Development and Optimization of a Novel Assay To Measure Neutralizing Antibodies against *Clostridium difficile* Toxins

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**Clostridium difficile** produces two major virulence toxins, toxin A (TcdA) and toxin B (TcdB). Antitoxin antibodies, especially neutralizing antibodies, have been shown to be associated with a lower incidence of *C. difficile* infection (CDI) recurrence, and antibody levels are predictive of asymptomatic colonization. The development of an assay to detect the presence of neutralizing antibodies in animal and human sera for the evaluation of vaccine efficacy is highly desired. We have developed such an assay, which allows for the quantification of the effect of toxins on eukaryotic cells in an automated manner. We describe here the optimization of this assay to measure toxin potency as well as neutralizing antibody (NAb) activity against *C. difficile* toxins using a design-of-experiment (DOE) methodology. Toxin concentration and source, cell seeding density, and serum-toxin preincubation time were optimized in the assay using Vero cells. The assay was shown to be robust and to produce linear results across a range of antibody concentrations. It can be used to quantify neutralizing antibodies in sera of monkeys and hamsters immunized with *C. difficile* toxoid vaccines. This assay was shown to correlate strongly with traditional assays which rely on labor-intensive methods of determining neutralizing antibody titers by visual microscopic inspection of intoxicated-cell monolayers. This assay has utility for the selection and optimization of *C. difficile* vaccine candidates.

*Clostridium difficile* is a leading cause of nosocomial diarrhea worldwide. Disease caused by the organism results from the disruption of the intestinal bacterial flora due to antibiotic treatment followed by exposure to and germination of *C. difficile* spores. The symptoms of *C. difficile* infection (CDI) range from mild diarrhea to pseudomembranous colitis and toxic megacolon. In acute situations, the mortality rate can be as high as 40%. CDI occurs most frequently in individuals following an initial bout of infection (1). CDI is becoming increasingly difficult to treat due to the emergence of hypervirulent, antibiotic-resistant strains, resulting in an increased need for new therapies (2, 3).

Pathogenic strains of *C. difficile* produce two potent exotoxins commonly referred to as TcdA and TcdB. These two toxins induce a broad range of local and systemic effects (inflammation and colonic epithelium damage) (4). The toxins, which are encoded in a 19-kb region of the genome referred to as the pathogenicity locus (PaLoc), function through glucosylation of GTPases of the Rho family, leading to cytoskeletal disruption and affecting the tight junctions of the colonic epithelium. This in turn causes a loss of trans-epithelial electrical resistance (TEER), leading to profound diarrhea in infected individuals. The exact role of TcdA and TcdB is not entirely clear; however, data suggest that both molecules are important for disease pathology in animal models (5, 6). TcdA and TcdB are members of the large clostridial toxin (LCT) family and contain a homologous structure. Both molecules consist of an N-terminal enzymatic domain which catalyzes the glucosylation of RhoA (at Thr37), Rac, and Cdc42 (both at Thr35) (7, 8). In addition, the proteins contain hydrophobic central cores that are thought to be involved in membrane translocation and a highly repetitive C-terminal domain (combined repetitive oligopeptides [CROPs]) that is critical for interaction with cell receptors. The toxins enter cells by receptor-mediated endocytosis and follow the classical endosomal pathway, requiring acidification for translocation. A conformational change in the molecules results in pore formation and translocation of the enzymatic domain into the cytosol. Subsequent autoproteolytic cleavage, activated by the presence of a cellular cofactor, results in the release of the enzymatic domain. This in turn leads to the glucosylation of small GTPases and the disaggregation of the cell cytoskeleton and cell death, which can be measured to determine functional activity (9–11).

Current treatment modalities for *C. difficile* infection include discontinuing the offending antibiotic and beginning empirical therapy with narrow-spectrum antimicrobial agents that preferentially target the *C. difficile* organism. New approaches to CDI prevention currently under evaluation are based upon the development of *C. difficile* vaccine candidates containing either chemically inactivated toxins or recombinant TcdA and TcdB fragments (12, 13). Vaccine efficacy is believed to be dependent upon the production of a potent humoral immune response containing antibodies that effectively neutralize the activity of these toxins. Therefore, vaccine development will require a functional assay with the ability to measure neutralizing responses in animal models and clinical trials.

Traditionally, microscopic evaluation of intoxicated cell monolayers has been used to evaluate neutralizing antibody re-
 sponses in vitro. These methods tend to be subjective and time-consuming because of the requirement for manual observation and counting of cells. The method described here measures the potential of hyperimmune sera raised against a toxoid vaccine to inhibit the action of TcdA and TcdB on susceptible cell lines in vitro and offers an alternative method to measure and evaluate antibody responses to potential vaccine candidates. We describe the optimization of this assay to enhance sensitivity for TcdA and TcdB as well as the miniaturization of the assay to a 384-well microtiter plate format that allows for the use of liquid-handling automation. We also demonstrate that this assay produces results comparable to those obtained by using the traditional method of visual observation of cell monolayers and verify that this assay can be used to evaluate immunogenicity of a C. difficile vaccine in animal models.

