The roles of toxin A and toxin B in *Clostridium difficile* infection

Insights from the gnotobiotic piglet model

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We recently published our findings indicating that anti-TcdB antibodies were effective as treatment for *C. difficile* infection, but that anti-TcdA actually worsened prognosis in the gnotobiotic piglet model. To further investigate the roles of the two toxins, we administered purified toxins separately or together, systemically, to piglets and found that both toxins, either alone or together, are able to elicit severe lesions systemically and are also able to cross into the gut lumen and cause large intestinal lesions typical of infection. We also found that anti-TcdA administered before systemic challenge with TcdA again did not protect from development of disease, but, in this case, did not appear to worsen prognosis. Further work is still needed, but these findings add to the growing knowledge regarding the roles of the *C. difficile* toxins.

Introduction

Determining the roles of the two *C. difficile* toxins, toxin A (TcdA) and toxin B (TcdB), in the pathogenesis of *C. difficile* infection (CDI) has long been a topic of interest in the field.¹⁻⁵ We recently published a paper of our experimental results with human monoclonal antibodies used to investigate roles of TcdA and TcdB in the pathogenesis of CDI.⁶ Interestingly, we found that only anti-TcdB was required in the piglet model to protect piglets from systemic and gastrointestinal lesions following oral inoculation with *C. difficile*, and that piglets treated with only anti-TcdA actually fared worse

than untreated controls. This finding drew into question the long-standing hypothesis that TcdA was actually more important in the pathogenesis of disease, but was in agreement with other recent work highlighting the importance of TcdB.^{1,2,7} Our work also confirmed that systemically administered antibodies were detectable not only in the blood, as expected, but were also detectable in the intestinal lumen.

Here we describe several experiments using systemic administration of purified TcdA and TcdB to examine the systemic effects of the two toxins in the absence of bacterial infection and other potential virulence factors present during colonic infection. Gnotobiotic piglets are used as an animal model of choice as we have found them to most closely compare with human *C. difficile* infection, as compared with the mouse or hamster models.⁸ Gnotobiotic piglets mimic the intestinal microflora altered state that makes human patients more susceptible to *C. difficile* infection, without the need for antibiotic treatment before inoculation, and piglets also develop a similar extent and range of lesions in the large intestine and clinical signs of disease compared with humans.⁸

Systemic Administration of TcdA and TcdB

Given the results of our findings and the yet unclear nature of the roles of TcdA and TcdB during the course of infection, we designed experiments to investigate the toxins alone, without

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C. difficile infection, as a way to separate the effects of the toxins from colonization and other potential virulence factors. Purified, recombinant toxins (rTcdA and rTcdB) were administered systemically to gnotobiotic piglets: 2 received rTcdA only, 2 received rTcdB only, and 2 received rTcdA and rTcdB. These recombinant toxins were produced by our laboratory, based on *C. difficile* strain VPI 10463, as previously described.⁹ We chose doses for the two toxins based on our previous measurements of toxins in the serum, as well as the relative cytotoxicity in cell-based assays, the standard for measurement of toxins in samples, which indicate that TcdB is more toxic to cells at lower concentrations than is TcdA.¹⁰ Based on both of these previous results, we administered 10 µg of TcdA and 1 µg of TcdB to piglets via intraperitoneal injection.

The 2 piglets that received only rTcdA and the 2 that received rTcdA and rTcdB died or were euthanized within 16 h of toxin administration. All of these piglets developed signs of severe systemic illness, including respiratory distress, that we have observed in *C. difficile* infected piglets previously and have associated with systemic toxin circulation.^{8,11} The 2 piglets that received rTcdB only had no clinical signs of disease 24 h after administration, so a second dose of 10 µg rTcdB was administered. Again, the piglets developed no signs of illness, so the dose was increased to 30 µg and then 100 µg of rTcdB. At 3 h after the 100 µg dose, both piglets began to show systemic signs of illness, including respiratory distress, and were euthanized.

Intestinal and systemic lesions associated with TcdA

Gross systemic lesions in both piglets treated with only rTcdA included severe abdominal and pleural effusions (Fig. 1A), as well as diffuse hyperemia of the lungs with petechiation of the lung surface (Fig. 1B). These piglets also developed hyperemia and dilatation of the large intestine, particularly in the spiral colon, but no mesocolonic edema was present (Fig. 1C). On histopathologic examination of lung tissues, the lungs were diffusely congested with blood, with most severe regions also having atelectasis

(Fig. 2A). No inflammation of the lung tissues was noted. Microscopic lesions of the large intestine included diffuse mucosal erosion and hemorrhages with focal ulcerations (Fig. 2B and C).

