Bacterial type AB₅ enterotoxins — structure, function and mechanism of action

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Abbreviations: CT — cholera toxin; LT — heat labile enterotoxin; ST — Shiga toxin; SLT — Shiga-like toxin; Gb₁ — globotetraosylceramide; Gb₂ — globotetraosylceramide; GM₁ — monosialotetrahexosylganglioside

ABSTRACT

One of the main factors causing bacterial diarrhea are AB₅ enterotoxins. This group is divided into four families: pertussis toxin, cholera toxin, shiga toxin and subtilase cytotoxin. In this review we will describe the activity, structure and function of the cholera and shiga toxin families. The AB₅ enterotoxins contain a catalytic subunit A and pentameric subunit B, which binds to the cell surface within lipid rafts. The cholera toxin family cause the constitutive activation of Gₛ protein, which results in CAMP production, an opening of the chloride channels and releases chloride ions into the lumen of the small intestine. In contrast, the shiga toxin family has a cytotoxic effect on epithelial cells. It can inhibit protein synthesis leading to cell death. Although AB₅ has a toxic activity, the B₁ subunits have a significant potential as a transporter for proteins with anticancer activity and as a tool for the visualization of lipid rafts and cancer cells.

INTRODUCTION

AB₂ toxins are important virulence factors for many bacterial species such as Escherichia coli, Vibrio cholerae and Shigella dysenteriae. There are four main groups of AB₂ toxins, characterized by the sequences of the A subunits and their catalytic properties: pertussis toxin, cholera toxin, shiga toxin and subtilase cytotoxin [1]. The AB₂ toxins contain a catalytic subunit A and a pentameric subunit B that binds to receptors on the cell surface. In this review, we will focus on the cholera toxin family containing cholera toxin produced by V. cholerae, the closely related heat-labile enterotoxins type I and II, produced by enterotoxigenic E. coli (ETEC) and the shiga toxin family, which is divided into three groups: Shiga toxin produced by S. dysenteriae and Shiga-like toxins types I and II produced by Shiga toxigenic E. coli (STEC). V. cholerae, S. dysenteriae and E. coli are responsible for many fatal cases of bacterial diarrhea. While many of these cases occur in developing countries, some rather devastating outbreaks do occur in the food industry of developed countries. In all cases, infections occur when patients ingest contaminated food or water, or by direct contact with infected animals or humans.

PATHOGENESIS

VIBRIO CHOLERAE

V. cholerae is a gram-negative, comma-shaped bacterium with a single polar flagellum. V. cholerae includes pathogenic and non-pathogenic strains, with the latter being predominant. Among 200 serogroups of V. cholerae only two (O139 and O1 also named classic and El Tor biotypes, respectively) are known to cause the very dangerous disease, cholera [2]. The major reservoirs of these pathogens are contaminated water and previously infected individuals. After ingestion of V. cholera, most of the bacteria are killed by gastric acid but some of them can survive to colonize the organism [3]. The essential virulence factor for the colonization of the small intestine is toxin coregulated pilus (TCP) [4], while cholera toxin is the major virulence factor causing diarrhea [5]. The accepted infectious dose of V. cholerae is 10⁶ cfu. However, this figure can be lower if the gastric acidity is reduced [6]. Cholera is a rapid and often fatal infection. The main symptoms are watery diarrhea (even up to 11 times per hour), vomiting, followed by acidosis, drowsiness and unconsciousness. In the worst case scenario patients may die within 2 h from circulatory collapse if no supportive treatment is provided. Left untreated, cholera results in about a 50% fatality rate but timely administration of oral rehydration solution (ORS — mixture of salt and sugar) can reduce the mortality rate to below 1% [6]. The highest risk of cholera outbreaks is related to overcrowded areas with poor sanitation and unsafe drinking water, such as found in India or developing countries but also during any natural disaster affecting large population [7]. In 2010 in Haiti, nine months after the earthquake, an outbreak of cholera started that quickly spread all across the country. Until 2014, 8,534 deaths and 697,256 cholera cases have been reported by the Haitian
Ministry of Public Health and Population. It was the largest cholera outbreak during recent years [8].

