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Toxicity assessment of *Clostridium difficile* toxins in rodent models and protection of vaccination

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ABSTRACT

Clostridium difficile is the leading cause of hospital-acquired diarrhea, also known as *C. difficile* associated diarrhea. The two major toxins, toxin A and toxin B are produced by most *C. difficile* bacteria, but some strains, such as BI/NAP1/027 isolates, produce a third toxin called binary toxin. The precise biological role of binary toxin is not clear but it has been shown to be a cytotoxin for Vero cells. We evaluated the toxicity of these toxins in mice and hamsters and found that binary toxin causes death in both animals similar to toxins A and B. Furthermore, immunization of mice with mutant toxoids of all three toxins provided protection upon challenge with native toxins. These results support the concept that binary toxin contributes to the pathogenicity of *C. difficile* and provide a method for monitoring the toxicity of binary toxin components in vaccines.

1. Introduction

Clostridium difficile (*C. difficile*), a noninvasive toxigenic bacterium and so-called hospital superbug, is responsible for 25–30% of antibiotic associated diarrhea in healthcare settings and nearly all cases of pseudomembranous colitis (PMC) and toxic megacolon [1,2]. The major virulence factors of toxigenic *C. difficile* are the large secreted glucosyltransferase protein toxins A (TcdA) and B (TcdB). TcdA and TcdB proteins share similar functional domains including: (1) cell surface receptor binding domain; (2) transmembrane domain that assists entry of the toxin into the intestinal epithelial cell cytoplasm; (3) cysteine protease domain that is activated by the host cytosolic cofactor inositol hexakisphosphate (InsP6) [3,4]; and (4) catalytic domain involved in binding and inactivation of intracellular Rho GTPases in intestinal epithelial cells, facilitating disruption of the cell cytoskeleton and necrosis and loss of the colonic monolayer integrity [5]. TcdA has also been shown to directly bind and induce apoptosis in monocytes [6,7]. In addition to TcdA and TcdB, some *C. difficile* strains such as BI/NAP1/027, produce an adenosine diphosphate ribosyltransferase binary toxin called, CDT, a member of the AB binary toxin group made up of an enzymatic (A) and a transport (B) component [8]. The precise role for binary toxin in pathogenesis is unclear, however, it has been shown to be toxic for Vero cells and may increase adherence of *C. difficile* to target cells, by the formation of microtubule protrusions [9,10].

Studies of prophylactic vaccines for *C. difficile* infection (CDI) have been performed for the last three decades [11,12]. The majority of vaccine candidates contain toxoid forms of TcdA (TxdA) and TcdB (TxdB) or recombinant mutants of TcdA (mTcdA) and TcdB (mTcdB) or, in some cases, chimeric constructs of the two molecules (cTxAB) [13–17]. These vaccines can induce high serum anti-TcdA and TcdB responses which are capable of neutralizing TcdA and TcdB and preventing cell rounding *in vitro* as well as protecting animals from morbidity and mortality in challenge models. Recently, the identification of binary toxin in highly virulent *C. difficile* isolates has suggested that it also contributes to CDI pathogenesis [18]. The

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value of including binary toxin in vaccines for *C. difficile* to enhance protection for binary toxin expressing strains such as BI/NAP1/027 is an ongoing question in the field. In order to better characterize the virulence of binary toxin *in vivo*, we compared the lethal dose (LD_{50}) values of TcdA, TcdB and binary toxin in a toxicity model in both mice and hamsters. Furthermore, we evaluated the protective efficacy of mutant toxoids of each toxin as antigens by immunizing mice followed by challenge with native toxin components in a mouse toxicity model.

2. Materials and methods

2.1. Animals

CD1 and C57BL/6 mice, female, 18–20 g, were purchased from Charles River Labs, Wilmington, MA. The mice were housed (in groups of 6–10) in cages with micro-isolator lids. Syrian golden hamsters, male, *Mesocricetus auratus*, 100–110 g, were purchased from Charles River Labs, Stonebridge, NY. Hamsters were housed individually in boxes with micro-isolator lids. Water and food were provided *ad libitum*. All animal experiments were approved by the Animal Care and Use Committee at Merck Research Laboratories, West Point, PA.

2.2. C. difficile *toxins and dosing preparation for toxicity assessment*

Purified toxin A (TcdA) and toxin B (TcdB) were purchased from Native Antigens, Co. (Oxfordshire, UK). Toxins were reconstituted in MilliQ water to yield 0.4 mg/ml solutions following the vendor's instructions. Binary toxin components CDTa (histidine-tagged 1mCDTa with C3S mutation) and CDTb (GST-tagged activated proCDTb) were expressed in *E. coli* and purified as described previously [19]. Activated proCDTb was obtained by chymotrypsin treatment to give CDTb [19]. The toxin doses were prepared in 20 mM HEPES, 100 mM NaCl, pH 7.0 at concentrations per 0.5 ml of: 100, 33, 11, 3.7, 1.2, and 0.4 ng for TcdA; 900, 300, 100, 33, 11, and 3.7 ng for TcdB; 800/14,000, 267/46,667, 89/1556, 29.6/519, 9.9/173, and 3.3 ng/57.6 ng for binary toxin (1mCDTa/CDTb, molar ratio = 1/7). All toxin preparations were stored at 4 °C until administration.