Intestinal and systemic lesions associated with TcdB

Gross systemic lesions in the 2 piglets treated with only rTcdB also included severe abdominal and pleural effusions, similar in appearance to piglets treated with only rTcdA (Fig. 1A). Lung lesions in these piglets differed, however, as the lungs displayed the areas of cranial-ventral consolidation we have previously noted in piglets inoculated with *C. difficile* spores⁸ (Fig. 1D). Besides the cranial-ventral regions, other areas of the lungs had a normal appearance and texture, and appeared to be well aerated. Microscopically, the cranial-ventral areas from the lungs of these piglets appeared similar to those given only rTcdA, with regional atelectasis, congestion, and no inflammation (Fig. 2D). On necropsy, mesocolonic edema was present around the spiral colon, and the large intestinal walls were thickened, but no hemorrhage was apparent (Fig. 1E). Histopathologic examination of the large intestine revealed extensive mesocolonic edema (Fig. 2E), with some areas having largely intact mucosa (Fig. 2E), but other areas having mucosal disruption and hemorrhages (Fig. 2F).

Intestinal and systemic lesions in piglets treated with TcdA and TcdB

Both of the piglets given both rTcdA and rTcdB developed acute, fatal disease, similar to those given only rTcdA. Given that the two piglets given only rTcdB did not develop any signs of illness with a 1 µg dose of rTcdB, we expect that the illness and lesions developed in the two piglets given both toxins were likely due mostly to rTcdA. The gross and microscopic lesions observed in these two piglets were identical to those in piglets given only rTcdA with abdominal and pleural effusions, hyperemia and petechiation of the lungs, and hemorrhage and erosion of the large intestinal mucosa.

Systemically administered toxin reaches the serum, body fluids, and gut

Detectable levels of rTcdA and rTcdB were present in serum, ascites, pleural

fluid, and large intestinal contents samples collected from all piglets. Toxin concentrations were greatest in the pleural fluid and ascites with up to 10 ng/ml of rTcdB and 100 pg/ml of rTcdA, and concentrations were least in the intestinal contents with 10 pg/ml of rTcdB and 10 pg/ml rTcdA measured.

Systemic Administration of TcdA with Human Monoclonal Anti-TcdA

Following the experiment to evaluate the roles of TcdA and TcdB after systemic administration, we performed another experiment to further study the interaction of TcdA and human monoclonal anti-TcdA. The human monoclonal antibodies used here were kindly provided by Merck, Inc and were developed using the *C. difficile* strain VPI 10463.¹² The human monoclonal anti-TcdA antibodies were administered to 2 gnotobiotic piglets at 5 d of age, and then 2 µg of purified rTcdA was administered the following day. The dose of rTcdA was reduced from the previous experiment given the extreme toxicity observed with the 10 µg dose and our desire to not overwhelm the antibodies. All 4 piglets developed severe, systemic signs of illness within 24 h and were euthanized, with no apparent worsening effect in the piglets treated with anti-TcdA in addition to rTcdA.

Summary

The experiments described here provide additional insights and follow-on from our recent work using human monoclonal anti-toxin antibodies to investigate the differential roles of TcdA and TcdB in pathogenesis of *C. difficile* infection.⁶ Systemic administration of purified toxins allowed us to isolate each toxin from the other and revealed that both are quite potent and elicit severe systemic and gastrointestinal lesions, with each causing differential lesions. In contrast to the in vitro knowledge that TcdB is generally toxic to cells in much lower concentrations, we found the reality in vivo to be the opposite, with rTcdA causing equally severe disease at least a 50-fold lower dose where 2 µg of rTcdA was

Figure 1. Necropsy images from piglets treated with TcdA and TcdB systemically. (A) The thoracic cavity with pleural effusion in a piglet treated with only rTcdA. (B) Hyperemia and petechiation of the lungs in a piglet treated with only rTcdA. (C) The spiral colon of a piglet treated with only rTcdA showing hyperemia and dilatation. (D) Cranial ventral lung consolidation in a piglet treated with only rTcdB. (E) Mild mesocolonic edema and thickening of the spiral colon in a piglet treated with only rTcdB. (F) The spiral colon from a normal, uninfected piglet.