**MATERIALS AND METHODS**

**Toxins.** TcdA and TcdB were purchased from List Biological Laboratories, Inc. (Campbell, CA) and tgcBIOMICS GmbH (Mainz, Germany). List Biological Laboratories toxins were purchased in vials containing 20 to 25 μg of hylified protein and stored at 4°C. Toxins were reconstituted on the day of the assay to ensure maximum activity. tgcBIOMICS toxins were stored at −20°C, and fresh aliquots were thawed as needed. Here, List Biological Laboratories is referred to as “supplier 1,” and tgcBIOMICS is referred to as “supplier 2.”

**Label-free quantitative mass spectrometry (MS): toxin digestion for bottom-up MS.** For each sample, 1 μg of toxin was dissolved in 100 mM NH₄HCO₃, and 6 M urea. Samples were then reduced for 15 min at 60°C in 20 mM TCEP [tris(2-carboxyethyl)phosphine] (Pierce, Rockford, IL). Alkylation was performed with iodoacetamide (Sigma) for 15 min at room temperature in the dark. Two sequential microwave-assisted protease digestions were performed with each sample (CEM microwave digester for 30 min at 50°C and 55 W). The first digestion was always performed with endoproteinase LysC (1:20 [wt/wt] enzyme-to-toxin ratio; Roche Diagnostics, Indianapolis, IN), and a second digestion was performed with either trypsin (Promega, Madison, WI), endoproteinase AspN (Roche), or endoproteinase GluC (Roche), each with a 1:20 [wt/wt] protease-to-toxin ratio after adding 100 mM NH₄HCO₃ to yield a 1.5 M urea concentration. Digestions were stopped by adjusting the pH with concentrated formic acid to 3.0.

**MS analysis.** Each toxin digest (0.5 μg) was analyzed on a linear ion trap instrument (LTQ XL; Thermo Fisher Scientific, Waltham, MA), using nano-liquid chromatography (LC)-tandem mass spectrometry (MS/MS). In brief, peptide digests were loaded for 15 min onto a C₁₈ trapping column (Cap Trap; Michrom Biosources, Auburn, CA) by using nanoflow high-performance liquid chromatography (HPLC) (Ultimate 3000; Dionex, Pittsburgh, PA) and then back-eluted onto a 10-cm analytical tip column (Biobasic C₁₈, with a 300-Å pore size, 5-μm particle size, and 75-μm internal diameter [ID]; New Objective, Woburn, MA). Peptides were eluted for 60 min by using a linear gradient from 5% acetonitrile and 0.1% formic acid to 40% acetonitrile and 0.1% formic acid. Eluting peptides were sprayed directly into the mass spectrometer. MS acquisitions were performed in profile mode. Each MS scan was followed by five MS/MS scans. Data-dependent collision-induced dissociation MS/MS spectra were acquired with dynamic exclusion enabled. The exclusion size list was set to 100. Label-free quantitation based on peptide MS peak heights was performed by using the Elucidator software package (in-house version 4.0; Rosetta Biosoft). SEQUEST peptide identifications from digests obtained with three protease combinations were combined. The protein false discovery rate was calculated by the Protein Teller algorithm and set to 0.2%. Relative protein abundance was approximated based on accumulated peptide MS signal intensities across the three analyses per toxin.

**Cell lines and cell culture.** Vero (African green monkey kidney; ATCC CCL-81), IMR90 (fetal rhesus monkey kidney epithelial; ATCC CCL-186), TH8 (human colorectal carcinoma epithelial; ATCC CCL-248), HT29 (colorectal adenocarcinoma; ATCC HTB-38), and CHO-K1 (Chinese hamster ovary; ATCC CCL-61) cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured as described by the ATCC. Briefly, Vero cells were cultured in Eagle’s minimum essential medium (EMEM) (ATCC 30-2003) supplemented with 10% heat-inactivated fetal bovine sera (FBS; HyClone, Logan, UT) and 100 units/ml of penicillin-streptomycin (Invitrogen, Grand Island, NY). IMR90 cells were cultured in EMEM supplemented with 10% FBS and 100 units/ml of penicillin-streptomycin, 2 mM L-glutamine, 1% minimal essential medium (MEM) nonessential amino acid (100× stock solution), 1 mM sodium pyruvate, and 1% sodium bicarbonate (7.5%). T84 cells were cultured in a 1:1 mixture of Ham’s F12 medium and Dulbecco’s modified Eagle’s medium supplemented with 5% FBS and 2.5 mM L-glutamine. HT29 cells were cultured in McCoy’s 5a medium supplemented with 10% FBS and 100 units/ml of penicillin-streptomycin. CHO-K1 cells were cultured in F-12K medium supplemented with 10% FBS and 100 units/ml of penicillin-streptomycin. All cell culture media were purchased from MediaTech (Manassas, VA) unless otherwise noted.

