Smart bacterial toxins

Autocatalytic cleavage of clostridial toxins – a target for novel therapeutic strategies?

Stefan Tenzer¹, Jessica Reineke²

¹ Institute for Immunology, University of Mainz, Germany
² Boehringer Ingelheim Vetmedica GmbH, Ingelheim am Rhein, Germany

• The discovery and clinical application of antibiotics is one of the most important developments in medical history. Salvarsan, introduced by Paul Ehrlich in 1910 for the treatment of syphilis, can be considered the first clinically relevant antibiotic. Since then, antibiotics have been routinely used, leading to an increased lifespan in the industrial world.

Today, antibiotics are the most frequently prescribed pharmaceuticals, with a total market share of almost 13%. However, even if most bacterial infections can be successfully treated with antibiotics nowadays, reports of infections with multi-resistant bacterial strains are on the increase. In addition, the clinical application of antibiotics may lead to severe and sometimes even life-threatening side effects, such as antibiotics-associated diarrhoea and pseudomembranous colitis (PMC). This was shown by the increase of these complications after the introduction of clindamycin in the 1970s. These side effects are not restricted to clindamycin, but can be induced by all clinically used antibiotics.

Pathology of C. difficile infections

• Clostridium difficile, a spore-forming, gram-negative bacterium, is responsible for 60% of the cases of antibiotics-associated diarrhoea and for almost 100% of the patients affected by pseudomembranous colitis. In particular, treatment with antibiotics such as cephalosporin compounds, clindamycin and fluoroquinolone compounds, as well as with broad-spectrum penicillins, is associated with a high risk of C. difficile-associated diarrhoea. The mechanism responsible for the outbreak of the disease is not yet fully understood. It might be related to both host and strain factors, since not all patients infected with C. difficile develop the disease. The clinical pattern can range from asymptomatic infection to life-threatening toxic megacolon. Case numbers are steadily increasing, e.g. in the USA from 82,000 cases in 1996 to 178,000 cases in 2003. In the last few years, several outbreaks were reported in Canada and in the USA in 2005 and recently in several European countries. Germany was first affected in March 2007. C. difficile is thus considered one of the most important nosocomial germs of developed countries.

C. difficile produces two toxins, Toxin A (TcdA) and Toxin B (TcdB) which cause antibiotics-associated diarrhoea or pseudomembranous colitis. They are very large (308 kDa and 269 kDa) bacterial proteins, which are members of the family of the so-called large clostridial cytotoxins (LCT’s) together with TcsH and TcsL of C. sordellii and TcnA of C. novyi. All these toxins display a high degree of sequence identity, a similar domain structure and harbour a glycosyltransferase moiety. Hypervirulent, ribotype 027 isolates of C. difficile recently caused some turmoil in the clinical setting, since the severe C. difficile disease...
was even affecting younger people who had not received antibiotic treatment\(^{16}\). Fluoroquinolone-resistant strains produce significantly higher toxin amounts, leading to more severe clinical symptoms\(^{16}\). There is an apparent risk that such strains could spread worldwide, which would have a serious impact on antibiotic treatment in general. We therefore need to gain a better understanding of the mode of action of TcdA and TcdB to combat \emph{C. difficile} infections.

**Biology of clostridial toxins**

- Both toxins are single-chained proteins characterized by a tripartite functional organization\(^{16}\). Their C-terminal domain is required for binding to the plasma membrane of the target cell\(^{17}\), the hydrophobic middle part is a putative translocation domain and the N-terminal catalytic domain of the proteins carries the glycosyltransferase site (Figure 1). The uptake process into the cytosol of the target cell has not yet been fully characterized. However, it is generally accepted that the toxins are endocytosed after binding to cell surface receptors\(^{18}\). After acidification of the endosomes, only the N-terminal domain of the toxin translocates into the cytosol\(^{19}\). The translocation is supposedly mediated by pore formation, since TcdA forms pores in artificial membranes at low pH\(^ {20}\).

Activation of the toxin requires proteolytic cleavage between the amino acids Leu543 and Gly544, which liberates a small fragment of 63 kDa that harbours the N-terminal catalytic domain into the cytosol\(^{20}\). The larger, 207 kDa C-terminal part of TcdB remains in the membrane fraction.

As shown by microinjection experiments, the N-terminal 63 kDa fragment displays full cytotoxic activity, while microinjection of the full-length toxin shows a delayed response. This confirms the requirement of proteolytic cleavage for catalytic activity of TcdB. Once liberated, the N-terminal glycosyltransferase domain can move freely in the cytosol to inactivate its target proteins, GTPases of the Rho/Rac family\(^{21}\). These proteins are involved in many cellular functions, e.g., organization of the actin cytoskeleton, control of transmembrane cell polarity and proliferation.

