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Clostridium difficile chimeric toxin receptor binding domain vaccine induced protection against different strains in active and passive challenge models

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ABSTRACT

Clostridium difficile is the number one cause of nosocomial antibiotic-associated diarrhea in developed countries. Historically, pathogenesis was attributed two homologous glucosylating toxins, toxin-A (TcdA) and toxin-B (TcdB). Over the past decade, however, highly virulent epidemic strains of *C. difficile* (B1/NAP1/027) have emerged and are linked to an increase in morbidity and mortality. Increased virulence is attributed to multiple factors including: increased production of A- and B-toxins; production of binary toxin (CDT); and the emergence of more toxic TcdB variants ($TcdB_{(027)}$). $TcdB_{(027)}$ is more cytotoxicity to cells; causes greater tissue damage and toxicity in animals; and is antigenically distinct from historical TcdB ($TcdB_{(003)}$). Broadly protective vaccines and therapeutic antibody strategies, therefore, may target TcdA, TcdB variants and CDT. To facilitate the generation of multivalent toxin-based *C. difficile* vaccines and therapeutic antibodies, we have generated fusion proteins constructed from the receptor binding domains (RBD) of TcdA, $TcdB_{(003)}$, $TcdB_{(027)}$ and CDT. Herein, we describe the development of a trivalent toxin (T-toxin) vaccine (CDTb/$TcdB_{(003)}$/TcdA) and quadravalent toxin (Q-toxin) vaccine (CDTb/$TcB_{(003)}$/TcdA/$TcdB_{(027)}$) fusion proteins that retain the protective toxin neutralizing epitopes. Active immunization of mice or hamsters with T-toxin or Q-toxin fusion protein vaccines elicited the generation of toxin neutralizing antibodies to each of the toxins. Hamsters immunized with the Q-toxin vaccine were broadly protected against spore challenge with historical *C. difficile* 630 (toxinotype 0/ribotype 003) and epidemic NAP1 (toxinotype III/ribotype 027) strains. Fully human polyclonal antitoxin IgG was produced by immunization of transgenic bovine with these fusion proteins. In passive transfer studies, mice were protected against lethal toxin challenge. Hamsters treated with human antitoxin IgG were completely protected when challenged with historical or epidemic strains of *C. difficile*. The use of chimeric fusion proteins is an attractive approach to producing multivalent antitoxin vaccines and therapeutic polyclonal antibodies for prevention and treatment of *C. difficile* infections (CDI).

1. Introduction

Clostridium difficile is a spore-forming, Gram-negative anaerobe that is the leading cause of infectious diarrheal disease in hospitals worldwide [1,2]. The clinical presentation of *C. difficile* infection (CDI) ranges from asymptomatic carriers to mild diarrhea to fulminant pseudomembraneous colitis. In the United States, CDI is responsible for 500,000 infections [3] and healthcare costs exceeding $3 billion [4]. Risk factors include antibiotic treatment of hospitalized patients receiving suppressive immunotherapy and chronic care elderly patients. In the US hospital setting, 10–25% of patients receiving antibiotic treatment develop CDI, relapse

Abbreviations: RBD, receptor binding domain; CDI, *C. difficile* infection; Q-toxin, quadravalent toxin vaccine; T-toxin, trivalent toxin vaccine; MLD, maximal lethal dose; HAC, human artificial chromosome.

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following antibiotic treatment is 20% (61,400 recurrences), and the death rate is >9% (27,000 deaths) [3].

C. difficile pathogenicity is mediated by two high molecular weight extotoxins, toxin-A (TcdA) and toxin-B (TcdB), which are secreted as single large polypeptides with similar functional domains [5]. The sequence homology between the toxins is 63% [6], however, TcdA and TcdB neutralizing antibodies are not cross-neutralizing [7]. The N-terminal domain of both toxins is an ADP-glucosyltransferase (GT) that mediates glycosylation of Rho GTPase resulting in disruption of colonic epithelial tight junction integrity with excessive fluid loss [8,9]. Adjacent to the GT domain is the autocatalytic cysteine protease (CP) domain, which functions to proteolytically cleave and activate GT within the acidic environment of endosomes [10,11]. The central hydrophobic domain (PT) is responsible for pore formation within endosomal membranes and aids in transport of activated GT into the cytosol of intoxicated cells [11]. The C-terminal domain consists of a series of repeating units that make up the receptor binding domains (RBD). The RBD binds toxins to cell-surface receptors on colonic epithelial cells which stimulate endocytosis, endosome formation, and uptake of holotoxin (Fig. 1A) [12,13].

The amino acid sequences of TcdA are highly conserved between the historical strain 630 (toxinotype 0/ribotype 03) and the more virulent epidemic strain B1/NAP1/027 (toxinotype III/ribotype 027), while TcdB has greater sequence differences (overall 92% identity). TcdB variant from NAP1 ($TcdB_{(027)}$) is 1000-fold more cytotoxic and 4-fold more lethal in mice than TcdB from strain 630 ($TcdB_{(003)}$) [14,15]. The greatest sequence differences occur within the RBD (overall 88% identity). Epitope mapping studies have identified 11 unique antigenic epitopes within the RBD between the two strains and antibodies are not cross-neutralizing [15].

Binary toxin (CDT) is a third virulence factor that is produced by the hypervirulent NAP1 strain. Although its role in pathogenesis is not fully understood, CDT is believed to contribute to increased morbidity and mortality [16–18]. CDT consists of two toxin components (Fig. 1A). CDTa is the enzymatically active component with ADP-ribosyltransferase activity which modifies G-actin and blocks polymerization of F-actin resulting in disruption of microtubule organization [16]. CDTb is the receptor binding component that is activated by serine protease to release two smaller peptides. The larger peptide forms a heptamer that binds the cell surface receptor and mediates internalization [16,19].

