Effective Sequestration of *Clostridium difficile* Protein Toxins by Calcium Aluminosilicate

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*Clostridium difficile* is a leading cause of antibiotic-associated diarrhea and the etiologic agent responsible for *C. difficile* infection. Toxin A (TcdA) and toxin B (TcdB) are nearly indispensable virulence factors for *Clostridium difficile* pathogenesis. Given the toxin-centric mechanism by which *C. difficile* pathogenesis occurs, the selective sequestration with neutralization of TcdA and TcdB by nonantibiotic agents represents a novel mode of action to prevent or treat *C. difficile*-associated disease. In this preclinical study, we used quantitative enzyme immunoassays to determine the extent by which a novel drug, calcium aluminosilicate, uniform particle size nonswelling M-1 (CAS UPSN M-1), is capable of sequestering TcdA and TcdB *in vitro*. The following major findings were derived from the present study. First, we show that CAS UPSN M-1 efficiently sequestered both TcdA and TcdB to undetectable levels. Second, we show that CAS UPSN M-1’s affinity for TcdA is greater than its affinity for TcdB. Last, we show that CAS UPSN M-1 exhibited limited binding affinity for nontarget proteins. Taken together, these results suggest that ingestion of calcium aluminosilicate might protect gastrointestinal tissues from antibiotic- or chemotherapy-induced *C. difficile* infection by neutralizing the cytotoxic and proinflammatory effects of luminal TcdA and TcdB.
ramine, colestipol, Synsorb 90, and tolevamer) have been examined in preclinical studies (25). Of these toxin-binding agents, only three have been tested in clinical studies. Unfortunately, none of these agents has proven to be as efficacious as traditional antibiotic therapies. Nevertheless, it is important to continue to develop new candidate therapies. In this article, we describe the characterization of calcium aluminosilicate uniform particle size nonswelling M-1 (CAS UPSN M-1), a novel calcium aluminosilicate agent that has been developed to selectively bind to and neutralize large clostridial protein toxins. Calcium aluminosilicate is recognized by the Food and Drug Administration (FDA) as a generally regarded as safe (GRAS) additive, which can be used as a supplement to foods at levels up to 2% (wt/wt) (27).

MATERIALS AND METHODS

Protein-based cytotoxic enzymes and reagents. Lyophilized C. difficile TcdA and C. difficile TcdB were stored according to the manufacturer’s specifications (Calbiochem, Gibbstown, NJ). TcdA and TcdB were resuspended in 10 mM 2,2-bis(hydroxymethyl)-2,2’,2’-nitrotritheanol (bis-Tris) buffer (Sigma-Aldrich, St. Louis, MO) and maintained on ice prior to being assayed. All other chemicals were molecular biology grade and stored as recommended by the manufacturer. A SevenMulti conductivity meter (Mettler Toledo, Columbus, OH) was used for pH measurements.

Putative toxin-binding agent. Calcium aluminosilicate uniform particle size nonswelling M-1 (CAS UPSN M-1), the novel sequestering agent used in this study, was provided by Salient Pharmaceuticals Incorporated (Houston, TX).