**C. difficile toxoid vaccine preparation.** Lyophilized C. difficile TcdA and TcdB from supplier 1 (selected due to lower cost and availability of material) were each reconstituted and then buffer exchanged by dialysis into morpholinoethanesulfonic acid (MOPS)-buffered saline (for monkey immunization) or into 20 mM HEPES-buffered saline (pH 7.4) (for hamster immunization) to obtain final concentrations of 0.3 to 0.4 mg/ml based on the toxin quantity measured by the manufacturer. The toxins were converted to the toxoid state by addition of formaldehyde to 0.43% and lysozyme to 4.2 mg/ml and incubation for 16 to 18 days at 2°C to 8°C. The toxoids were dialyzed against 20 mM HEPES buffer (pH 7.4) containing 100 mM sodium chloride and then sterilized by passage through a 0.22-μm filter. The toxoid concentration was then determined by UV-visible (UV-vis) spectrophotometry using extinction coefficients of 1.172 and 0.973 for TcdA and TcdB, respectively. These values were calculated by using the VPI 10463 ribotype sequence. Toxoids were combined with aluminum hydroxypolysulfate sulfate (AHPS) adjuvant (Merck & Co., Inc.), Iscomatrix adjuvant (CSL Biologicals, Inc., King of Prussia, PA), and saline, to produce a vaccine with final concentrations of 50 to 100 μg/ml toxoid A, 50 to 100 μg/ml toxoid B, 450 μg aluminum/mL, and 120 ISCO units/ml and a residual formaldehyde concentration of 0.016%.

**Rhesus macaque monkey immunization.** Adult rhesus macaques, approximately 4 to 5 years old, were housed at the New Iberia Research Center (NIRC) of the University of Louisiana at Lafayette. They were immunized intramuscularly in the deltoid muscle on weeks 0, 1, 2, and 26. Each dose of 500 μl vaccine contained 50 μg of toxoid A and 50 μg of toxoid B. Blood samples were collected before each immunization and on week 34 and evaluated for antibody titers. Blood samples from two monkeys from the week 34 collection were pooled (equal volumes), and this serum pool was used as a control for this study. The experimental protocol was approved by the Institutional Animal Care and Use Committee at both Merck & Co., Inc., and the NIRC, and the study was carried out at the NIRC.

**Hamster immunization.** Golden Syrian hamsters (male, 90 to 120 g) were obtained from Charles River Laboratories and were individually maintained in filter lid cages. The hamsters (eight per group) were immunized intramuscularly in the left quadriceps on days 0, 21, 42, and 63, and blood was collected via the retro-orbital technique immediately prior to each immunization and on day 77. Sera from individual hamsters were stored frozen. Each dose of 200 μl vaccine contained 10 μg of toxoid A and 10 μg of toxoid B. The control animals received adjuvant only. All hamster experiments were performed under Institutional Animal Care and Use Committee guidelines approved by Merck and Co., Inc.

**Evaluation of cytotoxicity by the IN Cell Analyzer 1000 imaging system.** Images of IMR-90 cells were acquired by using the IN Cell 1000 imaging system with a dichroic 505-nm filter for fluorescein isothiocya-
Cytotoxicity titration assay. The assay was run over a period of four consecutive days and comprised five steps: cell plating (step 1), toxin serial dilution and cell intoxication (step 2), cell staining (step 3), data acquisition (step 4), and data analysis (step 5). For cell plating (step 1), fifty microliters of a cell suspension of 15,000 cells/ml was seeded into a 384-well plate and incubated overnight in a humidified incubator with 5% CO2 at 37°C. For toxin serial dilution and cell intoxication (step 2), toxins were serially 2-fold diluted in complete culture medium (EMEM) and applied onto cells grown in 384-well plates. The cell plates were incubated in a CO2 incubator at 37°C for 48 h. For cell staining (step 3), cell plates were removed from the incubator and subjected to centrifugation at 450 × g in a swinging-bucket centrifuge for 5 min to pellet any free-floating cells. Cells were washed twice by using an Excel 405 microplate washer (BioTek, Winooski, VT) with angled liquid distributor pins to minimize the effect on the cell layer. Fifty microliters of fixation solution (Cytofix/Cytoperm Plus fixation permeabilization kit; Becton, Dickinson, San Diego, CA) was added to each well by using a MultiFlo microplate dispenser (BioTek), and plates were incubated at 4°C for 20 to 30 min. Cells were washed again as described above and stained with 50 μl/well of Alexa Fluor 488 phalloidin stain (Invitrogen) at a concentration of 0.016 μM diluted in PBS containing 1% BSA (Sigma-Aldrich Co., St. Louis, MO). Plates were incubated in the dark at room temperature for 1 h and washed twice as described above, and wells were filled with 80 μl PBS. For data acquisition (step 4), an image of the monolayer was acquired by using an ImageXpress Velos laser scanning cytometer (Molecular Devices, Sunnyvale, CA). The images were acquired by using a 488-nm laser, a 560 dichroic longpass (DRLP) mirror, and a 510- to 540-nm filter. Calibration was performed for each plate for photomultiplier tube (PMT) gain between 50 and 75% signal and scanning/collection focus. Acquisition parameters included a scan area of 3,800 by 3,800 μm and a scan resolution of 5 μm. Images were processed by using the following parameters: 10% baseline, offset of 5,000, formula average channels 1 and 2, split touching, and cell surface area of less than 600 μm² excluded. The total cell surface area in each well was calculated by the use of cytometer software and exported for data analysis. For data analysis (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 (TC50 [50% toxic concentration]) was the toxin concentration that caused 50% cytotoxicity in the well. The TC50 value was calculated by four-parameter logistic regression of the titration curve using GraphPad software.