Vaccination is a viable strategy for preventing CDI. Non-toxin based strategies include bacterial surface proteins such as flagellar components (FliC and FliD), adhesins (Cwp66) and surface polysaccharides (PSI, PSII and PSI) that are broadly expressed on the surface of different *C. difficile* ribotypes [20–22]. Strategies targeting the *C. difficile* toxins for prevention of CDI must take into consideration the role of multiple virulence factors and the emergence of toxin variants that are antigenically distinct from historical strains. To this end, we generated novel chimeric fusion proteins comprising the RBD of TcdA, $TcdB_{(003)}$, $TcdB_{(027)}$, and CDT. The immunogenicity and in vivo protection by multivalent vaccines and transgenically produced human antitoxin immunoglobulins were evaluated in mice toxin challenge and hamster CDI models.

2. Methods

2.1. Design of constructs

Chimeric fusion proteins were constructed to encode RBD of TcdA, $TcdB_{(003)}$, $TcdB_{(027)}$, and CDTb. The RBD amino acid sequence for TcdA was derived from *C. difficile* strain VPI 10463 (ATCC 43255), NCBI P16154 (toxinotype 0/ribotype 003); $TcdB_{(003)}$ from strain VPI 10463 (ATCC 43255), NCBI P18177 (toxinotype 0/ribotype 003); $TcdB_{(027)}$ from strain CD196, NCBI WP_009888442.1 (toxinotype III/ribotype 027); and CDTb from strain CD196, GenBank ABS57477.1 (toxinotype III/ribotype 027).

The coding sequences for TcdA RBD (truncated with 19 of 38 repeats), $TcdB_{(003)}$ and $TcdB_{(027)}$ RBDs (24 repeats each), and CDTb were codon optimized for expression in insect cells (GenScript).

The nucleotide sequences encoding the CDTb gene fragment (amino acids 1–835), TcdA RBD (1314 base pairs [bp], 6816–8130 bp), and $TcdB_{(003)}$ RBD (1608 bp, 5493–7098 bp) were PCR amplified from the synthesized gene. PCR-amplified gene fragments were digested with restriction enzyme: CDTb with BamHI/NheI; $TcdB_{(003)}$ RBD with NheI/XbaI; and TcdA RBD with XbaI/HindIII. After digestion, the three genes were ligated into the BamH1 and HindIII sites of pFastBac1 (Invitrogen). The plasmid encoding the three RBDs was used to construct a recombinant *Autographa californica* Multiple Nuclear Polyhedrosis Virus (AcMNPV) baculovirus using the Bac-to-Bac baculovirus expression system (Invitrogen) in *Spodoptera frugiperda* (Sf9) insect cells to express the trivalent fusion protein, hereafter referred to as T-toxin.

$TcdB_{(027)}$ RBD (1608 bp, 5493–7098) digested with SpeI/HinIII was fused to the C-terminus of the trivalent fusion gene to form the plasmid and baculovirus construct encoding the RBD of all four toxins, which was similarly expressed in Sf9 cells to produce the quadravalent fusion protein, hereafter referred to as Q-toxin.

2.2. Purification of T-toxin and Q-toxin chimeric fusion proteins from cell lystates

Baculovirus-infected Sf9 cells were lysed with 0.2% Tergiltol NP-9 in 25 mM Tris buffer (pH 8.0), 250 mM NaCl and 2 μg/mL leupeptin. Fusion proteins were purified with Fractogel EMD TMAE, phenyl HP and 30Q column chromatography. Purified T-toxin and Q-toxin were formulated in 25 mM Tris and 250 mM NaCl (pH 8.0) at 4.0 mg/mL.

2.3. SDS-PAGE and western blot

Purified T-toxin and Q-toxin were electrophoresed on 4–12% NuPAGE® gels (Invitrogen) and stained with SimplyBlue™ or used for Western blots. Blots were treated with chicken anti-TcdA (1:4000; US Biologicals), chicken anti-TcdB (1:10,000; US Biologicals), or rabbit anti-CDTb (1:5000; Covance) serum as primary antibodies and species-specific secondary antibodies, alkaline phosphatase-conjugated goat anti-chicken or rabbit IgG (1:5000; SeraCare).

2.4. Spore preparation

C. difficile strain 630 (BAA-1382, toxinotype 0/ribotype 003) and strain B1/NAP1/027 (BAA-1805, toxinotype III/ribotype 027) were obtained from American Type Culture Collection (ATCC). Each strain was grown on trypticase soy + 5% sheep blood agar plates for five days under anaerobic conditions at 37 °C. Spores were harvested and heat-shocked at 60 °C for 15 min. Inoculum was prepared by diluting stock spore suspensions in DMEM to $1–2 \times 10^8$ spores/mL (NAP1) or 1×10^3 spores/mL (strain 630). Titers were verified by dilution plating on *C. difficile* spores overnight under anaerobic conditions at 37 °C. Spore concentrations were 3.5×10^7 CFU/mL (NAP1) and 3.0×10^2 CFU/mL (strain 630).

2.5. Toxins

TcdA and $TcdB_{(003)}$ were purchased from List Biological Laboratories and $TcdB_{(027)}$ from Native Antigen Company. Binary toxin (CDTa + CDTb) was produced by Novavax.

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