ESCHERICHIA COLI

E. coli is a gram-negative, rod-shape bacterium. Most E. coli strains are not harmful for humans and are a part of the normal gut flora. Some of them, however, have an ability to cause gastrointestinal diseases. According to the mechanism of pathogenesis, E. coli strains can be divided into at least six groups.

Enterotoxogenic E. coli (ETEC) are classified as pathogenic strains. ETEC infection is usually transmitted through the consumption of contaminated water or food. 10^8 CFU is sufficient to initiate infection. The major virulence factors of ETEC are the production of heat-stable and heat-labile enterotoxins [9]. ETEC strains are associated with weaning diarrhea among children in the developing world and traveler’s diarrhea [9].

Shiga-toxin producing E. coli (STEC) includes two serotypes: O157 and non-O157 and is a major food-borne pathogen worldwide. The main reservoirs of STEC are animals, as well as water and food contaminated by their feces [10]. STEC can produce two kinds of Shiga-like toxins – type I and II, which are major virulent factors responsible for bloody diarrhea and hemolytic uremic syndrome (HUS) [11]. STEC production is acquired due to infection by a prophage carrying the DNA coding for the toxin and nonproducing strains can be transformed into producing ones after incubation with STEC strains. The largest reported outbreak of STEC with 3,842 cases and 53 fatal outcomes happened in 2011 in Germany. Most of the deaths were related with HUS, which is clinically defined by thrombocytopenia, nonimmune microangiopathic hemolytic anemia and acute kidney failure [13].

SHIGELLA DYSENTERIAE

S. dysenteriae is a rod-shape bacterium closely related to E. coli species. S. dysenteriae induces shigellosis, which leads to bloody diarrhea. This symptom is related with the destructive effect of Shiga toxin on intestinal epithelial cells. Shigella is spread by direct contact with an infected person, contaminated food and water [14]. S. dysenteriae can cause HUS but less frequently than STEC [13].

STRUCTURE OF AB_5 ENTEROTOXINS

Enterotoxins such as cholera toxin (CTX, CT), shiga toxin (STX, ST) and heat-labile enterotoxins (LT-I, LT-II) belong to the AB_5 toxin family. The AB_5 toxins consist of two main structural subunits: heterodimeric subunit A (CTA) and homopentameric subunit B (CTB). Subunit A consists of two polypeptide chains — A_1 and A_2, linked by a single disulfide bridge. CTA is linked to both CTB and CTA, CTA, has an enzymatic function. Subunit B consists of five monomers, arranged in ring-like (doughnut-like) configuration with binding sites for the plasma membrane receptor [5]. Despite their similar structure and function, the amino acid composition of AB_5 proteins is different. The structures of cholera, shiga and heat-labile enterotoxins are shown in Fig. 1.

CHOLERA TOXIN FAMILY

Cholera toxin belongs to type I of the heat-labile enterotoxins and is produced mainly by two serogroups of V. cholerae – O1 and O139 [15]. The subunit A consists of a 22 kDa A_1 fragment and 5.4 kDa A_2 fragment. The A_2 fragment provides an anchor between the A_1 and pentameric subunit B. Subunit A_1 has an enzymatic (toxic) function. Subunit B of cholera toxin consists of five identical 11.6 kDa B monomers, formed in a donut-like, ring shape [5].

Heat-labile enterotoxins are produced by enterotoxigenic E. coli (ETEC) and belong to two subfamilies of cholera toxins — heat-labile enterotoxins type I and type II. Heat-labile enterotoxins type II contain three variants: LT-IIa, LT-IIb [16] and LT-IIc [17], which differ in their amino acid sequences. In terms of both structure and function, LT and LT-II are closely related to CT [18] but there are some differences in the amino acid composition. In both types, the A subunits are highly homologous. In contrast, the amino acid sequences of the B subunits are less well conserved [19]. While the amino acid sequences of subunits B of CT and LT-I exhibit over 80% identity, the B polypeptides of LT-II have little homology (<14%) to CTB and LTB amino acid sequences [20]. The structures of LT and CT are shown in Fig. 1A and B.