Cell-based neutralization antibody assay. The cell-based neutralization antibody (NAb) assay was run over a period of four consecutive days and comprised the same five-step procedure used for the cytotoxicity titration assay described above. Exceptions for steps 2 and 5 are as follows. In step 2, test sera were 2-fold serially diluted, followed by incubation with the respective toxins for specified times at 37°C (referred to as serum-toxin preincubation). The mixture was then applied onto cells grown in 384-well plates and incubated in humidified CO2 incubators at 37°C for 48 h. The cells were fixed, permeabilized, and stained with Alexa Fluor 488 phalloidin, and an image of the monolayer was acquired by using the ImageXpress Velos scanning cytometer as described above. In step 5, the total cell surface area data were imported into an Excel workbook to calculate the titer. The titer of a test sample (ED50 [50% effective dose]) was the dilution at which cytotoxicity was decreased by 50%, ED50 was calculated by linearly interpolating between the consecutive dilutions whose signals bracket the midpoint signal. The midpoint signal is the average of the total cell surface area of medium-only control wells and that of the toxin-only control wells.

### Table 1: Experimental design of the antitoxin NAb assay DOE

| Run order | Analyst | Day | Block | Cell density (no. of cells/well) | Toxin concn^a | Preincub. time (h) |
|-----------|---------|-----|-------|---------------------------------|---------------|-------------------|
| 1         | 1       | 1   | 1     | 1,000                           | High          | 2                 |
| 2         | 1       | 1   | 1     | 500                             | High          | 0.5               |
| 3         | 1       | 1   | 1     | 1,000                           | Low           | 0.5               |
| 4         | 1       | 1   | 1     | 500                             | Low           | 2                 |
| 5         | 1       | 1   | 1     | 750                             | Medium        | 1                 |
| 6         | 1       | 1   | 1     | 750                             | Medium        | 1                 |
| 7         | 2       | 1   | 2     | 500                             | High          | 2                 |
| 8         | 2       | 1   | 2     | 500                             | Low           | 0.5               |
| 9         | 2       | 1   | 2     | 1,000                           | Low           | 2                 |
| 10        | 2       | 1   | 2     | 1,000                           | High          | 0.5               |
| 11        | 2       | 1   | 2     | 750                             | Medium        | 1                 |
| 12        | 2       | 1   | 2     | 750                             | Medium        | 1                 |
| 13        | 1       | 2   | 3     | 750                             | Low           | 1                 |
| 14        | 1       | 2   | 3     | 500                             | Medium        | 1                 |
| 15        | 1       | 2   | 3     | 1,000                           | Medium        | 1                 |
| 16        | 1       | 2   | 3     | 750                             | Medium        | 0.5               |
| 17        | 1       | 2   | 3     | 750                             | Medium        | 1                 |
| 18        | 2       | 2   | 4     | 750                             | Medium        | 0.5               |
| 19        | 2       | 2   | 4     | 750                             | High          | 1                 |
| 20        | 2       | 2   | 4     | 750                             | Medium        | 2                 |
| 21        | 2       | 2   | 4     | 750                             | Medium        | 1                 |
| 22        | 2       | 2   | 4     | 750                             | Medium        | 1                 |

^a For the anti-TcdA NAb assay, low, medium, and high toxin concentrations were 3, 4, and 5 ng/ml, respectively. For the anti-TcDB NAb assay, low, medium, and high toxin concentrations were 20, 40, and 80 pg/ml, respectively.