2.3. LD_{50} test

Each dose of toxin was administered to six CD1 mice by intraperitoneal (ip) injection using a 25 gauge needle. Monitoring of mice was performed every 2 h on the first day following injection and twice a day on d2 and d3. Animals were euthanized at 72 h. Hamster LD_{50} evaluation was conducted by the same procedure with adjusted concentrations of each toxin. The monitoring time for hamsters was extended to 168 h in order to collect additional information. The LD_{50} of each toxin was calculated based on survival rate at 72 h using the Spearman–Karber method and results are reported as toxin dose/100 g bodyweight.

2.4. Immunization and challenge

C57BL/6 mice were immunized with formalin inactivated 5mTcdA (5mTxdA) or 5mTcdB (5mTxdB) or 3mCDTa/proCDTb or the combination of 5mTxdA/5mTxdB/3mCDTa/proCDTb (for further description of these molecules see Xie et al. [19,20]) formulated with AAHS (amorphous aluminum hydroxyphosphate sulfate, Merck, West Point PA, 225 μg/ml). C57BL/6 mice were immunized with *C. difficile* vaccine candidates individually and in combination at d0, d7, d14 and d21. A total of 100 μL of each formulation was injected intramuscularly to two quadriceps, at 50 μL/each. Mouse blood was collected post-dose 4 on d28 and neutralization antibody titer was analyzed in a cell-based assay as previously described [19,20]. On d35, the immunized mice were challenged i.p. with lethal doses of either native TcdA or TcdB or activated binary toxin or a combination of TcdA/TcdB/binary toxin. The protection of the challenge is reported as the number of surviving animals per test group (see Table 2).

3. Results and discussion

3.1. LD_{50} *of* C. difficile *toxins in rodent models*

The LD_{50} is a commonly used method to assess acute toxicity *in vivo*. We evaluated the toxicity of all three known toxins produced by *C. difficile*, TcdA, TcdB and CDTa/CDTb, in rodent models to compare their relative potencies. In this test, CD1 mice were administered six dose levels of each toxin in 3-fold serial dilutions, in 0.5 ml per dose by i.p. injection, with 6 mice per dose level. The toxin dose range was designed to span the predicted toxicity of each of the molecules. The LD_{50}s were calculated using the Spearman–Karber method from lethality observed by 72 h post-challenge and the results are presented as nanogram of toxin per 100 g body weight in order to compare the data from mice and hamsters. The LD_{50} of binary toxin is reported here as the dose of 1mCDTa for comparison purposes as this is the active component and since neither CDTa nor activated proCDTb alone showed toxicity (data not shown). Fig. 1 illustrates the LD_{50} were 34.3 ng/100 g for TcdA, 247.8 ng/100 g for TcdB, and 623.3 ng/100 g for binary toxin (1mCDTa) in this mouse model (see Table 1). Among the toxins, TcdA was 7.2-fold more potent than TcdB and 18.1-fold more

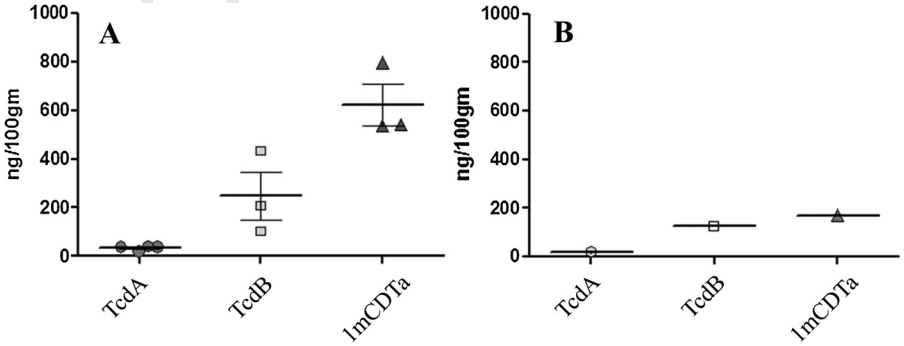

Fig. 1. Acute toxicity of *C. difficile* toxins in (A) CD1 mice and (B) hamsters (LD_{50} ng toxin/100 gm bodyweight). The LD_{50} calculation is based on 72 h lethality for each serially diluted toxin. The LD_{50} is calculated as the concentration of each toxin resulted in 50% death of animals in the test group.

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