Figure 1. The 3D ribbon structures of (A) cholera toxin, (B) heat-labile enterotoxin type I and (C) shiga toxin. Subunit A is labelled yellow. Each monomer of the pentameric subunit B is labelled with different colours: red, grey, blue, green and pink. From [25], modified.
SHIGA TOXIN FAMILY

Shiga toxin (STX, ST), Shiga toxin I (SLTI) and II (SLTII) belong to the Shiga toxin family [1] STX is produced by *Shigella dysenteriae*, SLTI and SLTII by *E. coli* (STEC — Shiga toxin producing *E. coli*). Subunit A of Shiga toxin is 32.2 kDa in size and consists of a 27.5 kDa A1 fragment and 4.5 kDa A2 fragment linked by a disulfide bridge. The subunit B consists of five identical 7.7 kDa monomers. The structure and amino acid composition of STX and STXI are almost identical (92–100% homology) whereas SLTII is identical only at 56% of amino acid residues [11]. The structure of ST is shown in Fig. 1C.

BINDING TO RECEPTORS

In nature, the enterotoxin subunit B recognizes and binds to gangliosides (sialylated glycosphingolipids) in the apical membrane of intestinal epithelial cells [21]. Structurally, gangliosides are oligoglycosylceramides that contain N-acetylneuraminic acid (sialic acid or NeuAc) residues or less commonly N-glycolyl-neuraminic acid (NeuGc) joined via glycosidic linkages to one or more of the monosaccharide units [20]. Gangliosides are present in all cell types but especially abundant in neurons. They play important roles in many cellular physiological processes, including differentiation, memory control, cell signaling, neuronal protection, neuronal recovery, and apoptosis. Gangliosides can also be anchors and entry points for toxins, bacteria, viruses, and autoantibodies [22]. The types of gangliosides vary significantly between different species and different cell types within a single species. This diversity arises from the various arrangements by which the sugar groups are arranged on the ceramide core [23]. The receptors for CT, LT and LT-II are one or more gangliosides that are bound by the B subunit. CT is able to bind only GM1 (monosialotetrahexosylganglioside), LT has the highest affinity for GM1 but can bind also to polyglycosylceramides, asialo-GM1, GM2, and polylactosamine-containing glycoproteins. LT-II binds to GD1a (LT-IIa) and GD1b (LT-IIb) [20]. Each B pentamer has five binding sites for its respective ganglioside receptor, thus enabling the holotoxin to crosslink multiple gangliosides. Each receptor-binding site on the toxin is found to lie primarily within a single B subunit, while there is a single solvent-mediated hydrogen bond from residue Gly33 of an adjacent subunit. The large

![Diagram of cholera toxin mechanism of action](image)

Figure 2. Scheme of cholera toxin mechanism of action. Cholera toxin (CTX) binds to GM1 and is endocytosed from the plasma membrane by different types of endocytic mechanisms. Next, it is transported to the Golgi apparatus (GA) and then to the endoplasmatic reticulum (ER). In the ER, subunit A (CTA) dissociates from subunit B (CTB), where it is next unfolded and translocated across the membrane by Sec61 protein. In the cytosol, refolded CTA forms a complex with ARF6. The CTA/ARF6 complex causes an activation of the Gα-subunit of protein G (Gsα) that results in the activation of adenylate cyclase (AC) and continuous production of cAMP. The high concentration of cAMP in the cytosol results in the opening of cAMP-dependent chloride channels (cystic fibrosis transmembrane conductance regulator — CFTR), which in turn causes secretion of chloride ions into the lumen of the small intestine. Images by M. Komiazzyk.

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majority of interactions between the receptor and the toxin involve the two terminal sugars of GM₃, galactose and sialic acid, with a smaller contribution coming from the N-acetyl galactosamine residue. Both the B subunit and the holotoxin, bind to the gangliosides, while monomer B is not capable to bind single molecules of GM₃ [21].