Design of the *Clostridium difficile* NAb assay optimization experiment. The objective of the assay optimization experiment was to identify optimal conditions for three critical assay parameters by evaluating their combined effects. The factors evaluated included 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, including cell seeding density (500, 750, and 1,000 cells/well), preincubation time (0.5, 1, and 2 h) and toxin concentration (3, 4, and 5 ng/ml for TcdA and 20, 40, and 80 pg/ml for TcDB). Titre concentrations were selected so that the center point was approximately 8- to 10-fold higher than the geometric mean (GM) TC50 of the respective toxin (14). Design-Expert version 7.1.3 software was used to generate a face-centered cube design consisting of 22 runs (one 384-well plate per run) performed in four blocks of five or six runs. The 22 runs were divided between two analysts and 2 days of testing, with the four combinations of analyst and day defining the four blocks. The 22 runs were comprised of the eight combinations of the three factors at their low and high settings, the six face-centered axial points, and the center point replicated twice within each block (total of eight times) (Table 1). An identical set of samples was tested on each of the 22 plates. The sample set included medium-only and toxin-only controls, a titration of the toxin, four human sera that were negative by antitoxin enzyme-linked immunosorbent assays (ELISAs), a high-titer positive serum sample from a vaccinated monkey, and three samples created by spiking the positive monkey sera into a pool of negative human sera at 2.5%, 10%, and 40% spike levels. Monkey serum-spiked negative human serum was used to test the matrix effect of human sera in the NAb assay. Dilutions of the human and monkey samples were tested twice within each plate. 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 (TC50), the level and variability of the NAb titers (ED50) for the human and monkey samples, and the linearity of the NAb titers across the range of monkey spikes tested. Optimal conditions were identified by using Design-Expert software.
Visual endpoint titer assay. A visual endpoint titer assay was performed according to the above-described protocol except that plates were examined microscopically by two analysts independently prior to fixation and staining using the hamster sera. Sera from eight hamsters in the vaccine group with five time points were tested. The percentage of cells that were protected by the hamster immune sera was estimated for each well. The endpoint titer was determined as the first serum dilution that protected fewer than 90% of the cells (15). The individual titer determined by the two analysts was used for the data analysis.

Statistical data analysis. All analyses were performed on the natural-log-transformed responses. Differences among factor levels were assessed by using a multifactor analysis of variance (ANOVA) model containing all main-effect and two-way interactions 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 factor levels was assessed based on the differences of the least-squares means. P values of less than 0.05 were considered significant. Precision of the NAb titer for the monkey positive control was assessed by variance component analysis (VCA). The VCA was performed on the log-transformed titers by using the MIXED procedure in SAS software (version 9.1.3).

RESULTS

Evaluation of toxin-mediated cytotoxicity using an ImageXpress Velos laser scanning cytometer. Addition of TcdA or TcdB to IMR90 cell monolayers led to a characteristic F-actin depolymerization and cell rounding phenotype. Preincubation of toxins with hyperimmune monkey sera protected the cells from the cytotoxic effects (Fig. 1A). Cell morphology changes were captured by an ImageXpress Velos laser scanning cytometer, using a 384-well plate that could be scanned and analyzed in 5 to 10 min. The cytometer software is capable of quantifying the cell surface area labeled by the fluorescent signal emitted by the Alexa Fluor 488 phalloidin dye (Fig. 1B). The total cell surface area was inversely correlated with cytotoxicity and was used to calculate the TC_{50} of toxin molecules and ED_{50} of immune sera, suggesting that an automated imaging approach could be useful for evaluating antibody responses to vaccine.

Screening of cell lines and toxin sources. Vero, IMR-90, T84, HT29, and CHO cells, which were previously described in the literature as used for cytotoxicity assays, were screened for sensitivity to TcdA and TcdB obtained from supplier 1 (Fig. 2A and B) (14–17). Compared to Vero cells, IMR-90 and CHO cells had similar sensitivity to TcdB-induced killing but were 18- and 380-fold less sensitive to TcdA, respectively. T84 and HT29 cells had similar sensitivity to TcdA and were 290- and 70-fold less sensitive to TcdB than Vero cells. Vero cells were found to be sensitive to both TcdA and TcdB and were selected for further optimization of the assay.

We next evaluated TcdA and TcdB purchased from two different suppliers (supplier 1 and supplier 2) for their ability to kill Vero cells (Fig. 2C and D). Both manufacturers purify the toxins following anaerobic culture of C. difficile. TcdB from supplier 2 was found to be approximately 100 times more potent than that obtained from supplier 1 and comparable to TcdB evaluated from other sources (data not shown). Similarly, TcdA from supplier 2 was found to be more potent than that obtained from supplier 1 (approximately 3-fold). In order to determine the relative purity of toxin preparations from the two suppliers, label-free quantitative mass spectrometry analysis was performed. We were unable to detect TcdB contamination in TcdA preparations from either supplier; however, TcdA was detected at a level of 0.4% in TcdB sourced from supplier 1 (data not shown). SDS-PAGE analysis also demonstrated greater proteolytic degradation of TcdB from supplier 1 than that of TcdB from supplier 2. Therefore, TcdA and TcdB from supplier 2 were chosen for further optimization of the assay.