Quantitative enzyme immunoassay for toxin quantification. The concentration of C. difficile TcdA or TcdB was measured using the Premier Toxins A&B-enzyme immunoassay (EIA) according to the manufacturer’s instructions (Meridian Bioscience, Inc., Cincinnati, OH), except that a series of assay positive-control samples (i.e., TcdA and TcdB reference standards at a range of known concentrations) was incorporated into each repeated measurement; the concentrations of these reference standards typically ranged from 5 to 20 ng/ml. In brief, the resultant quantitative enzyme immunoassay (qEIA) uses C. difficile TcdA- and TcdB-specific polyclonal antibodies to capture TcdA and TcdB and to noncovalently anchor them to the solid-phase EIA support matrix. The matrix-bound toxins were subsequently complexed with horseradish peroxidase (HRP)-conjugated mouse anti-toxin A (monoclonal) or goat anti-toxin B (polyclonal) antibodies, respectively. After the removal of the unbound HRP-antibody conjugates, the degradation of urea hydrogen peroxide by toxin-bound horseradish peroxidase was assayed in the presence of the reducing cosubstrate 3,3’,5,5’-tetramethyl-(1,1’-biphenyl)-4,4’-diamine (TMB). Phosphoric acid (1 M) was used to arrest the reaction. The terminal chromophore benzidine-4,4’-diamine (BZDI), an oxidized derivative of TMB, was measured in arbitrary units (AU) at an optical density of 450 nm (OD450) using an Infinite M200 microplate reader (Tecan US, Inc., Durham, NC). The resultant pellet eluate, including any proteins eluted from the calcium aluminosilicate, was carefully transferred to a fresh tube. Negative-control samples (i.e., SeeBlue Plus2 devoid of calcium aluminosilicate) were otherwise treated identically to the experimental samples.

The original supernatant and the pellet eluate were subjected to PAGE. Samples (15 µl) were loaded into the 1-mm wells of a NuPAGE Novex Tris-acetate gel (Invitrogen) using 1× lithium dodecyl sulfate (LDS) sample buffer (Invitrogen). The protein electrophoresis was carried out (150 V for 1 h) in an Xcel SureLock mini cell (Invitrogen) and 1× NuPAGE Tris-acetate SDS running buffer (Invitrogen). Proteins were stained using the SimplyBlue SafeStain (Invitrogen), according to the manufacturer’s instruction. The results were captured using the FluorChem HD2 documentation system with a 3-MHz cooled digital charge-coupled-device camera (Alpha Innotech).

Biosatistics. Raw qEIA data were captured using Infinite M200 i-Control software, exported to Excel (Microsoft Corporation, Redmond, WA), and analyzed using Prism (GraphPad Software, Inc., La Jolla, CA). Unless otherwise indicated, the data represent at least three repeated measurements. The data are expressed as the mean (±) or either the standard error of the mean (SEM) or the standard deviation (SD), as noted. Calibration curves were generated by least-squares regression. Analysis of variance (ANOVA) was used to determine the statistical significance of the measured differences between treatments. When significant differences were detected by ANOVA, Bonferroni tests were performed post hoc in order to explore these differences. An associated P value of <0.05 was considered statistically significant. The base 10 logarithm (log10) of each data point was calculated, and a calibration curve for the interpolation of unknowns was generated using least-squares linear regression.

RESULTS

Optimization of a qEIA to detect TcdA and TcdB. Samples containing known concentrations of TcdA or TcdB were used to generate standard curves. The standard curves from each of six randomly selected vials of TcdB were plotted in Fig. 1. A one-factor ANOVA found that toxin vial-specific effects were statistically significant (P < 0.0051); however, post hoc comparisons using the Bonferroni test indicated that, with the exception of one obvious outlier (P < 0.05), the differences between the remaining five curves were statistically nonsignificant (P > 0.05). For this experimental subset (n = 5), the Pearson product-moment correlation coefficient (r) indicated a strong, colinear relationship between the toxin concentration and optical density that was statistically significant between interexperimental EIA replicates (r = 0.9078, P = 0.0047). Similar results were seen for TcdA (n = 5), although the differences between the individual vials of TcdA were statistically nonsignificant (P = 0.8707). As seen with TcdB, a strong...
colinear relationship between the toxin concentration and optical density was observed. This colinear relationship was statistically significant between interexperimental qEIA replicates \((r = 0.9973, P = 0.0027)\). In separate experiments that examined the effect of toxin thermostability, the differences in the qEIA reactivity following short-term (e.g., 8 h) incubation on ice were found to be statistically nonsignificant \((P > 0.05)\). As a result, individual toxin vials were used to conduct multiple sequestration assays within a single workday and were then discarded.

