCDTb, and CDTb were expressed in *Escherichia coli* (Table 1). CDTa, 1mCDTa, and GST_ProCDTb were used as reagents in the binary toxin functional assay. The binary toxin constructs 3mCDTa (S_{345}F, E_{385}Q, E_{387}Q) and ProCDTb were expressed in insect cell expression systems to improve the yield and reduce the endotoxin level for vaccine products. The three mutations in 3mCDTa were introduced to reduce the enzymatic activity of binary toxin (24). We optimized the binary toxin cytotoxicity assay first. Based on the result, we then optimized the neutralization antibody (NAb) assay using control serum samples pooled from monkeys immunized with insect cell-expressed binary toxin. Furthermore, we demonstrated that the optimized NAb assay can be used to measure the neutralizing antibody response in the serum samples of hamsters immunized with *E. coli*-expressed binary toxins. The methods of expression, purification, and SDS-PAGE analysis of these toxins can be found in the supplemental material.

The antigens 5mTcdA (W_{101}A, D_{287}A, E_{514}Q, W_{519}A, C_{700}A) and 5mTcdB (W_{102}A, D_{288}A, E_{515}Q, W_{520}A, C_{698}A) used in vaccine preparation were also expressed in insect cells. The details of expression and purification can be found in International Patent Application publication no. WO2013112867 (23).

Rhesus macaque immunization. The antigens 5mTcdA, 5mTcdB, 3mCDTa, and ProCDTb were used in the vaccine. The 5mTcdB antigen was treated with formaldehyde, followed by dialysis in 50 mM HEPES buffer (pH 7.0, 100 mM NaCl) and sterile filtration (0.22 μm). Vaccine formulation then involved mixing aluminum adjuvant, antigens, and Iscomatrix adjuvant (CSL Biotherapies, Inc., King of Prussia, PA). Adult rhesus macaques (4 to 10 years of age) were housed at the New Iberia Research Center (NIRC) of the University of Louisiana at Lafayette. They were immunized intramuscularly with 0.5 ml of the vaccine preparations containing 20 μg of 5mTcdA, 20 μg of 5mTcdB, 5 μg of 3mCDTa, and 5 μg of ProCDTb in the deltoid muscle on days 0, 7, and 30. Blood samples were collected before each immunization and on day 45. The animals were observed daily for any abnormal clinical signs of illness or distress. Twenty milliliters of hyperimmune serum collected on day 45 from two monkeys was pooled and used as a reagent for assay development. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at both Merck & Co., Inc. and the NIRC, and the study was carried out at the NIRC.

Golden Syrian hamster immunization. The antigens 5mTcdA, 5mTcdB, CDTa, and CDTb were used in the vaccine. All four antigens were treated with formaldehyde. Excess formaldehyde was removed by dialysis in 50 mM HEPES buffer (pH 7.0, 100 mM NaCl). The antigens were then mixed with aluminum adjuvant and Iscomatrix adjuvant to produce the vaccines listed in Table 2. Golden Syrian hamsters (male, 90 to 120 g) were obtained from Charles River Laboratories and individually housed in filter-lid cages at Merck's West Point site. The hamsters in groups 1 to 4 were immunized intramuscularly with 0.2 ml of vaccine, as described in Table 2. The hamsters in group 5 received adjuvant only. The hamsters were immunized on days 0, 21, 42, and 63, and approximately

TABLE 2 Compositions of vaccines used for hamster immunization

Group	Dose (μg/injection) for antigen[a]:			
	5mTcdA	5mTcdB	CDTa	CDTb
1	10	10	0	0
2	10	10	0.6	0
3	10	10	0	4.4
4	10	10	0.6	4.4

[a] 5mTcdA and 5mTcdB were immunized in the left leg; CDTa and CDTb were immunized in the right leg. The hamsters in group 5 (negative-control group) were immunized with adjuvant alone.

0.5 ml of blood was collected from each hamster via the retroorbital technique immediately prior to each immunization and on day 77. The serum samples were stored in a frozen state. Ten hamsters were used in each group. The hamster experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Merck and Co., Inc.

Activation of GST_ProCDTb. GST-ProCDTb was activated by digestion with immobilized chymotrypsin (Princeton Separations, Inc., Princeton, NJ) at a 10:1 protein-to-chymotrypsin mass ratio. The GST_ProCDTb-chymotrypsin mixture was incubated at 37°C with shaking (150 rpm) for 30 min. After incubation, the resin was removed by centrifugation for 2 min at 10,000 × *g* at room temperature, and the activated GST_ProCDTb (a_CDTb) was collected and stored at −70°C until use.

