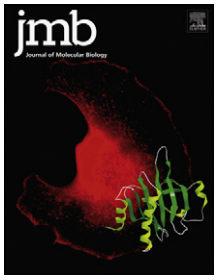

Epitopes and Mechanism of Action of the *Clostridium difficile* Toxin A-Neutralizing Antibody Actoxumab

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http://dx.doi.org/10.1016/j.jmb.2017.02.010
Edited by M. Guss

Abstract

The exotoxins toxin A (TcdA) and toxin B (TcdB) are produced by the bacterial pathogen *Clostridium difficile* and are responsible for the pathology associated with *C. difficile* infection (CDI). The antitoxin antibodies actoxumab and bezlotoxumab bind to and neutralize TcdA and TcdB, respectively. Bezlotoxumab was recently approved by the FDA for reducing the recurrence of CDI. We have previously shown that a single molecule of bezlotoxumab binds to two distinct epitopes within the TcdB combined repetitive oligopeptide (CROP) domain, preventing toxin binding to host cells. In this study, we characterize the binding of actoxumab to TcdA and examine its mechanism of toxin neutralization. Using a combination of approaches including a number of biophysical techniques, we show that there are two distinct actoxumab binding sites within the CROP domain of TcdA centered on identical amino acid sequences at residues 2162–2189 and 2410–2437. Actoxumab binding caused the aggregation of TcdA especially at higher antibody:toxin concentration ratios. Actoxumab prevented the association of TcdA with target cells demonstrating that actoxumab neutralizes toxin activity by inhibiting the first step of the intoxication cascade. This mechanism of neutralization is similar to that observed with bezlotoxumab and TcdB. Comparisons of the putative TcdA epitope sequences across several *C. difficile* ribotypes and homologous repeat sequences within TcdA suggest a structural basis for observed differences in actoxumab binding and/or neutralization potency. These data provide a mechanistic basis for the protective effects of the antibody *in vitro* and *in vivo*, including in various preclinical models of CDI.

Introduction

Clostridium difficile is a Gram-positive, spore-forming bacterium that infects the gastrointestinal tract of both humans and animals. In humans *C. difficile* infection (CDI) can cause mild symptoms such as a low-grade fever, watery stools, and minor abdominal cramping, as well as more severe symptoms such as bloody diarrhea, pseudomembrane colitis, toxic megacolon, and death [1]. Individuals whose normal gut flora has been compromised by treatment with antibiotics are most at risk for CDI. Over the past few decades, the incidence of CDI has increased throughout the developed world and is now a major health concern. Most often transmitted in a healthcare facility setting, *C. difficile* has become the most commonly reported pathogen in hospitals in the United States [2] and causes over 14,000 deaths per year[†]. Currently,