Data distribution and transformation. The descriptive statistic skewness \((g_1)\) and the Kolmogorov-Smirnov (K-S) normality test were used to examine the data distribution within the TcdA and TcdB data sets. While both data sets skewed right \((TcdA, g_1 = 0.6626; TcdB, g_1 = 1.331)\), the K-S test indicated that neither exhibited significant differences from the Gaussian distribution \((P > 0.10)\). Nevertheless, the \(\log_{10}\) of each data point was calculated to normalize the data. The linear regressions of the mean TcdA \((n = 4; R^2 = 0.968)\) and TcdB \((n = 5; R^2 = 0.966)\) reference curves are plotted in Fig. 2. Signal response plateaus were observed at very high toxin concentrations and very low toxin concentrations (data not shown).

Calcium aluminosilicate does not affect the pH of the qEIA system. In order to determine if calcium aluminosilicate might artificially diminish the sensitivity of the qEIA by affecting the pH of the buffer system, calcium aluminosilicate was supplemented to a final working concentration of 0.5 mg/ml in 100 mM Tris (pH 6.5), and the pH was measured \((n = 3)\) once the mixture was equilibrated to 37°C. The incorporation of calcium aluminosilicate up to 0.5 mg/ml did not affect the pH of the buffer system \((\bar{x} = 6.52; SD, 0.01)\) compared to that of a buffer control that was devoid of calcium aluminosilicate \((\bar{x} = 6.50; SD, 0.001)\), as expected.

Effective, dose-dependent sequestration of large \(C.\ difficile\) cytotoxins by calcium aluminosilicate. Calcium aluminosilicate was assessed for its ability to reduce the concentration of large clostridial protein-based cytotoxic enzymes \textit{in vitro}. During these dose-response experiments, the concentration of TcdA or TcdB (Fig. 3) was fixed at 10 ng/ml, while the calcium aluminosilicate concentration was varied 100-fold (i.e., 0.05 mg/ml, 0.075 mg/ml, 0.1 mg/ml, 0.3 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml, and 5 mg/ml). Calcium aluminosilicate efficiently sequestered TcdA and TcdB \textit{in vitro}. The differences in the mean endpoint OD\textsubscript{450} values between the assay negative-control (i.e., vehicle devoid of TcdA) samples \((\bar{x}, 0.044 AU; SD, 0.001 AU)\) and the experimental samples supplemented with calcium aluminosilicate up to 2 mg/ml \((\bar{x}, 0.049 AU; SD, 0.004 AU), 3 mg/ml \((\bar{x}, 0.049 AU; SD, 0.005 AU), and 5 mg/ml \((\bar{x}, 0.048 AU; SD, 0.004 AU)\) were statistically nonsignificant \((P > 0.05)\).

The raw qEIA measurements obtained from the experimental samples were converted to residual toxin concentrations using the intraexperimental EIA calibration curves. The differences in the residual TcdA concentrations between the assay positive-control (i.e., TcdA-containing samples devoid of calcium aluminosilicate) samples and the experimental samples containing calcium aluminosilicate supplemented up to 0.05 mg/ml \((\bar{x}, 11.11 ng/ml; SD, 1.82 ng/ml), 0.075 mg/ml \((\bar{x}, 11.35 ng/ml; SD, 0.22 ng/ml), and 0.1 mg/ml \((\bar{x}, 10.93 ng/ml; SD, 0.06 ng/ml)\) were statistically nonsignificant (Fig. 3). In contrast, statistically significant differences in the residual TcdA concentrations were measured between the assay positive-control and experimental samples containing calcium aluminosilicate supplemented to 0.3 mg/ml \((\bar{x}, 8.54 ng/ml;
SD, 1.19 ng/ml; SD, 0.80 ng/ml; P < 0.05), 0.75 mg/ml (SD, 0.65 ng/ml; P < 0.001), 1 mg/ml (SD, 0.71 ng/ml), 2 mg/ml (below the lower limit of detection [bLLD]; P < 0.001), 3 mg/ml (SD, bLLD; P < 0.001), and 5 mg/ml (SD, bLLD; P < 0.001). The lower limits of detection for this qEIA are approximately 1.4 ng/ml for TcdA and 2.4 ng/ml for TcdB.