Binary toxin cytotoxicity titration assay. The assay was run over a period of three consecutive days and comprised five steps: (i) cell plating, (ii) serial dilution of toxin and cell intoxication, (iii) cell staining, (iv) data acquisition, and (v) data analysis. For step 1, 50 μl of a cell suspension of Vero cells at 30,000 cells/ml was seeded into 384-well plates and incubated overnight in a humidified incubator with 5% CO_2 at 37°C. Vero cells (ATCC CCL-81) were obtained from ATCC and cultured according to ATCC's instructions. For step 2, binary toxin mixtures consisting of 1mCDTa and a_CDTb were serially diluted 2-fold in complete culture medium, and 40 μl was then applied to cells grown in 384-well plates. The cells were incubated in a CO_2 incubator at 37°C for 20, 24, or 48 h. For steps 3 and 4, cell staining and data acquisition were performed according to the methods described previously (25). For step 5, the total cell surface area in each well was plotted against the toxin concentration in each dilution. The potency of a test toxin (50% toxic concentration [TC_{50}]) was determined as the toxin concentration that caused 50% cytotoxicity. The TC_{50} was calculated by linearly interpolating between the consecutive dilutions whose signals bracketed the midpoint signal. The midpoint signal was defined as half of the total cell surface area of the medium-only control wells. For ease of comparison, we describe the TC_{50} for binary toxin preparations in terms of CDTa concentrations only.

A design-of-experiment methodology was used to further optimize four critical assay parameters, including GST_ProCDTb chymotrypsin activation time, cell-seeding density, toxin intoxication time, and the ratio of CDTa to a_CDTb in the binary toxin. Five cell-seeding densities (500, 750, 1,000, 1,500, and 2,000 cells/well), three toxin intoxication times (20, 24, and 48 h), the GST_ProCDTb chymotrypsin activation time (20, 30, and 40 min), and the CDTa-to-a_CDTb ratio (1:4, 1:7, and 1:10) were evaluated in the optimization experiment based on results from screening experiments. Cell-seeding densities of 1,000, 1,500, and 2,000 cells/well were evaluated at the 20-h and 24-h toxin intoxication times (six combinations), and cell-seeding densities of 500, 750, and 1,000 cells/well were evaluated at the 48-h toxin intoxication time (three combinations). The toxin intoxication time was chosen so the cells were between 50% and 90% confluence at the end of the assay. The alternative combinations of cell-seeding density and toxin incubation time resulted in nine 384-well plates. The experiment was performed independently by two analysts across these nine plates. Within each plate, the nine different combinations of chymotrypsin activation times (three levels) and CDTa-to-CDTb ratio (three levels) were each tested in two independently prepared 16-point 2-fold dilution series beginning at the 1:2 dilution. The remaining 6 columns of each plate contained a medium-only control and 3 different toxin control arms: CDTa only, a_CDTb only, and CDTa plus GST_ProCDTb. The toxin control arms were each tested in a single 16-point 2-fold dilution series beginning at the 1:2 dilution.

Cell-based neutralization antibody assay. The NAb assay was run over a period of three consecutive days and comprised, with a few exceptions, the same five-step procedure used in the cytotoxicity titration assay described above. The exceptions in steps 2 and 5 were that in step 2, 1mCDTa and a_CDTb were combined at a 1:7 CDTa-to-CDTb molar ratio using either 4-, 8-, or 16-fold TC_{50} for 1mCDTa. The control and test sera were 2-fold serially diluted, followed by incubation with the binary toxin mixture for either 0.5, 1, or 2 h at 37°C. This incubation time is referred to as serum-toxin preincubation. Forty microliters of the serum-toxin mixture was then applied to cells grown in 384-well plates and incubated in humidified CO_2 incubators at 37°C for 22 to 24 h. The cells were fixed, permeabilized, and stained with Alexa Fluor 488 phalloidin, and an image of the monolayer was acquired using the ImageXpress Velos scanning cytometer (25). In step 5, the total cell surface area data were imported into a Microsoft Excel workbook to calculate the neutralization titer. The neutralization titer of a test sample (50% effective dose [ED_{50}]) was defined as the dilution at which cytotoxicity was decreased by 50%. The ED_{50} was calculated by linearly interpolating between the consecutive dilutions whose signals bracketed the midpoint signal. The midpoint signal was defined as the average of the total cell surface area of medium-only control wells ($n = 16$) and the total cell surface area of the toxin-only control wells ($n = 16$).

A design-of-experiment methodology was used to further optimize three critical assay parameters, including toxin concentration, cell-seeding density, and serum-toxin preincubation time. Three levels (low, medium, and high) were chosen for each factor based on preliminary data, cell-seeding density (1,000, 1,500, and 2,000 cells/well), preincubation time (0.5, 1, and 2 h) and toxin concentration (242, 484, and 968 pM). Throughout the testing, GST_ProCDTb chymotrypsin activation was performed for 30 min at 37°C, the molar ratio of CDTa to a_CDTb was 1:7, and the toxin incubation time was fixed at 22 h, based on the cytotoxicity DOE data. The Design-Expert version 7.1.3 software was used to generate a face-centered cube design consisting of 18 runs (one 384-well plate per run) performed in four blocks of four or five runs (see Table S1 in the supplemental material). An identical set of samples was tested on each of the 18 plates. The sample set included medium-only and toxin-only controls, a titration of the toxin, positive and negative hamster and human sera, low- and high-titer positive serum samples from vaccinated monkeys, and three samples created by spiking the high-positive monkey serum into a pool of negative human serum at 12.5%, 25%, and 50% spike levels. The monkey serum spiked into negative human serum was used to assess the matrix effect of human serum in the NAb assay. The toxin titration and the serum samples were each tested in a 16-point 2-fold titration series across the plate rows. The assay conditions were evaluated in terms of the signal-to-noise ratio (ratio of medium-only response to toxin-only response), the toxicity of the toxin (TC_{50}), the level and variability of the NAb titers (ED_{50}) for the human and monkey samples, and the linearity of the NAb titers across the range of monkey spikes tested.