Figure 2. Histopathology images from piglets treated with TcdA and TcdB systemically. All images are from tissues fixed in formalin, imbedded in paraffin and stained with hematoxylin and eosin, 200x magnification. (A) Lung section from a piglet treated with only rTcdA, showing regional atelectasis and congestion. (B) Colon section from a piglet treated with only rTcdA showing mucosal erosion and hemorrhage. (C) Colon section from a piglet treated with only rTcdA showing mucosal erosion and hemorrhage. (D) Lung section from a piglet treated with only rTcdB showing regional atelectasis and congestion. (E) Colon section from a piglet treated with only rTcdB showing mesocolonic edema with a largely intact mucosa. (F) Colon section from a piglet treated with only rTcdB showing a focal area of mucosal disruption.

as toxic as 100 µg of rTcdB. rTcdA caused diffuse petechiation and congestion of the lungs and diffuse mucosal erosion and hemorrhage in the large intestine, while

rTcdB caused cranial-ventral congestion of the lungs and extensive mesocolonic and submucosal edema in the large intestine.

Our experiment combining systemically administered rTcdA with human monoclonal anti-TcdA antibodies was performed to further

identify possible negative interaction between TcdA and antibodies. While no worsening effect was noted in this experiment, as reported in our previous work,⁶ we found it interesting to note that anti-TcdA again provided no protection from disease, even against a physiologic level of rTcdA administered systemically with antibodies already present in circulation. We realize that with such a small experiment in a few animals, we cannot draw firm conclusions, but clearly anti-TcdA antibodies used for treatment of CDI is a topic deserving of further research to ensure that we do not make assumptions and can provide safe and effective treatments.

While these experiments need to be further expanded to include other combinations of antibody treatment and toxin administration or inoculation with spores and repeated in the future, this additional work provides important information regarding the roles of the *C. difficile* toxins. This work indicates that TcdA is toxic in lower concentrations, both are able to elicit differential systemic and gastrointestinal lesions, both can act alone or together, and both can cross into the gut lumen from the systemic circulation and vice versa.

Methods

Animals, toxin, and antibody dosing

A total of 6 gnotobiotic piglets were derived via Cesarean section and maintained in sterile isolators for the duration of the experiment, as we have previously described.⁸ The piglets were

divided into 3 groups and recombinant TcdA and/or TcdB (rTcdA or rTcdB) were administered via intraperitoneal injection. The rTcdA and rTcdB were expressed in *Bacillus megaterium* and purified in our laboratory using previously described methods.⁹ Two piglets were treated with 10 µg of rTcdA, 2 piglets were treated with 1 µg of rTcdB, and 2 piglets were treated with 10 µg of rTcdA + 1 µg of rTcdB. Toxin dosing was based on in vitro cell based assays with purified rTcdA and rTcdB, which indicate that the toxicity of rTcdB is at least 10-fold greater than rTcdA.⁹ The human monoclonal anti-TcdA antibodies used in this study were developed by Massachusetts Biologic Laboratories and Medarex, Inc.,¹² and were provided for this study and currently licensed by Merck, Inc. The anti-TcdA antibodies were administered to piglets at a dose of 10 mg/kg, based on the dosing in human studies.^{13,14} All animals were cared for and monitored according to institutional animal care and use committee protocol.

Necropsy and histopathology

All piglets received a complete necropsy following death or euthanasia to observe any gross gastrointestinal or systemic lesions. Sections of the small intestine, cecum, colon, liver, spleen, kidneys, pancreas, lung, and heart tissues were collected and immediately fixed in buffered formalin for histopathologic examination. Samples were then imbedded in paraffin and stained with hematoxylin and eosin. Blood and intestinal contents were collected from all piglets, and pleural effusion and ascites

were collected at the time of necropsy, if present.

Cytotoxicity assay

We used the ultrasensitive immunocytotoxicity assay developed by our laboratory¹⁰ to measure toxin in serum, body fluids and intestinal contents. Briefly, mRG1-1 cells expressing the FCγR1-α chain were added to wells of a standard 96 well plate, and incubated overnight before use. Samples plus the A1H3 antibody, which enhances cytotoxicity of TcdA, were added to the cells. Alpaca anti-TcdA and TcdB antibodies were used to neutralize the toxins, and serial dilutions of rTcdA and rTcdB were used as positive controls and to obtain a standard curve to determine toxin concentration. Results from the 96 well plates were evaluated for percentage of cell rounding at regular intervals for up to 24 h.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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References