The efficiency by which the calcium aluminosilicate sequestered TcdB was also explored (Fig. 3). The differences in endpoint OD450 measurements between the assay negative-control (i.e., vehicle devoid of TcdB) samples (x, 0.047 AU; SD, 0.004 AU) and the experimental samples supplemented with calcium aluminosilicate up to 3 mg/ml (x, 0.043 AU; SD, 0.001 AU) and 5 mg/ml (x, 0.041 AU; SD, 0.002 AU) were statistically nonsignificant (P > 0.05). As performed with TcdA, the raw qEIA measurements were converted to residual toxin concentrations. The differences in the residual TcdB concentrations between the assay positive-control (i.e., TcdB-containing samples devoid of calcium aluminosilicate) samples and the experimental samples containing calcium aluminosilicate supplemented up to 0.05 mg/ml (x, 9.07 ng/ml; SD, 0.06 ng/ml), 0.075 mg/ml (x, 8.75 ng/ml; SD, 0.05 ng/ml), 0.1 mg/ml (x, 9.02 ng/ml; SD, 1.38 ng/ml), and 0.3 mg/ml (x, 8.44 ng/ml; SD, 0.8 ng/ml) were statistically nonsignificant (P > 0.05). In contrast, statistically significant differences in residual TcdB concentrations were measured between the assay positive-control and the experimental samples containing calcium aluminosilicate supplemented up to 0.5 mg/ml (x, 6.27 ng/ml; SD, 0.36 ng/ml; P < 0.001), 0.75 mg/ml (x, 5.47 ng/ml; SD, 0.64 ng/ml; P < 0.001), 1 mg/ml (x, 4.99 ng/ml; SD, 1.23 ng/ml; P < 0.001), 2 mg/ml (x, 3.75 ng/ml; P < 0.001), 3 mg/ml (x, bLLD; P < 0.001), and 5 mg/ml (x, bLLD; P < 0.001).

Protein-binding activity of calcium aluminosilicate. The selectivity of calcium aluminosilicate’s protein-binding activity was explored further using a variation of the C. difficile toxin-binding assay described above. During these experiments, calcium aluminosilicate (5 mg/ml) was challenged with a commercial protein cocktail that contained a number of nontarget proteins, including myosin, bovine serum albumin, and glutamate dehydrogenase. Representative SDS-PAGE gels of the supernatant and the pellet eluate can be found in Fig. 4. CAS UPSN M-1 bound nontarget proteins but did so with various efficiencies. Indeed, CAS UPSN M-1 bound myosin and bovine serum albumin inefficiently, while glutamate dehydrogenase was bound efficiently. Attempts to elute proteins bound to CAS UPSN M-1 were unsuccessful (Fig. 4, lanes 4 and 5), which suggests that proteins bound to CAS UPSN M-1 are bound tightly.

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DISCUSSION

The initial toxin concentrations used in this study were selected because they approximated the median concentration of TcdA (4.3 ng/ml) that is typically found in the stools of patients with C. difficile-associated diarrhea (range, 0.6 ng/ml to 19 pg/ml) (28). As such, the qEIA protocol developed in this study enabled C. difficile TcdA and TcdB quantitation at clinically relevant concentrations. No hook effect was observed for either toxin at toxin concentrations between 5 and 15 ng/ml, which constituted the linear range for this qEIA. The high-dose hook effect occurs when the antigen negatively affects the binding capacity of the reporter antibody or when it is added in excess of the reporter antibody (29). The intraexperimental EIA calibration curves generated using this qEIA protocol enabled toxin quantification via interpolation and, thus, facilitated the conversion of optical density measurements to residual toxin concentrations. Furthermore, given the high degree of reproducibility, this qEIA supported interexperimental comparisons between repeated measurements (e.g., randomized block experiments).