Statistical analysis of the *C. difficile* binary toxin cytotoxicity or NAb assay optimization experiments. All analyses were performed on the natural log-transformed TC_{50} or ED_{50} data. Differences among the factor levels in the cytotoxicity assay DOE were assessed using an analysis of covariance (ANCOVA) model in which toxin incubation time and cell-seeding density were treated as categorical factors, and chymotrypsin activation time and the ratio of CDTa to a_CDTb were treated as continuous factors. The differences among the factor levels in the NAb assay DOE were assessed using a multifactor analysis of variance (ANOVA) model containing all main-effect and two-way interaction terms. None of the two-way interaction terms were significant at the 5% level, and therefore, the interaction terms were dropped from the final model. Statistical significance between the categorical factor levels was assessed based on the differences of the least-squares means. *P* values of <0.05 were considered significant. The precision of the NAb titer was assessed by variance component analysis (VCA) across the set of positive-test and -control samples. All analyses were performed using the MIXED procedure in SAS (version 9.3).

RESULTS

Purification and characterization of CDTa and ProCDTb for use in neutralizing antibody assays. We initially sought to express both components of binary toxin as fully functional proteins using recombinant production in *E. coli*. CDTa and 1mCDTa (49 kDa) were purified to a purity of >98% as estimated by densitom-

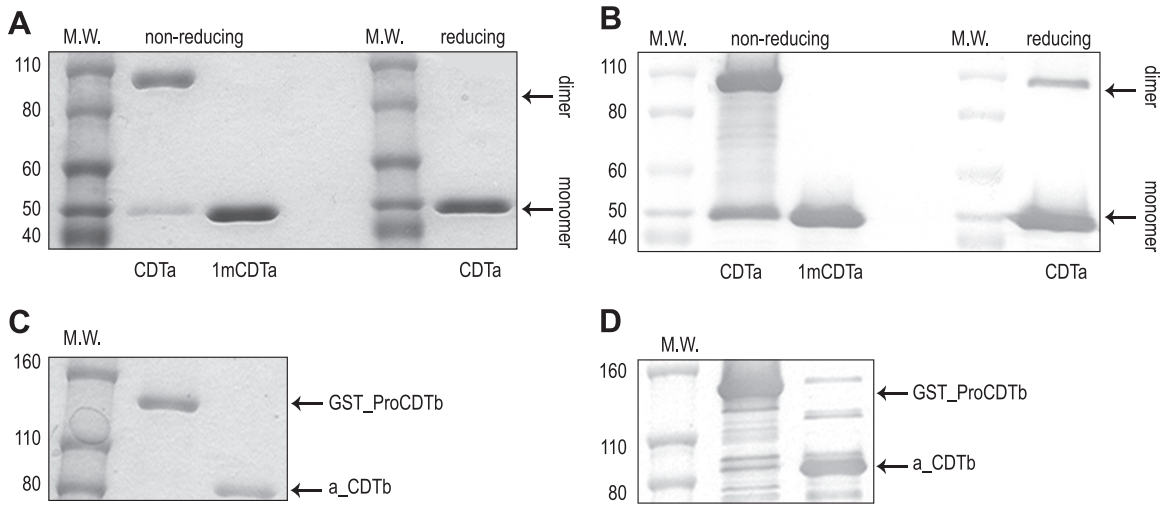

FIG 1 Identification and characterization of purified CDTa and GST_ProCDTb. (A) SDS-PAGE of CDTa and 1mCDTa. Proteins were stained with Coomassie blue. (B) Western blot of CDTa and 1mCDTa using anti-CDTa monoclonal antibody. (C) SDS-PAGE of GST_ProCDTb and a_CDTb. Proteins were stained with Coomassie blue. (D) Western blot of GST_ProCDTb and a_CDTb using anti-CDTb monoclonal antibody. Some degradation of GST_ProCDTb was seen in the Western blot but not in the Coomassie blue-stained gel. Two micrograms of each protein was loaded onto the SDS-PAGE gels. M.W., molecular weight, in thousands.

etry on SDS-PAGE gels. Although 80% of CDTa formed a dimer on nonreduced gels, the CDTa dimer was reduced to a monomer by adding dithiothreitol (DTT) to the SDS-PAGE loading buffer. This suggested that the dimerization was a result of disulfide bond formation, and in an attempt to alleviate this phenomenon, we engineered a mutation in the molecule that resulted in a change at position two of cysteine to alanine to yield 1mCDTa. This mutation completely eliminated the propensity of the molecule to dimerize. Both CDTa and 1mCDTa were recognized by an anti-CDTa monoclonal antibody, as shown by Western blot (Fig. 1A and B), confirming the identities of these two proteins.