Effect of cell seeding density and incubation time at 37°C on toxin potency. We first evaluated the effect of cell seeding density on toxin activity. To do this, TcdA and TcdB were individually titrated on Vero cell monolayers that were seeded at 500, 750, or 1,000 cells/well (Fig. 3). These cell seeding densities were found to have a negligible effect on toxin potency. The geometric mean (GM) TC_{50}s of TcdA and TcdB were calculated to be approximately 0.40 ng/ml (range, 0.25 to 0.75 ng/ml) and 5.6 pg/ml (range, 4.1 to 7.0 pg/ml), respectively. We also evaluated the stability of toxin preparations by incubation for up to 2 h at 37°C and found that this did not affect either toxin’s potency (data not shown).

Optimization of the NAb assay by using design-of-experiment methodology. Cell seeding density, toxin concentration, and serum-toxin preincubation time were optimized by the design-of-experiment (DOE) methodology. The results of the TcdA NAb assay DOE are shown in Fig. 4. The ED_{50} of the hyperimmune monkey sera decreased and the signal-to-noise ratio increased as the TcdA concentration increased (Fig. 4A and B). The ED_{50} was ~17,000 with 0.5 h of preincubation and stabilized at ~21,000 to 22,000 with 1 to 2 h of preincubation (Fig. 4C). The ED_{50} correlated strongly with the concentration of spiked monkey sera in negative human sera (R^2 = 0.99). This suggests that the assay could be used to detect neutralizing antibodies in human sera (Fig. 4D).

Results of the TcdB NAb assay DOE are shown in Fig. 5. Similar to what was observed for the TcdA NAb assay described above, the ED_{50} of the monkey sera decreased and the signal-to-noise ratio increased as the TcdB concentration increased (Fig. 5A and B). The ED_{50} was ~7,500 with 0.5 h of preincubation and stabilized at ~8,000 to 8,500 with 1 to 2 h of preincubation (Fig. 5C). The ED_{50} correlated well with the concentration of spiked monkey sera in negative human sera (R^2 = 0.94) (Fig. 5D).

Cell seeding densities tested in the experiments were found to have little effect on the ED_{50} in either assay (data not shown). Based on the NAb assay DOE results, the optimized NAb assay conditions were obtained and are summarized in Table 2. The total geometric coefficient of variation (GCV) was 21.0% for the TcdA NAb assay DOE (Table 3).

Measurement of NAb titers in hamster sera. Animal models of immunogenicity and protection from pathogen challenge are typically used for assessment of vaccine efficacy during preclinical development. The most frequently utilized model for C. difficile infection is the golden Syrian hamster challenge model. We evaluated the ability of a model (toxoid) vaccine, containing formaldehyde-detoxified TcdA and TcdB, to induce serum antibodies with neutralizing activity. Following immunization of hamsters with either the toxoid vaccine or an adjuvant control, we assessed the ability of serum to neutralize either TcdA or TcdB in the optimized Vero cell assay (Fig. 6). There were no detectable neutralizing antibodies in the adjuvant control group. However, neutralizing antibodies for TcdA were readily detectable following a single immunization, and titers increased following subsequent vaccinations. Similarly, neutralizing antibodies for TcdB were first measured following two immunizations, and titers increased over subsequent immunizations.
We also compared our imaging-based assay with the traditional assay, which relies on microscopic examination of cell rounding following intoxication of cell lines. The ED$_{50}$ determined by using the method employing the scanning cytometer correlated closely with that obtained by using the microscopic cytotoxicity determination (Fig. 7). The $R^2$ values were 0.98 and 0.99 for the anti-TcdA NAb assay and the anti-TcdB NAb assay, respectively.

**DISCUSSION**

Toxin neutralization is defined as the ability of antibodies raised against vaccine components to inhibit the action of toxins on a susceptible cell substrate *in vitro*. Current epidemiological, preclinical, and clinical research supports the concept that the humoral immune response plays a critical role in protection against CDI (18–21). In particular, serum anti-TcdA IgG was convincingly shown to correlate with protection against the disease (likely through toxin neutralization) (19). A toxoid vaccine was shown to induce antitoxin levels comparable to or higher than those observed in convalescent CDI patients and asymptomatic carriers after repeated immunization in young, healthy human subjects (mean age, 23 years) (14, 18). Furthermore, administration of this vaccine to three patients with recurrent *C. difficile* infection resulted in resolution of disease following cessation of antibiotic