Shiga toxins have the highest affinity to Gb₃ and Gb₄. Each B monomer has three potential binding sites, so theoretically the holotoxin binds to fifteen Gb, or Gb₄ molecules [11]. The Gb₃ binding affinity for SLTI (or ST) is stronger than for SLTII although SLTII is suggested to form a more stable Gb₃ complex [11].

MECHANISM OF ACTION OF AB₅ ENTEROTOXINS

CHOLERA TOXIN FAMILY

The example of cholera toxin will be described to explain the pathway of enterotoxin entry into the cell cytoplasm. Subunit B of cholera toxin binds to five molecules of monoganglioside GM₃. CT is internalized from the plasma membrane by endocytosis. This process occurs by different pathways (caveolin- or clathrin-dependent pathway but also in a number of cell type caveolin- or clathrin-independent pathways), which can function simultaneously [23]. The CT then undergoes retrograde trafficking via a glycolipid-dependent pathway through the trans-Golgi network to the lumen of the endoplasmic reticulum (ER). Here, the A1 subunit is dissociated from CT, unfolded by protein disulfide isomerase (PDI), translocated across the Sec61 channel of the ER membrane and released into the cytosol, where it quickly refolds. In the cytosol, ARF6 binds to CTA1. The active complex of CTA1/ARF6 stimulates proprotein disulfide isomerase-dependent pathway through the trans-Golgi network to the lumen of the endoplasmic reticulum (ER). Here, the A1 subunit is dissociated from CT, unfolded by protein disulfide isomerase (PDI), translocated across the Sec61 channel of the ER membrane and released into the cytosol, where it quickly refolds. In the cytosol, ARF6 binds to CTA1. The active complex of CTA1/ARF6 stimulates protein G by ADP-ribosylation of the α-subunit thus inhibiting the hydrolysis of GTP to GDP and leaving adenylate cyclase (AC) constitutively activated [24]. The activated AC catalyzes the conversion of ATP to cAMP. The high concentration of cAMP results in the opening cAMP-dependent chloride channels (cystic fibrosis transmembrane conductance regulator – CFTR), which results in the secretion of chloride ions into the lumen of the small intestine [25,26] (Fig. 2).

Accumulation of Cl⁻ results in the secretion Na⁺ into the lumen of the small intestine across the tight junction. Accumulation of NaCl in the small intestine lumen creates an osmotic gradient that results in water outflow into the small intestine lumen across the tight junction (Fig. 3) [21].

SHIGA TOXIN FAMILY

Trafficking via the cell membrane and transport into the cytoplasm is similar for both cholera and shiga toxin families. The main difference between these two toxin families is the enzymatic activity of subunit A. Unlike the cholera toxin, subunit A of Shiga toxin has cytotoxic activity. STA inactivates the 60S ribosomal subunit, which affects the inhibition of protein synthesis and death of the host cell. Shiga toxins are also known to induce programmed cell death, or apoptosis, in many cell types, which seems to be related to the activation of the ribotoxic stress response or induction of the unfolded protein response (UPR) [11].

GENETICS AND SECRETION OF AB₅ ENTEROTOXINS

The majority of pathogenic bacteria contain bacteriophages integrated into their DNA (prophages). In fact prophages can form even 3–10% of the genome of many bacterial strains [21]. Many of these bacteriophages encode virulence factors, e.g. botulin toxin or enterotoxin A produced by Clostridium botulinum and Staphylococcus aureus, respectively. AB, enterotoxins are also virulence factors encoded by prophages [24]. The most studied virulence factors are genes encoding choler toxin (ctxAB). ctxAB genes are carried by a filamentous, lysogenic bacteriophage known as CTXφ, which is integrated into the bacterial chromosome. The CTXφ DNA is found integrated at either one (in El Tor V. cholerae) or two (classical O1 V. cholerae) sites within the V. cholerae genome [27]. The CTXφ integrated with DNA El Tor strains, in contrast to classical strains (O1), can produce virions that can then infect other bacterial strains [28]. Expression of ctxAB genes is regulated by bacterial proteins activated by quorum sensing or, in some cases, by phage replication. CT is transported to the periplasm and then secreted by the type II secretion system [28].