To facilitate the solubility of ProCDTb, this protein was expressed in *E. coli* as a fusion with glutathione *S*-transferase (GST). This molecule, GST_ProCDTb (121 kDa), was purified to >95%, as estimated by densitometry on SDS-PAGE gels. The GST_Pro domain was removed by incubation with immobilized chymotrypsin for 30 min at 37°C (Fig. 1C), and the resulting protein is referred to as a_CDTb (94 kDa) to indicate that it is in an active form. Using electrospray ionization–time of flight (ESI-TOF) mass spectrometry, the activation site of GST_ProCDTb was identified to be the peptide bond between Met_{169} and Ser_{170}. Both the GST_ProCDTb and a_CDTb were recognized by an anti-CDTb monoclonal antibody (Fig. 1D), confirming their identities.

Measurement of binary toxin cytotoxicity. Next, we developed a method to evaluate the function of the binary toxin preparations in a Vero cell-based cytotoxicity assay. Vero cells were used in the cytotoxicity assay, as they have been shown to be sensitive to binary toxin (26). HT29, T84, Caco-2, HepG2, and IMR-90 cells were also compared to Vero cells to determine whether these cell lines were sensitive to binary toxin as well (see supplemental material). Vero cells were selected for use in the binary toxin NAb assay due to their favorable characteristics for high-throughput assays, including rapid cell growth and low variation between cell passages (data not shown).

CDTa and a_CDTb need to be combined in a proper molar ratio in order to have cytotoxic activity. Upon entry into the cell, CDTa ADP-ribosylates G-actin and depolymerizes the filamentous actin (F-actin) filament (24), and F-actin filaments can be visualized using Alexa 488 phalloidin staining (Fig. 2A). Vero cells that had been treated with binary toxin demonstrated dramatically lower levels of F-actin staining (Fig. 2B). Preincubation of binary toxin with hyperimmune monkey serum protected the cells from the cytotoxic effect of the toxin (Fig. 2C and D), suggesting that this toxicity was specific to binary toxin and was not a result of nonspecific killing. The changes in F-actin filament quantity can be determined in a rapid manner using an automated Velos scanning cytometer. The total cell surface area was inversely correlated with cytotoxicity and was used to calculate the TC_{50} values of the toxin molecules and the ED_{50} values of the immune sera.

Binary toxin cytotoxicity assay optimization. Several critical factors were screened in the cytotoxicity assay, such as the required incubation time of GST_ProCDTb with chymotrypsin to form a_CDTb, the equivalency of 1mCDTa and CDTa, the optimal 1mCDTa-to-a_CDTb molar ratio, and the cell-seeding density. GST_ProCDTb was activated by chymotrypsin for 10, 20, 30, 60, 120, 180, or 240 min, with a 10:1 protein-to-chymotrypsin mass ratio. SDS-PAGE analysis revealed that the GST_Pro domain was fully cleaved following incubation with chymotrypsin for ≥10 min (Fig. 3A). The TC_{50} of binary toxin was found to be similar when GST_ProCDTb was activated for 20, 30, or 60 min. However, the TC_{50} was slightly higher when GST_ProCDTb was activated for 10, 120, 180, or 240 min (<2-fold higher, Fig. 3B). This was further confirmed by the DOE data showing that altering the GST_ProCDTb activation time from 20 to 40 min had only a minimal effect on the observed TC_{50} values (data not shown).

Next, 1mCDTa was evaluated to determine whether it had activity similar to that of CDTa in the cytotoxicity assay. We found that the TC_{50} for 1mCDTa was only 1.2-fold higher than that obtained for CDTa, and therefore we used 1mCDTa in the assay to ameliorate aggregation problems.