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**FIG 1** Morphology changes caused by toxins and measurement of cytotoxicity in IMR90 cells. (A) IN Cell 1000 microscopic images of IMR90 cells treated with medium only, toxin only, and toxin plus hyperimmune monkey sera raised against toxoid. Cells were stained with Alexa Fluor 488 phalloidin (green) and Hoechst 33342 (blue). Although the IN Cell analyzer provides high-resolution images, it is time-consuming and low throughput. Ab, antibody. (B) ImageXpress Velos image of IMR90 cells acquired by a laser scanning cytometer in a 384-well plate. Panels a, b, and c are representative well images of IMR-90 cells treated with a high toxin concentration, a low toxin concentration, and medium only. Panels a’, b’, and c’ are the same images processed by cytometer software. The yellow outlines highlight the surface area identified by the software, which correlates with the surface area of the cells. The low toxin concentration is close to the TC$_{50}$ value of the respective toxin. The high toxin concentration is at least 8-fold higher than the TC$_{50}$ value of the respective toxin.
therapy (21). Although there is a strong association of TcdB with disease, no epidemiological or clinical data have yet established a protective threshold for anti-TcdB. Additionally, a recent study reported that geometric mean concentrations of anti-TcdB antibodies targeting the receptor binding domain of TcdB were significantly lower in the serum of subjects with recurrent CDI than in those without recurrence. Also, anti-TcdB neutralization antibody levels at days 14 and 28 correlated with protection against recurrent CDI in humans. A lower concentration of neutralizing anti-TcdA antibody was also associated with recurrence of CDI (20).

An automated assay readout that correlates with cytotoxicity is therefore needed for vaccine development. Intoxication of cells leads to actin depolymerization and subsequent cell rounding and an overall decrease in cell size. We found that the total surface area of the cells inversely correlated with toxin cytotoxicity. A laser scanning cytometer enables automated acquisition of the cell surface area data. Several alternative methods have also been evaluated for quantifying toxin activity on susceptible cell lines. We evaluated other markers of viable cells, such as the ATP level in the cells, measured by Cell Titer Glo (Promega Co.), and compared them to the assay results described here. We determined that C. difficile toxin-intoxicated cells retain substantial levels of ATP de-

| TABLE 2 | Optimized conditions for anti-TcdA and anti-TcdB NAb assays |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cell line | Cell seeding density (no. of cells/well) | Toxin concn (ng/ml) | Mean preincubation time (min) ± SEM | Mean cell intoxication time (h) ± SEM |
| Anti-TcdA Vero | 750 | 4 | 75 ± 15 | 46 ± 2 |
| Anti-TcdB Vero | 750 | 0.04 | 75 ± 15 | 46 ± 2 |
spite the fact that they are undergoing actin depolymerization and cell rounding. Because of this residual ATP, the signal-to-noise ratio was low, and the Cell Titer Glo assay was not very robust (data not shown).

Differential sensitivity of cell lines to TcdA and TcdB has been reported in the literature (17), and consequently, cell line selection plays an important role in the sensitivity of this assay. The quantity of toxin receptors on the cell surface likely contributes to the sensitivity of each cell line. TcdA has been shown to bind to receptors containing a trisaccharide \(\text{Gal}_3\text{H}_2\text{Gal}_3\text{H}_2\text{GlcNAc}\) in hamster and rabbit brush border membranes and erythrocytes (22, 23). Carbohydrate receptors for TcdA are also found on human intestinal epithelial cells (24). Higher-level binding of TcdA to rat basophilic leukemia (RBL) cells than to IMR-90 cells correlates with the higher sensitivity of the RBL cells (25). Specific receptors for TcdB have not been identified; however, it is likely that the distribution of receptors to TcdB will play a similar role as the receptors to TcdA in determining cell sensitivity to the toxins. The differential susceptibility of T84 and CHO cell lines to TcdA and TcdB allows for the evaluation of toxin activity in complex media such as \textit{C. difficile} culture filtrates and underlines the importance of selecting a cell substrate with optimal sensitivity for the toxin to be evaluated (16).

Toxin concentrations used in the NAb assay have an inverse correlation with the serum titer determined. To increase the sensitivity of this assay, it is critical to use the minimum amount of toxin required to intoxicate more than 90% of the cells. Typically, four times the minimum concentration of toxin resulting in cell rounding of 100% of cells has been used for NAb assays reported in the literature (15, 26). Our quantitative assay allows accurate determination of the TC\textsubscript{50} of individual toxin preparations. We determined that eight times the geometric mean TC\textsubscript{50} was the minimum concentration of toxin that resulted in 100% cell rounding, and we selected this amount of toxin to allow for enhanced reproducibility and sensitivity in the assay. The toxin concentration is also positively correlated with the signal-to-noise ratio, which affects the robustness of the assay. Our experiences showed that the assay was more variable and less robust if the ratio was less than 10. The toxin concentration that we selected resulted in a ratio that was consistently greater than 10.