- Kuehne SA, Cartman ST, Heap JT, Kelly ML, Cockayne A, Minton NP. The role of toxin A and toxin B in *Clostridium difficile* infection. *Nature* 2010; 467:711-3; PMID:20844489; <http://dx.doi.org/10.1038/nature09397>
- Lyras D, O'Connor JR, Howarth PM, Sambol SP, Carter GP, Phumoonna T, Poon R, Adams V, Vedantam G, Johnson S, et al. Toxin B is essential for virulence of *Clostridium difficile*. *Nature* 2009; 458:1176-9; PMID:19252482; <http://dx.doi.org/10.1038/nature07822>
- Triadafilopoulos G, Pothoulakis C, O'Brien MJ, LaMont JT. Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. *Gastroenterology* 1987; 93:273-9; PMID:3596162
- Tschudin-Sutter S, Widmer AF, Perl TM. *Clostridium difficile*: novel insights on an incessantly challenging disease. *Curr Opin Infect Dis* 2012; 25:405-11; PMID:22614522; <http://dx.doi.org/10.1097/QCO.0b013e32835533a2>
- Wu D, Joyee AG, Nandagopal S, Lopez M, Ma X, Berry J, Lin F. Effects of *Clostridium difficile* toxin A and B on human T lymphocyte migration. *Toxins (Basel)* 2013; 5:926-38; PMID:23645153; <http://dx.doi.org/10.3390/toxins5050926>
- Steele J, Mukherjee J, Parry N, Tzipori S. Antibody against TcdB, but not TcdA, prevents development of gastrointestinal and systemic *Clostridium difficile* disease. *J Infect Dis* 2013; 207:323-30; PMID:23125448; <http://dx.doi.org/10.1093/infdis/jis669>
- Hamm EE, Voth DE, Ballard JD. Identification of *Clostridium difficile* toxin B cardiotoxicity using a zebrafish embryo model of intoxication. *Proc Natl Acad Sci U S A* 2006; 103:14176-81; PMID:16966605; <http://dx.doi.org/10.1073/pnas.0604725103>
- Steele J, Feng H, Parry N, Tzipori S. Piglet models of acute or chronic *Clostridium difficile* illness. *J Infect Dis* 2010; 201:428-34; PMID:20039803; <http://dx.doi.org/10.1086/649799>
- Yang G, Zhou B, Wang J, He X, Sun X, Nie W, Tzipori S, Feng H. Expression of recombinant *Clostridium difficile* toxin A and B in *Bacillus megaterium*. *BMC Microbiol* 2008; 8:192; PMID:18990232; <http://dx.doi.org/10.1186/1471-2180-8-192>
- He X, Wang J, Steele J, Sun X, Nie W, Tzipori S, Feng H. An ultrasensitive rapid immunocytotoxicity assay for detecting *Clostridium difficile* toxins. *J Microbiol Methods* 2009; 78:97-100; PMID:19393695; <http://dx.doi.org/10.1016/j.mimet.2009.04.007>
- Steele J, Chen K, Sun X, Zhang Y, Wang H, Tzipori S, Feng H. Systemic dissemination of *Clostridium difficile* toxins A and B is associated with severe, fatal disease in animal models. *J Infect Dis* 2012; 205:384-91; PMID:22147798; <http://dx.doi.org/10.1093/infdis/jir748>

12. Babcock GJ, Broering TJ, Hernandez HJ, Mandell RB, Donahue K, Boatright N, Stack AM, Lowy I, Graziano R, Molrine D, et al. Human monoclonal antibodies directed against toxins A and B prevent *Clostridium difficile*-induced mortality in hamsters. *Infect Immun* 2006; 74:6339-47; PMID:16966409; <http://dx.doi.org/10.1128/IAI.00982-06>
13. Lowy I, Molrine DC, Leav BA, Blair BM, Baxter R, Gerding DN, Nichol G, Thomas WD Jr., Leney M, Sloan S, et al. Treatment with monoclonal antibodies against *Clostridium difficile* toxins. *N Engl J Med* 2010; 362:197-205; PMID:20089970; <http://dx.doi.org/10.1056/NEJMoa0907635>
14. Taylor CP, Tummala S, Molrine D, Davidson L, Farrell RJ, Lembo A, Hibberd PL, Lowy I, Kelly CP. Open-label, dose escalation phase I study in healthy volunteers to evaluate the safety and pharmacokinetics of a human monoclonal antibody to *Clostridium difficile* toxin A. *Vaccine* 2008; 26:3404-9; PMID:18502001; <http://dx.doi.org/10.1016/j.vaccine.2008.04.042>