Toxins A and B of Clostridium difficile

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Abstract: The toxins produced by Clostridium difficile share several functional properties with other bacterial toxins, like the heat-labile enterotoxin of Escherichia coli and cholera toxin. However, functional and structural differences also exist. Like cholera toxin, their main target is the disruption of the microfilaments in the cell. However, since these effects are not reversible, as found with cholera toxin, additional mechanisms add to the cytotoxic potential of these toxins. Unlike most bacterial toxins, which are built from two structurally and functionally different small polypeptide chains, the functional and binding properties of the toxins of C. difficile are confined within one large polypeptide chain, making them the largest bacterial toxins known so far.

Key words: Clostridium difficile; Toxin A; Toxin B; Mechanism; Receptor

Introduction

The disease caused by the toxins of Clostridium difficile is known as antibiotic-associated diarrhea (AAD) and the clinical picture ranges from a mild diarrhea to a life threatening pseudo membranous colitis (PMC). PMC is mainly observed in patients treated with antibiotics [1-5] and is suspected when patients present with watery or bloody diarrhea. C. difficile produces at least two toxins which clearly contribute to the development of PMC [6]. Some investigators suggest the existence of additional toxins produced by C. difficile, but their existence has to be confirmed, or is strain-specific, and their action and importance in relation to PMC is not yet established [7-11]. The main toxins are designated as toxin A, an enterotoxin [11,12], and toxin B, a cytotoxin [13], according their elution sequence on an ion-exchange resin [14,15]. Although several molecular masses have been reported ranging from 50 kDa to 300 kDa, DNA sequence determination unambiguously showed that the molecular mass for toxin A is 308 kDa [16,17] and for toxin B 270 kDa [18,19]. The amino acid sequence of both toxins shows 63% homology [18,20], but significant differences also exist between the two toxins. Still, it remains difficult to assign specific functions to these toxins. History has shown that in several cases incorrect assignments were made: glutamate dehydrogenase was used in a test to detect toxin A [21], enolase was reported as a property of toxin B [22] and ADP-ribosylation was later shown not to be a characteristic of toxin B [23,24].
Toxin A

Toxin A is the largest bacterial toxin hitherto identified [25]. Toxin A forms homodimers under physiological conditions. This enables toxin A to bind via a multivalent binding mechanism to carbohydrate groups present on target cells. Subsequently, the toxin is concentrated in coated pits and internalized. After being processed in an acidic compartment, it exerts its effect on the cytoskeleton. Disrupting the cytoskeleton ultimately causes the death of the intoxicated cell.

For toxin A four major biological activities were reported [25]. Toxin A is enterotoxic, cytotoxic [13], presumably cytotonic [25–27] and causes agglutination of rabbit red blood cells [25]. No classical enterotoxicity similar to cholera toxin was observed, but a hemorrhagic exudate containing sodium, potassium, chloride and protein [12,28]. Unlike cholera toxin, toxin A causes cell death [29] and does not influence the cAMP level, the main toxic property of cholera toxin [30]. For toxin B, only lethal and cytotoxic properties are described [25]. The main properties of these toxins are summarized in Table 1. The cytotoxic mechanism of toxin A is different from that of toxin B since an antiserum to the latter did not inhibit cytotoxicity of toxin A [26]. However, it is possible that this antiserum to toxin B inhibits the binding of toxin B to the cell surface and consequently the cytotoxicity of toxin B. If toxin A uses different binding strategies, it still can bind to its target cell after treatment with the antiserum to toxin B. In such a case the cytotoxic principles could still be similar. Besides the differences, large homologies exist between the two toxins, mostly exemplified by the neutralization of both toxins by polyclonal anti-Clostridium sordellii anti-toxin [31]. Additionally, both toxins are neutralized by modification of only one arginine residue [32]. It is not yet known at which step this residue is involved.