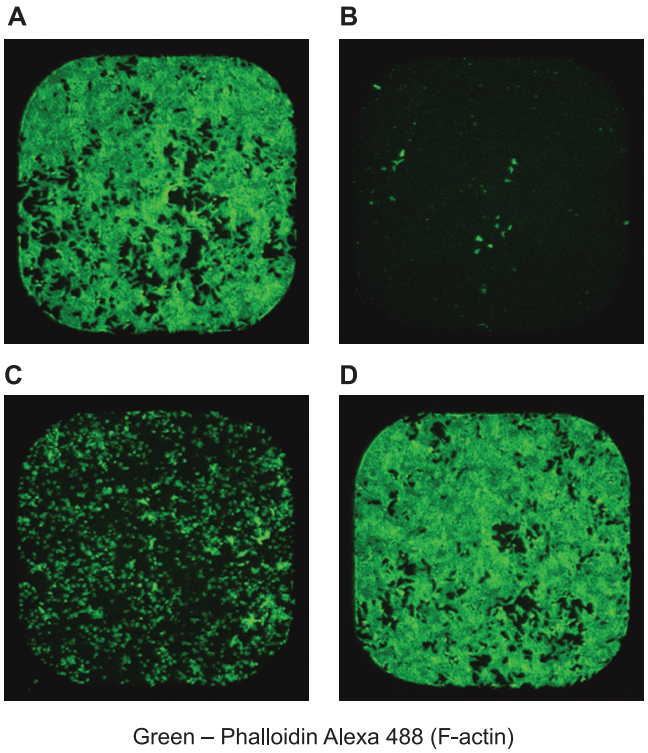

FIG 2 Measurement of binary toxin cytotoxicity of Vero cells in a 384-well plate using an ImageXpress Velos scanning cytometer. Shown are the representative well images of Vero cells treated with different reagents. (A) Medium only (100%); (B) binary toxin consisting of 484 pM of CDTa with a 1:7 CDTa-to-a_CDTb molar ratio (1%). F-actin filaments were completely depolymerized by the binary toxin. (C) Binary toxin in panel B was preincubated with an equal volume of 1- to 640-fold diluted hyperimmune monkey serum. The cells were partially protected from binary toxin cytotoxicity (16%). (D) Binary toxin in panel B was preincubated with hyperimmune monkey serum. The cells were fully protected from binary toxin cytotoxicity (98%). Percentages listed in the figure legend refer to the amount of the cell area that is fluorescently labeled relative to that of the medium-only well.

It has been proposed that CDTa binds to a heptamer of CDTb on the cell surface (7). However, it is not clear how many CDTa molecules bind to the heptamer of CDTb. Therefore, we evaluated 1mCDTa-to-a_CDTb molar ratios of 5:1, 1:1, 1:4, 1:7, and 1:10 by altering the amount of a_CDTb while maintaining the 1mCDTa concentration. The TC_{50} of 1mCDTa decreased from 1,150 pM at the 5:1 molar ratio to 56 pM at the 1:7 molar ratio. Molar ratios of 1:7 and 1:10 had similar TC_{50} values for 1mCDTa (data not shown). The DOE data confirmed that the TC_{50} of 1mCDTa significantly decreased as the 1mCDTa-to-a_CDTb molar ratio increased from 1:4 to 1:10 (Fig. 3C). A 1:7 molar ratio was selected for the optimized assay because CDTa is predicted to bind to a_CDTb heptamer on the cell surface.

Using the 1:7 molar ratio, the TC_{50} also increased from 44 pM to 68 pM as the cell-seeding density increased from 500 cells/well to 2,000 cells/well (Fig. 3D). Despite the marginally higher TC_{50} at 1,500 cells/well than that at 500 cells/well (1.3-fold), we chose 1,500 cells/well as the optimal density for the assay because it reduced the cell intoxication time from 48 h, as required for lower cell-seeding densities, to 20 to 24 h. The total geometric coefficient of variation was calculated to be 22.9% for the cytotoxicity assay. The addition of individual toxin components (1mCDTa alone or a_CDTb alone) or the combination of 1mCDTa and nonactivated GST_ProCDTb to the cells did not result in observable toxicity at concentrations 1,000-fold higher than the TC_{50} of the active components in binary toxin (data not shown).

Optimization of binary toxin NAb assay using design-of-experiment methodology. Based on the optimized cytotoxicity assay, we further optimized the binary toxin NAb assay by evaluating the joint effects of binary toxin concentration, serum-toxin preincubation time, and cell-seeding density using DOE methodology. The ED_{50} titer of a monkey control serum sample decreased from 1,894 to 452 as the binary toxin concentration increased from 242 pM to 968 pM (Fig. 4A). On the other hand, the signal-to-noise ratio increased from 92 to 788 as the binary toxin concentration increased over this same range (Fig. 4B). To provide for a suitably rugged assay and to strike an acceptable balance between assay sensitivity and specificity, the optimized binary toxin concentration was selected to be 448 pM, which is the minimum binary toxin concentration required to cause 100% cytotoxicity. Varying serum-toxin preincubation times from 30 to 120 min had only a minimal effect on the ED_{50} titer (Fig. 4C). Changing the cell-seeding density from 1,000 cells/well to 2,000 cells/well was found to have little effect on the ED_{50} (data not shown). Therefore, the midpoints of the serum-toxin preincubation times (60 min) and cell-seeding densities (1,500 cells/well) were chosen as the optimal conditions for the NAb assay. The ED_{50} correlated strongly with the concentration of monkey serum spiked into normal human serum ($R^2 = 0.99$, Fig. 4D). The total geometric coefficient of variation was 5.3% for the NAb assay.

Measurement of neutralizing antibody titers in hamster serum. The Syrian hamster challenge model is the most frequently utilized model for studying *C. difficile* infection. We evaluated the ability of a vaccine containing formaldehyde-detoxified 5mTcdA and 5mTcdB with either CDTa, CDTb, or both binary toxin components to induce serum antibodies with neutralizing activity. A method for measuring the NAb titer against TcdA and TcdB was described previously (25). Here, we determined the NAb titer that was specific for binary toxin. There were no detectable neutralizing antibodies for binary toxin in the adjuvant group and the group that received 5mTcdA and 5mTcdB preparations only. However, a neutralizing antibody titer was detectable postdose 3 with a vaccine containing CDTa or postdose 2 with a vaccine containing either CDTb or both binary toxin components. The combination of CDTa and CDTb produced a more potent neutralizing antibody response than that from either protein alone (Fig. 5A).