![FIG 5 Optimization of toxin concentration and preincubation time in the anti-TcdB NAb assay using the DOE methodology. (A and B) Effect of TcdB concentration on the NAb titer of monkey sera and signal-to-noise ratio. (C) Effect of preincubation time on the NAb titers of monkey sera. The relatively large spread in monkey titers within each preincubation time is due chiefly 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 22 DOE runs. Midpoint lines in panels A to C indicate the geometric mean value. Error bars indicate the 95% confidence interval of the geometric mean. (D) Correlation of NAb titer with the concentration of spiked monkey sera in negative human sera at 40 pg/ml of TcdB. Each point represents geometric mean titer ± standard error of the mean of 12 runs at 40 pg/ml of TcdB. Signal-to-noise ratio is the ratio of the medium-only response to the toxin-only response. *, \(P \leq 0.05\); **, \(P \leq 0.01\); ***, \(P \leq 0.001\).](http://cvi.asm.org/)

Differential sensitivity of cell lines to TcdA and TcdB has been reported in the literature (17), and consequently, cell line selection plays an important role in the sensitivity of this assay. The quantity

![FIG 6 Measurement of anti-TcdA (A) and anti-TcdB (B) NAb titers in hamster sera after immunization with TcdA and TcdB toxoid vaccine. Each point in the vaccine group represents geometric mean titer ± standard error of the mean of eight hamster sera. Pre, preimmune sera; PD, postdose.](http://cvi.asm.org/)

![FIG 7 Correlation of endpoint titers using a traditional visual endpoint titer assay with NAb titers (ED\textsubscript{50}) using an imaging-based assay for anti-TcdA (A) and anti-TcdB (B) NAbs. Each point represents the NAb titer of one of 40 hamster sera analyzed by two analysts independently (n = 80).](http://cvi.asm.org/)

![TABLE 3 Variance component analysis of the optimized NAb assays](http://cvi.asm.org/)
enhancing assay sensitivity and robustness. As toxin preparations can contain both enzymatically active and inactive molecules, it is important to select a supply of toxin with maximal activity at a given concentration, as inactive toxin will likely compete for binding of antibodies and affect the neutralizing titer of any antiserum preparation.

ELISA data demonstrated that antitoxin binding antibody titers increase quickly after dose 1 in hamsters, while neutralizing antibody titers increase more slowly (data not shown). We have determined that NAB titer levels following four immunizations correlate with protection of hamsters from lethal C. difficile VPI 10463 challenge (data not shown). A correlate of protection has not yet been fully established for humans but will likely be important for demonstrating efficacy of several vaccine molecules currently being investigated. Due to the large number of serum samples generated during clinical evaluation of vaccines, a semi-automated assay, such as the one described here, will be highly useful.

Cell-based assays are capable of measuring neutralizing antibodies targeting the CROPs, enzymatic, or transmembrane domains. Babcock et al. (27) speculated that neutralizing antibodies targeting the CROPs domain could prevent the binding of toxins to cell surface receptors. However, neutralizing antibodies targeting the enzymatic domain may function through direct inhibition of enzymatic activity, and neutralizing antibodies targeting the transmembrane domain could prevent the internalization of toxins (16, 27). Since not all neutralizing antibodies are able to protect mice from in vivo toxin challenge, it is important to consider both in vivo protection as well as in vitro neutralizing activity when selecting vaccine candidates.

One limitation of our study is that we have not evaluated immunization of animals with either TcdA or TcdB alone. Because of this, we cannot fully evaluate the potential of cross-neutralization afforded by either toxin preparation. It is likely that cross-reactive epitopes exist for TcdA and TcdB due to the homology of the CROPs regions of the two proteins. However, due to the fact that both proteins are expressed by the majority of pathogenic C. difficile strains, we believe that any vaccine developed for this infection would require inclusion of both of these molecules. Therefore, we have not directly evaluated the contribution of neutralizing antibodies targeting the individual components of the vaccine.

In conclusion, we have developed and optimized a novel assay which is sensitive, robust, and linear. The assay can be used to quantify neutralizing antibody titers to TcdA and TcdB in hamster and monkey hyperimmune sera. No matrix effect was seen when animal sera were spiked into human sera, indicating that it will be possible to use the assay to detect both endogenous and hyperimmune neutralizing antibodies in clinical studies. The value of this assay is 2-fold: first, it serves as a useful tool to evaluate potential C. difficile vaccine candidates in various animal models, and second, it provides a diagnostic tool for future clinical studies. The measurement of concentrations of neutralizing antibodies against TcdA and TcdB in CDI cases will provide useful information on antibody threshold levels required for protection from this disease and may allow for the establishment of an immune correlate of protection for comparison of vaccines.

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