**Inositolphosphates are required for autocatalytic activation of TcdB**

- Proteolytic processing has been described for different bacterial toxins. For example, diphtheria toxin is cleaved by the endoprotease furin after binding to its specific receptor\(^ {22}\). Furin is a ubiquitous endopeptidase, which normally catalyses the cleavage and concurrent activation of proproteins and prohormones\(^{23}\). It is also required for cleavage of the binding component of anthrax toxin, known as the protective antigen\(^ {24}\). Again, this proteolytic cleavage is required for activation and uptake of the toxin via the endocytic pathway\(^{25}\).

But which protease catalyses the activation of TcdB? Until recently, this was believed to be the work of a pepstatin A-sensitive host protease\(^ {26}\). We therefore set out to identify the host protease required for activation of TcdB. In pilot experiments, TcdB was efficiently cleaved when co-incubated with cytosolic extracts from porcine splenocytes\(^ {28}\). After several chromatographic purification steps, including ion exchange and gel filtration chromatography, we obtained a highly active fraction that displayed a pronounced cleavage activity when co-incubated with highly purified toxin B. However, biochemical analysis of this fraction showed several inconsistencies with our initial hypothesis. The active fraction showed no significant UV absorption at 280 nm and was still active after being heated to 96 °C for 30 min. Furthermore, even treatment with proteases or phenolchloroform extraction did not affect cleavage activity.

Finally, by analysing the active fraction by nano-electrospray-mass spectrometry in negative-ion mode, we were able to obtain high-quality fragmentation spectra. The comparison of the acquired spectra with the literature enabled us to identify inositolphosphates as the active component purified from the splenocyte extracts. Once inositolphosphates had been identified as an essential co-factor for proteolytic activation of TcdB, and in view of the fact that cleavage activity has never been observed for inositolphosphates themselves, we hypothesized that the cleavage activity may, in fact, not be attributed to a host protease, but intrinsic to TcdB.

Cleavage of TcdB is inhibited by pepstatin A, an inhibitor for aspartyl proteases. We decided to use the aspartyl protease inhibitor 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP) to inactivate and identify the active protease site of TcdB by covalent modification\(^ {28}\). This treatment completely abolished not only *in-vitro* cleavage of the toxin in the presence of inositolphosphates, but also its cellular toxicity when tested in cell culture. This effect was not seen when the toxin was microinjected directly into the cytosol of target cells. This experiment showed that EPNP-treatment modified only the protease site of
TcdB, leaving the N-terminal glycosyltransferase activity intact.

**Characterization of the active protease of TcdB**

- Using mass spectrometric analysis of a tryptic digest of the EPNP modified toxin, we succeeded in identifying a peptide fragment carrying the EPNP-modified active site which was localized to the motif DSG at position 1,665(28). However, the DSG motif at position 1,665 may not be the only active protease site of TcdB(29). Egerer et al.(30) compared the amino acid sequence of TcdB and RTX toxin of *Vibrio cholerae*, which harbours a putative cysteine protease site (Figure 1). In contrast to our data, they observed that autocatalytic cleavage is inhibited by the cysteine protease inhibitors N-ethyl-maleimide and iodoacetamide. They then expressed recombinant toxins, which led to mutation in all three amino acids to form the catalytic triad of the homologous active cysteine protease site. They observed that, in contrast to recombinant wild-type toxin, neither of the three mutants showed autocatalytic cleavage in the presence of inositolphosphates. Furthermore, Rac-glucosylating activity and cellular toxicity were detected only for the recombinant wild-type toxin, indicating that both asparyl and cysteine protease functions may be required for the activation of TcdB.

**New treatment strategies for C. difficile infections**

- By and large, these findings pave the road for the development of novel treatment strategies for *C. difficile* infections. Since toxin activity is one of the key virulence factors of *C. difficile*(35), one innovative treatment strategy would be not to combat the pathogen itself, but to inactivate its main weapons. Some naturally occurring strains of *C. difficile* lack TcdA and TcdB and can act as a protection from antibiotic-induced diarrhoea and PMC. This makes the inactivation of the toxins an attractive strategy.

All current treatment strategies for the antibiotic-induced *C. difficile* infections are, paradoxically, based on the application of different antibiotics(34,36). A novel treatment strategy without the use of antibiotics could be based on directly inactivating the toxins by exploiting their need for autocatalytic activation. There are two main targets for this strategy. The proteolytically active site of the toxin could be inactivated by the application of specific protease inhibitors, a principle already converted into a therapy for HIV infections. The second, and possibly even more fascinating strategy, is to inactivate the toxin by autocatalytic cleavage in the gut before it can reach the target cell. Autocatalytic cleavage occurs very quickly, even in the presence of low micromolar concentrations of InsP₆, and only full-length toxin displays cellular toxicity. For this reason, therapeutic delivery of InsP₆ – which is already being used as a nutritional supplement in the USA – into the colon could efficiently block toxin action and therefore prevent damage to the intestinal epithelium.

**Acknowledgement**

- This work was supported by the Sonderforschungsbereich ‘Invasion
und Persistenz bei Infektionen’ und die Immunologie Cluster of Excellence at the University of Mainz.

References