The mode of action of toxin A can be divided into several phases. The cell membrane receptors are disputed. Several studies were performed using animal cells as target at 4°C. For those cells, the Galα1-3Galβ1-4GlcNac carbohydrate structure is the target structure for the toxin. However, the binding to this carbohydrate is temperature-dependent, a feature used to isolate the toxin. The toxin does not bind to thyroglobulin at 37°C, which has 11 Galα1-3Galβ1-4GlcNac groups on its surface [33]. No data are available showing binding of the toxin at 37°C to cells that express a large number of Galα1-3Galβ1-4GlcNac residues. This Galα1-3Galβ1-4GlcNac carbohydrate is not expressed on human nucleated cells [34,35] and therefore cannot serve as the receptor. One other carbohydrate structure, expressed on human cells, was reported to bind to toxin A at 37°C [36]. The C-terminal part of the toxin contains repetitive carbohydrate binding sites [37]. The structural make-up of the toxin shows 38 putative carbohydrate binding domains, 76 in total for the native dimeric protein. This enables toxin A to bind with an enormous number of binding sites to a cell, which expresses over 1000 carbohydrate structures on its surface. This

| Structural and functional properties of toxin A, toxin B and cholera toxin |
|---|---|---|
| **Molecular mass** | Toxin A | 308 kDa | Toxin B | 270 kDa | Cholera toxin | 27 kDa (A chain) |
| Subunit structure | A₂ | 11.6 kDa (B chain) |
| **Target ligand** | Carbohydrate | Probably A | AB₅ |
| **Valency of binding** | Up to 76 | Not known | Ganglioside GM₁ |
| **Main functional properties** | Enterotoxin | Cytotoxin | Enterotoxin |
| **Influen of granulocytes** | Yes | Unknown | No |

* A₂, a dimer of two identical monomers; AB₅, a heterohexamer consisting of one A subunit and five B subunits.
permits a low affinity of a single binding site, because of complete compensation by a very high valency. This enables the toxin to bind firmly to carbohydrate structures present on the cell. However, binding to single carbohydrate structures will be minimal due to the low affinity. Such a multivalent binding phenomenon is not unique. Cholera toxin uses the same strategy, although to a much lesser degree [38]. Cholera toxin binds ganglioside GM₁ by an oligomer composed of five identical low-molecular mass subunits [38,39], and it has the property to create additional accessible receptor sites by production of neuramidase that transforms higher-order gangliosides into GM₁, the target ganglioside for cholera toxin [38].

After binding to the cell membrane, which is dependent on Ca²⁺ [25], toxin A is evenly distributed [27]. At this stage it is sensitive to papain and chymotrypsin [40] but not to trypsin. This is remarkable because of the large number of lysine and arginine residues present in the toxin. The fact that those residues are shielded from trypsin attack after binding to the cell surface suggests that those residues are either involved in binding or are in close proximity to the binding sites. In the next step the toxin is found in coated pits and internalized [27]. This internalization is inhibited by an anti-secretory factor produced by the pituitary gland [41] and is released after oral challenge with toxin A. A fluid response is caused [42] due to a permeability increase and is located in the tight junctions [43]. Prostaglandin E₂, together with leukotriene B₄ is secreted in the lumen [44]. This is different as found for cholera toxin [38]. The amount of prostaglandin is a measure for the permeability. However, its release is independent from the fluid secretion or the inflammatory response. Recently, the effect of toxin A (and toxin B) on signal transduction pathways was studied [45,46]. Of the pathways studied, the effect of toxin A was mainly directed to phospholipase A₂, resulting in the generation of arachidonic acid. Phospholipase A₂, specific for arachidonic acid, is involved in the arachidonic acid cascade, which generates several metabolites like prostaglandins and leukotrienes, explaining the release of these mediators after toxin treatment. Toxin A is chemotactic and chemokinetic towards granulocytes and a marked influx is observed [44]. This influx of granulocytes is not seen in the case of cholera toxin. It is not known either at which stage the influx takes place. The internal Ca²⁺ concentration of the granulocytes is raised, reflecting that those cells were activated [47]. This influx and the subsequent death of the enterocyte causes the release of leukotriene B₄. The membrane of the intoxicated cell forms blebs [30]. Both effects could be mediated by the action of phospholipase A₂. The internalized toxin, present in an endosome, fuses with a lysosome, because lysosomotropic agents prevented the cytopathogenic effect [48]. The cytotoxic effect could also be inhibited by quinacrine, an inhibitor of phospholipase A₂, resulting in the inhibition of the fusion of the endosome with a lysosome [45]. Monensin, a drug able to raise the pH of intracellular acidic vesicles, blocked the effects of toxin A [49], while no influence was found on the cellular effects by toxin A of Ca²⁺-dependent cytosolic protease inhibitors like leupeptin and antipain [30,49]. Additionally, inhibitors of lysosomal proteases could not prevent the appearance of cytopathogenic effect [48]. Others showed no influence of lysosomotropic agents [12]. An acidic compartment and the action of a serine protease seem essential for the activity of toxin A [49]. After intracellular processing the toxin exerts its effect on several proteins of the cytoskeleton. Vinculin is not expressed at all, while vimentin and α-tubulin were diffused in intoxicated cells [49]. The amount of F-actin is decreased [43], probably due to the phosphorylation of tropomyosin [50].