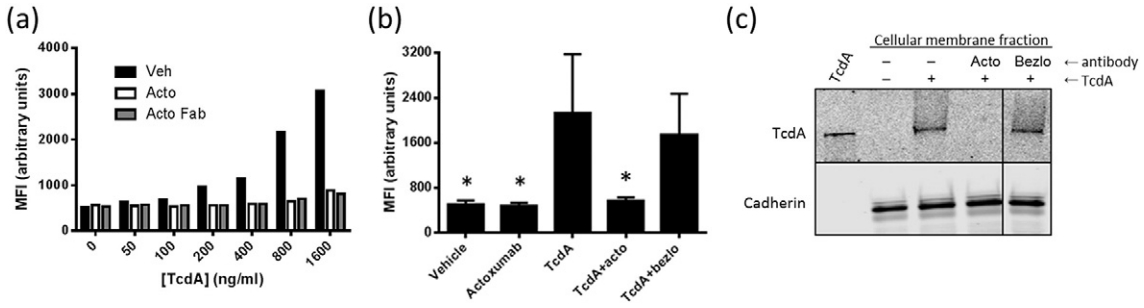

Fig. 1. Actoxumab prevents the binding of TcdA to HT29 and Vero cells. (a) Flow cytometry analysis of HT29 cells preincubated with increasing concentrations of TcdA-Atto488 at 4 °C in the presence or absence of vehicle, actoxumab (200 μg/ml), or actoxumab-Fab (200 μg/ml). Following incubation, MFI was measured with excitation and emission wavelengths of 488 nm and 530 nm, respectively. A representative experiment is shown. (b) Flow cytometry analysis of HT29 cells preincubated with 800 ng/ml TcdA-Atto488 at 4 °C in the presence or absence of vehicle, actoxumab, or bezlotoxumab. MFIs were calculated as per panel (a). Values are means ± standard deviation of two independent experiments. acto = actoxumab; bezlo = bezlotoxumab. *$p < 0.05$ compared to TcdA alone by paired two-tailed *t*-test. (c) Western blot of membranes isolated from Vero cells following incubation with 1 μg/ml TcdA in the presence of vehicle, actoxumab, or bezlotoxumab (200 μg/ml). The top panel shows TcdA and the bottom panel shows cadherin (loading control).

CDI is treated with standard of care antibiotics vancomycin, metronidazole, and fidaxomicin. Despite the high efficacy of these agents in treating an initial episode of CDI, 25 to 30% of patients will suffer a recurrence within 3 months [3], with subsequent recurrences occurring at an even higher rate. Thus, there is a great need to develop novel therapies that will reduce the risk of recurrence.

The symptoms of CDI are primarily caused by the exotoxins toxin A (TcdA) and toxin B (TcdB), which are produced by the bacterium during the infection [4–7]. TcdA and TcdB are structurally similar proteins, each having four separate domains: an amino-terminal glucosyltransferase domain (GTD), internal autoprotease and translocation domains, and a combined repetitive oligopeptide (CROP) domain at the carboxy-terminus. The CROP domains of TcdA and TcdB are composed of multiple short repeats (SRs; 32 in TcdA and 20 in TcdB) interspersed with a smaller number of long repeats (LRs; 7 in TcdA and 4 in TcdB) and have been presumed to play a role in receptor binding [8]. Both toxins bind to intestinal epithelial cells, and possibly other mucosal cells, and are internalized through receptor-mediated endocytosis [9]. The low pH environment of the endosome triggers a conformational change in the protein, resulting in the translocation of the GTD across the endosomal membrane and into the cytoplasm [10–12]. The autoprotease domain then cleaves the GTD [13], allowing it to diffuse through the cytoplasm and inactivate small GTPases of the Ras superfamily (particularly the Rho subfamily but also Rap and Ras) through covalent glucosylation [14,15], resulting in actin depolymerization, inflammatory cytokine production, and cell death [16–18].

While much is known about the trafficking of TcdA and TcdB and their mechanisms of action once internalized into target cells, exactly how the toxins bind to cells and through which receptors is less clear. Because different cell types show different levels of susceptibility to each toxin, it is believed that TcdA and TcdB bind to different receptors. Truncated versions of TcdA and TcdB lacking the CROP domain are still capable of intoxicating cells, albeit with lower potency than intact toxins, showing that regions outside the CROP domain are also involved in receptor binding [19,20]. Recently, poliovirus receptor-like protein 3, chondroitin sulfate proteoglycan 4, and members of the Wnt receptor frizzled family have been identified as putative cellular receptors for TcdB [21–23]. The TcdB CROP domain appears to be not necessary for binding to poliovirus receptor-like protein 3 or frizzled family protein members. While the potential receptors for TcdB identified thus far are membrane proteins, the receptor for TcdA is thought to be a cell surface carbohydrate [24]. The LRs in the CROP domain may serve as receptor binding sites, since a crystal structure of a C-terminal fragment of the TcdA CROP domain in complex with α-Gal-(1,3)-β-Gal-(1,4)-β-GlcNAcO$(CH_2)_8CO_2CH_3$ shows binding of the carbohydrate to residues located around the LR regions [25].

The need for more effective treatments for CDI has led to the development of alternative non-antibiotic therapies. Foremost among these are the two fully human monoclonal antibodies actoxumab and bezlotoxumab, which target TcdA and TcdB, respectively. The combination of actoxumab and bezlotoxumab is highly protective in primary and recurrent animal models of CDI [26–29]. In clinical trials, bezlotoxumab alone or in combination with actoxumab significantly