In addition, we compared the imaging-based assay with the traditional visual endpoint assay, which relies on microscopic examination of cell rounding following the intoxication of cell lines. The ED_{50} determined using the method employing the scanning cytometer correlated closely with the ED_{50} obtained using the microscopic cytotoxicity determination ($R^2 = 0.96$, Fig. 5B).

DISCUSSION

Binary toxin-expressing *C. difficile* strains have emerged recently as the cause of severe outbreaks of CDI. Among these, strains of the NAP1 type accounted for 67 to 82% of the clinical isolates identified during CDI outbreaks in Montreal, Canada in 2003 and 2004 (27), and 61% of all clinical isolates from 25 acute health care facilities in Chicago in 2009 (28). Goldenberg and colleagues (29) showed that patients infected with *C. difficile* strains containing binary toxin not only had significantly higher peripheral white

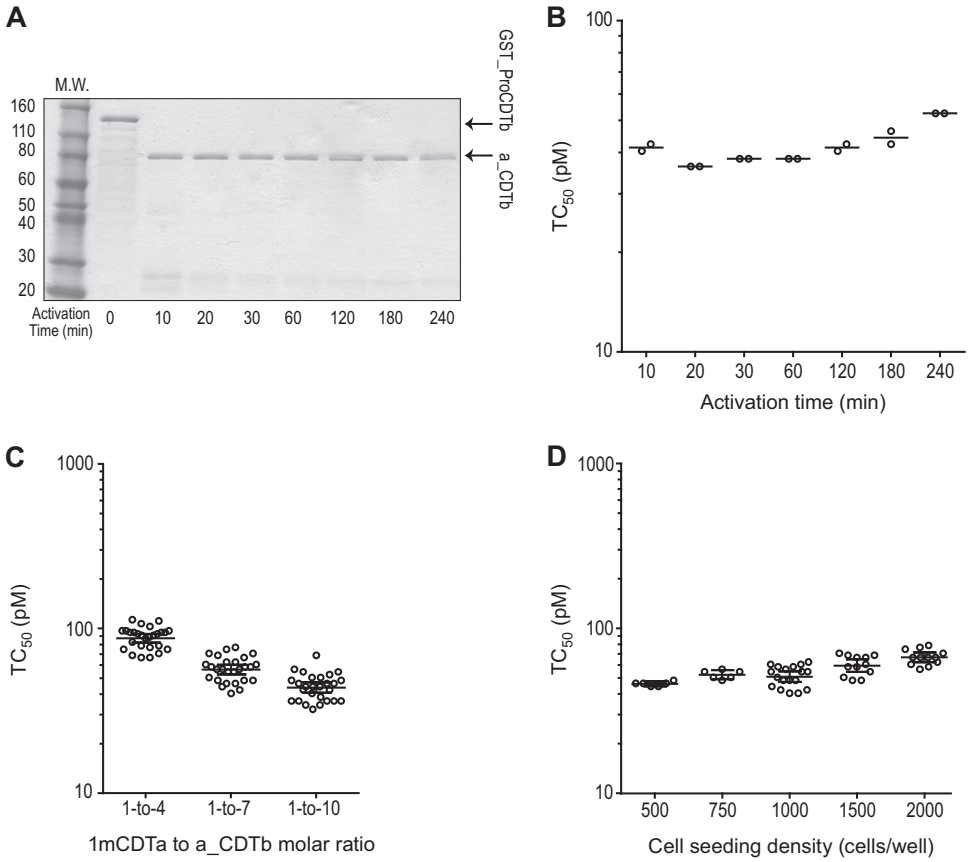

FIG 3 Optimization of the binary toxin cytotoxicity assay. (A) SDS-PAGE gel of GST_ProCDTb treated with immobilized chymotrypsin for different times from 0 min to 240 min. (B) TC_{50} of CDTa in binary toxin. CDTa was mixed with a_CDTb activated with different chymotrypsin activation times at a 1:7 molar ratio. The midpoint lines indicate the geometric mean value of two independent tests. (C) TC_{50} of 1mCDTa at 1:4, 1:7, and 1:10 1mCDTa-to-a_CDTb molar ratios in the DOE. The decreasing trend in TC_{50} with increasing molar ratio was statistically significant ($P = 0.002$). The error bars indicate the 95% confidence interval of the geometric mean. (D) TC_{50} of 1mCDTa in binary toxin with a 1:7 1mCDTa-to-a_CDTb molar ratio at different cell-seeding densities in the DOE. The increasing trend in TC_{50} with increasing cell-seeding density was statistically significant ($P < 0.001$). The midpoint lines indicate the geometric mean values. The error bars indicate the 95% confidence interval of the geometric mean.

blood cell counts but also had a significantly higher 30-day all-cause mortality rates (31% versus 14%). These data suggest that binary toxin may contribute to the pathogenesis of CDI in humans. A high-throughput functional assay is highly desirable for the further study of the role of binary toxin in the pathogenesis of CDI and the role of anti-binary toxin neutralizing antibody in protection against CDI.