Remarkably, a monoclonal antibody that does not prevent cytotoxicity in vitro completely inhibited the secretory and tissue-damaging effect in the intestine [12].

**Toxin B**

Toxin B binds to membrane receptors that are yet unknown. Unlike toxin A it does not bind to the Galα₁-3Galβ₁-4GlcNac carbohydrate structure present on animal cells. The separation of the two toxins on bovine thyroglobulin that bears
11 Galα1-3Galβ1-4GlcNac carbohydrate chains is based on this difference in binding behavior. Contrary to toxin A, no fluid response is observed [42] by toxin B-treated cells. Toxin B-treated cells show an arborized phenotype [29,51]. The binding is reversibly inhibited by 2,4-dinitrophenol, an inhibitor of the energy metabolism [52]. Dissimilar to toxin A, toxin B is sensitive to trypsin after binding to the cell [53]. The internalization of the toxin is dependent on Ca²⁺ ions [54] and can be blocked with calcium channel blockers verapamil and LaCl₃ [55]. Cytopathogenic effects are thwarted by inhibitors of the energy metabolism after the endocytosis is completed [53]. ADP-ribosylation was reported as a property of the toxin [23]. This would mean that the toxin has similar properties as the cholera toxin A subunit and E. coli heat-labile enterotoxin [38]. Indeed, the in vivo fluid accumulation is similar for all those toxins. However, the same authors could separate the ADP-ribosylation from the cytotoxicity after extensive purification [24]. Enolase activity was also assigned to toxin B [22]. However, Green et al. [56] and our group [57] could independently prove that enolase and toxin B are different entities, although they form a complex and co-purify in several purification methods. Treatment with tumor-promoting phorbol esters or dibutyryl-cAMP prevents the toxin-induced morphological changes [54]. The cytopathogenic effect of toxin B was inhibited by monensin [55], chloroquine and ammonium chloride [53]. Furthermore, mutant CHO cells that are defective in acidification of its endosomes were resistant to toxin B [58]. At conditions when the fusion between endosomes, containing toxin B, and lysosomes is impeded by KCl or benzyl alcohol the cytopathogenic effect is reversely inhibited [58]. Inhibition of lysosomal proteases also blocks the cytopathogenic effect [58]. All these experiments point to a processing of the toxin in an acidic compartment before it exerts its effects leading to the degradation of F-actin [59–62]. Several other proteins of the adhesion plaque proteins, like vinculin and talin, are relocated in the intoxicated cell [50,61]. Cells treated with the toxin form membrane protrusions or blebs [51]. The formation of these blebs has been assigned to the activation of phospholipase A₂ [46]. The blebs are free of β₂-microglobulin, actin, vimentin and keratin, while tubulin, filamin, α-actinin and calmodulin are found in the blebs. Disruption of the F-actin seems to be mediated by the incorporation of anorganic phosphate in tropomyosin and vimentin [50]. Phosphorylation of tropomyosin results in the severing of microfilaments and capping of short filaments. Many of the actions of toxin B are analogous to the mechanism as was described for cytochalasin B [63]. However, cytochalasin is not toxic for the cells and its effects are fully reversible. Thus, using a slightly different intracellular routing than toxin A it also breaks down the cytoskeleton. The disruption of the cytoskeleton leads finally to the death of the cell. An additive effect to the death of the cell is the activation of phospholipase A₂ and C, leading to the formation of several inflammatory mediators [46]. This activation is independent from the microfilament disorganization [46] and is not observed for cholera toxin.