This assay revealed that calcium aluminosilicate efficiently removed both TcdA and TcdB at physiologically relevant concentrations. Indeed, calcium aluminosilicate neutralized TcdA to subclinical levels in vitro. As for tolevamer, protein binding by calcium aluminosilicate does not occur in a general or otherwise indiscriminate fashion and, thus, displays a degree of target specificity. Tolevamer is an anionic, high-molecular-weight polymer (>400 kDa) that was developed to neutralize TcdA and TcdB. Tolevamer has been shown to ameliorate CDI-like symptoms in hamsters (30). In addition, tolevamer has demonstrated therapeutic efficacy in a number of phase II and phase III clinical studies (25, 26). While effective, tolevamer’s cure rate was found to be inferior to those of vancomycin and metronidazole (26, 31). Surprisingly, however, the rate of CDI reoccurrence was generally lower with tolevamer than with either vancomycin or metronidazole (26).

TcdA and TcdB are postulated to be paralogs (32, 33). As a result, these proteins share significant amino acid sequence similarity to one another, especially at their amino- and carboxy-terminal regions. The two toxins share approximately 47% identity to each other and approximately 68% sequence similarity (data not shown). Both proteins are composed of three well-characterized functional domains (15). The amino terminus of the protein encodes a peptidase C80-type glycosyltransferase domain and a proximal substrate recognition domain. The hydrophobic middle region is putatively involved in membrane translocation. The carboxy terminus of the protein encodes the clostridial repetitive oligopeptides (CROPS) (also known as cell wall-binding [CWB] domains). The carboxy-terminal CROPS facilitate calcium-dependent host cell recognition (33) and may also play a role in the sequestration of TcdA and TcdB by calcium aluminosilicate. Proteins that are evolutionarily and/or structurally related to TcdA and TcdB might also be viable therapeutic targets for calcium aluminosilicate; however, additional research is required to test this hypothesis.

In addition to the similarities noted above, a number of toxin-specific differences were also observed. For example, the lowest experimental concentration of calcium aluminosilicate for which there was no observable effect was 0.1 mg/ml for TcdA but 0.3 mg/ml for TcdB. The minimum effective concentration (i.e., the threshold dose) for calcium aluminosilicate was 0.3 mg/ml for TcdA but 0.5 mg/ml for TcdB. Under these conditions, the calcium aluminosilicate concentration that achieved the maximum efficacy (EC100) was 2 mg/ml for TcdA, but the concentration of calcium aluminosilicate that provided approximately 50% of the maximum effect (EC50) for TcdA was 0.5 mg/ml. In contrast, the EC100 and EC50 for TcdB were 3 mg/ml and 1 mg/ml, respectively. While calcium aluminosilicate sequesters both protein-based cytotoxins, these results suggest that its affinity for TcdA is greater than its affinity for TcdB.

As antibiotic-resistant pathogens continue to emerge, the development of nonantibiotic treatment options represents a timely therapeutic approach to CDI management. Calcium aluminosilicate exhibited potent C. difficile TcdA- and TcdB-neutralizing activity and selective protein binding in vitro. Given the well-documented safety profile of calcium aluminosilicate (34), these studies provide in vitro evidentiary support of our hypothesis that ingestion of calcium aluminosilicate might protect gastrointestinal tissues and accelerate a patient’s recovery from antibiotic- or chemotherapy-induced C. difficile-associated diarrhea by neutralizing the cytotoxic effects of luminal TcdA and TcdB. Depending on its relative effectiveness and tolerability during downstream clinical studies, CAS UPSN M-1, the novel sequestration agent described in this study, may be used to complement or, possibly, replace existing antibiotic therapies for the treatment of CDI. However, it is beyond the scope of this current study to examine the biological effects of calcium aluminosilicate in vivo.

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