Binary toxin enters the cells through LSR receptor-mediated endocytosis. LSR is a type I single-pass transmembrane protein and is mainly expressed in the liver, intestines, kidney, ovaries, and testes but not in muscle or heart (30, 31). LSR is thought to be involved in the clearance of triglyceride-rich lipoproteins and in the organization of tricellular tight junctions (32). To optimize the binary toxin Nab assay, we screened colon cancer cell lines (HT-29, Caco-2, and T84), a hepatocarcinoma cell line (HepG2), a kidney epithelial cell line (Vero), and a fibroblast cell line (IMR-90) for sensitivity to binary toxin, and we also looked at LSR expression in cell lysates (see Fig. S1 in the supplemental material). The results show that the colon cancer cells are the most sensitive to binary toxin among those tested, which is consistent with the high LSR protein level shown by the anti-LSR Western blot for these cell lines.

In addition to the binary toxin complex, we demonstrated that a_CDTb alone causes cytotoxicity in HT-29 cells (data not shown). Previously, it was described that the related *C. perfringens* iota toxin b (Ib) alone causes cytotoxicity by necrosis in A431 human epithelial carcinoma cells and A549 human lung adenocarcinoma cells (33). It has also been reported that in monolayers of Caco-2 cells, transepithelial resistance (TER) is decreased by the formation of pores by Ib oligomer insertion into the cell membrane (34). Papatheodorou et al. (35) have shown by fluorescence microscopy that a_CDTb can induce the clustering of LSR into subcompartments of the plasma membrane (lipid rafts), but they were not able to conclude whether the activated B component forms a heptamer that subsequently binds to the LSR receptor or whether the monomer first binds to the LSR receptor and then oligomerizes. We speculate that a_CDTb causes a high degree of LSR clustering on the HT-29 cell membrane, which might cause cytotoxicity through necrosis or disruption of the cell membrane similar to the cytotoxicity caused by Ib toxin. HT-29 cells were not selected for the assay because of the toxicity caused by a_CDTb alone.

To date, it is unknown how many molecules of CDTa bind to the CDTb heptamer on the cell surface. We demonstrate here that

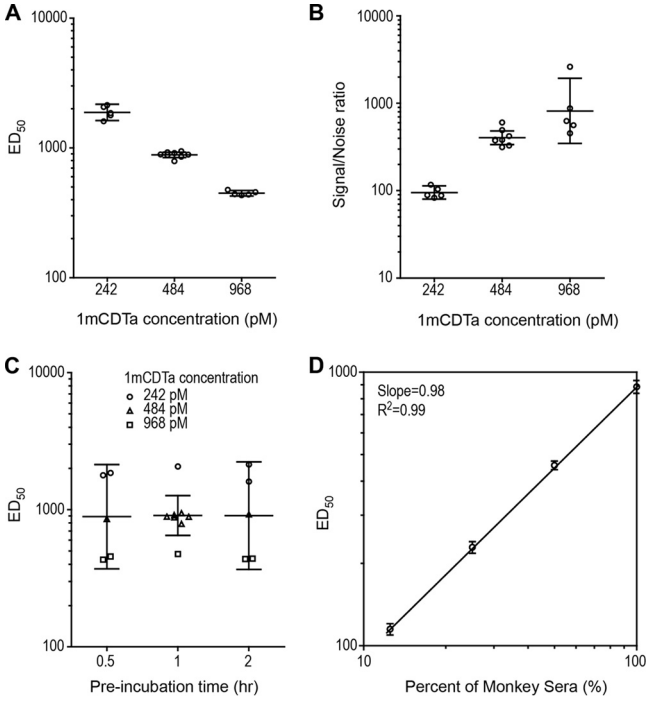

FIG 4 Optimization of binary toxin NAb assay using design-of-experiment methodology. (A) Effect of binary toxin concentration on the NAb titers of the monkey serum. The decreasing trend in the NAb titer of the monkey serum with increasing concentration of binary toxin was statistically significant ($P < 0.001$). (B) Effect of binary toxin concentration on the signal-to-noise ratio. The increasing trend in the signal-to-noise ratio with increasing concentration of binary toxin was statistically significant ($P < 0.001$). The midpoint line indicates the geometric mean value. (C) Effect of serum-toxin preincubation time on the NAb titers of monkey serum. No statistically significant difference was observed among the three time points ($P = 0.65$). The relatively large spread on monkey serum titers within each preincubation time is due to the effect of toxin concentration. Each point in panels A and C represents the geometric mean titer of duplicate data from one of the DOE runs. The midpoint lines in panels A to C indicate the geometric mean value, and the error bars indicate the 95% confidence interval of the geometric mean. (D) Correlation of NAb titer with the concentration of spiked monkey serum in negative human serum with 484 pM of CDTa in the binary toxin NAb assay. Each point represents the geometric mean titer ± standard deviation of eight runs. The signal-to-noise ratio is the ratio of the medium-only response to the toxin-only response.