Toxin B is able to inhibit protein synthesis in several intestinal cell types, which could add to the pathophysiological effects seen in patients suffering from antibiotic-associated colitis [64]. The action of toxin B is not restricted to enterocytic cells. Mononuclear phagocytes show similar cytoskeletal rearrangements after treatment with the toxin but the cells remain viable [65]. Mononuclear phagocytes play an important role in inflammation through the production of cytokines. The production of cytokines like tumor necrosis factor after treatment with toxin B and interferon has been demonstrated [65] and may add to the pathogenesis.

References

10 Popoff, M.R., Rubin, E.J., Gill, D.M. and Boquet, P.


Clostridium difficile binds to the human carbohydrate anti-
logous repetitive C-terminal carbohydrate-binding sites of Clostridium difficile toxins and Streptococcus mutans glucosyltransferases. J. Bacteriol. 174, 6707–6710.
41 Torres, J., Jennische, E., Lange, S. and Lönroth, I. (1991) Clostridium difficile toxin A induces a specific antisecretory factor which protects against intestinal mucosal dam-
44 Triadafilopoulos, G., Pothoulakis, C., Weiss, R., Gi-
46 Shoshan, M.C., Florin, I. and Thelestam, M. (1993) Activi-
47 Pothoulakis, C., Sullivan, R., Melnick, D.A., Tri-
adafilopoulos, G., Gadenne, A.S., Meshulam, T. and La-
lar internalisation of Clostridium difficile toxin A. Microb. Pathog. 2, 455–463.
49 Fiorentini, C., Malorni, W., Paradisi, S., Giuliano, M., Mastrandono, P. and Donelli, G. (1990) Interaction of Clostridium difficile toxin A with cultured cells: cytoskele-
51 Malorni, W., Fiorentini, C., Paradisi, S., Giuliano, M., Mastrandono, P. and Donelli, G. (1990) Surface blebbing and cytoskeletal changes induced in vitro by toxin B from Clostridium difficile: an immunochemical and ultrastruc-
52 Florin, I. and Thelestam, M. (1981) Intoxication of cul-
58 Florin, I. and Thelestam, M. (1986) Lysosomal involve-
ment in cellular intoxication with Clostridium difficile toxin B. Microb. Pathog. 1, 373–385.
62 Wedct, N., Toselli, P., Pothoulakis, C., Faris, B., Oliver, P., Fran
gblau, C. and LaMont, T. (1983) Ultrastructural ef-
63 Shoshan, M.C., Ómáin, P., Skog, S., Florin, I. and The-
64 Pothoulakis, C., Triadafilopoulos, G., Clark, M., Franzblau, C. and LaMont, J.T. (1986) Clostridium difficile cytotoxin inhibits protein synthesis in fibroblasts and intestinal mu-
cosa. Gastroenterology 91, 1147–1153.
65 Siffert, J.-C., Baldacini, O., Kuhry, J.-G., Wachsmann, D., Benabdelloumene, S., Faradji, A., Monteil, H. and Poi
dron, P. (1993) Effects of Clostridium difficile toxin B on human monocytes and macrophages: Possible relation-