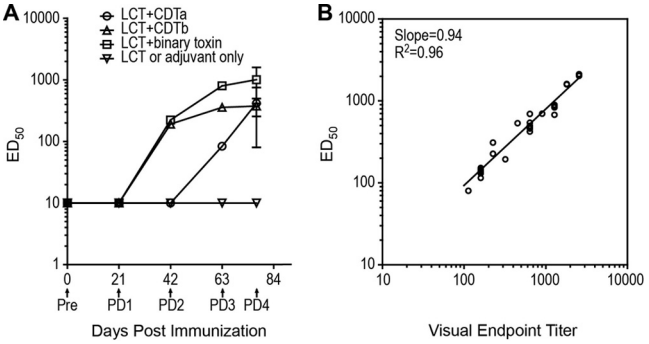

FIG 5 Measurement of anti-binary toxin NAb titers in hamster serum. (A) Preimmune (Pre), post-dose 1 (PD1), PD2, and PD3 sera were pooled for the testing. Each point at PD4 in the vaccine group represents the geometric mean titer ± standard deviation of the 10 hamster serum samples. LCT, large clostridial toxin, i.e., 5mTcdA and 5mTcdB. (B) Correlation of endpoint titers using a traditional visual endpoint titer assay with NAb titers (ED_{50}) using the imaging-based assay for anti-binary toxin NAb assay. Each point represents the geometric mean NAb titer of one of the 30 hamster serum samples analyzed independently by two analysts.

a 1:7 CDTa-to-a_CDTb molar ratio approaches the lowest TC_{50} value for binary toxin. This suggests that one CDTa molecule is bound to the CDTb heptamer and becomes endocytosed into the endosome. Therefore, 1:7 was determined to be the optimal CDTa-to-CDTb ratio for binary toxin. One limitation of this approach is that we have not purified native binary toxin from *C. difficile* and therefore do not know whether recombinant binary toxin has the same potency as native binary toxin.

Unlike TcdA and TcdB, there is currently no established method for purifying binary toxin from *C. difficile* bacterial culture, likely due to the low expression levels of these proteins (36). Here, we demonstrate that recombinant CDTa lacking its signal peptide is fully active, and mutation of the Cys_2 to Ala has little effect on the activity of the protein but eliminates the dimer formed through disulfide bonding of this residue. However, we have determined that three mutations at the enzymatic activity center ($S_{345}F$, $E_{385}Q$, and $E_{387}Q$) (24) eliminate the cytotoxicity of the binary toxin (S. Secore, S. Wang, J. Zorman, J. Xie, M. Miezeiewski, M. Horton, R. Xoconostle, B. Wang, C. Lancaster, J. Wagner, A. Kristopeit, S. C. Wang, S. Christanti, S. Vitelli, R. Rustandi, I. Rogers, M.-P. Gentile, A. Goerke, J. Skinner, E. Strable, D. S. Thiriot, J. L. Bodmer, S. Subramanian, and J. Heinrichs, unpublished data). In contrast to CDTa, ProCDTb needs to be activated by chymotrypsin in order to have activity (26). The experiments reported here show that the GST_Pro domain is specifically cleaved off by chymotrypsin at the peptide bond between Met_{169} and Ser_{170}; hence, there is no need to remove the GST tag before activation. In addition to *E. coli* expression, the insect cell was also demonstrated to be capable of expressing proteins, which are suitable for vaccine development.

Although serum anti-TcdA and anti-TcdB IgG titers have been convincingly shown to correlate with protection against CDI (likely through toxin neutralization) (20, 37), the role of anti-binary toxin neutralizing antibodies is still elusive. We show here, for the first time, a high-throughput assay that measures neutralizing antibodies against *C. difficile* binary toxin. We demonstrate that the serum NAb titer has an inverse correlation with binary toxin concentration and that the utilization of a binary toxin concentration of 8- to 10-fold the TC_{50} resulted in a robust and sensitive assay. Hamsters immunized with either CDTa or CDTb alone or with both components of binary toxin generated neutralizing antibodies against the binary toxin complex. Combining CDTa and CDTb had an additive effect in boosting the neutralizing antibody titer. We speculate that neutralizing antibodies targeting CDTa might block the binding of CDTa to the CDTb heptamer. Likewise, neutralizing antibodies targeting CDTb might block the binding of CDTa to CDTb heptamers or might block the binding of CDTb to the cell surface receptor. Both scenarios might prevent binary toxin from entering the cell. We have also shown that the assay was able to detect neutralizing antibodies spiked into human serum samples that did not contain endogenous antibodies to binary toxin and that the titer obtained correlated very well with the amount of hyperimmune monkey serum added. This suggests that this assay may be useful for quantifying neutralizing antibodies in human sera.

In conclusion, we have developed and optimized high-throughput binary toxin cytotoxicity and neutralizing antibody assays that are sensitive, robust, and linear. These assays have utility for quantifying the potency of binary toxin preparations and neutralizing antibody titers to binary toxin in hamster and monkey hyperimmune sera, which are essential for elucidating further the role of binary toxin in the pathogenesis of CDI. These assays will also be very useful in epidemiology studies and vaccine development for the prevention of *C. difficile* infection.

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