The nucleotide sequence of CTX is similar to LT-I produced by E. coli. According to evolutionary data, it genes are foreign genes that were acquired by E. coli probably from V. cholerae. Unlike the ctx genes, it genes are found on an extrachromosomal virulence plasmid called pEnt. In addition, entire pEnt plasmids can be transferred to non-pathogenic strains of E. coli, rendering them toxigenic [18].

Figure 3. Scheme of cAMP dependent ion transport in epithelial cells results in massive secretion of water and electrolytes from interstitial fluid to lumen of small intestine. From [21], modified.
Genes encoding all types of LT-II (lt-II genes) are located on the chromosome and are flanked by homologous phage-related genes upstream and phage-related sequences downstream, which suggest that LT-II may be phage-encoded. The mechanism of LT-II secretion is still unknown, but probably it is the same as cholera toxin [29].

Shiga toxins are encoded by genes localized on the genomes of functional or defective, genetically heterogeneous groups of lambdoid bacteriophages termed Stx-phages [30]. In both cases, stx genes are carried by a prophage integrated with the chromosome. Shiga toxin produced by S. dysenteriae is encoded by a prophage, which has no essential structural phage genes required for the production of infective phage particles. In this case, the mechanism of secretion for the toxin is similar to V. cholerae and requires secretion system type II [11]. Unlike stx, genes encoding SLT-I and SLT-II are regulated by phage promoters. The intensified production of SLT is correlated with the induction of the phage lytic cycle [11]. Following the host cell destruction, toxin molecules and virions are released. These complete virions can infect other, non STEC cells [11]. Usually, the phage lytic cycle is caused by the bacterial SOS response. This mechanism is activated by the accumulation of damaged DNA, e.g. resulting from the application of antibiotics. Therefore, the application of antibiotic therapy during STEC infection is controversial [12].

APPLICATION OF AB$_2$ ENTEROTOXINS

Although destructive and dangerous for human life, modified enterotoxins are used in molecular biology and medicine. For instance fluorescent subunit B of cholera toxin is widely used as a marker for lipid rafts (Fig. 4) [31,32]. The presence of a toxic subunit A is not crucial for binding cholera toxin to GM$_1$. Thus, this ability allows the non-toxic subunit B to be exploited as a raft-associated protein. The holotoxin, as well as subunit B, has strong, mucosal, immunogenic properties. This activity results in the possibility of using CTB as an immunomodulatory and anti-inflammatory agent [33]. CTB has an ability to bind receptors on the intestinal mucosal surface, which can improve the oral delivery of other vaccine-relevant antigens [33]. The oral, nasal or sublingual delivery of antigens and CTB conjugates has been found to induce tolerance with much higher efficiency in comparison to antigen alone. In animal models, this conjugate dramatically suppresses autoimmune disorders and allergies caused by the application of the antigen. A clinical trial of this kind of therapy was prepared for patients with Behcet’s disease and the results are promising [34].

The application of Shiga toxin is also widely investigated. The potential application of Shiga and Shiga-like toxins is related with Gb$_3$ gangliosides, which are overexpressed in many cancer types. The injection of subunit B of Shiga toxin fused with an imaging agent may allow the imaging of these kinds of cancers [35]. The non-toxic STB potentially can be used to deliver drugs to the same cancer cells. For example, radio-isotope-coupled STB might kill monocyte-derived macrophages and dendritic cells. The Conjugate of StB with a drug was shown to have a therapeutic effect only after endocytosis and the subsequent release of the drug to the cytosol. Although StB is nontoxic in vitro, the short- and long-term effects of StB and its derivatives need to be tested further in animal models [35].

CONCLUSIONS

The goal of this review was to give a brief description about enterotoxins causing diarrhea. The main pathogens producing these toxins are widespread and can be easily transmitted, which results in dangerous outbreaks of disease. The main problem occurs in overcrowded, developing countries where the sanitary system is poor. But as we saw a few years ago in Germany and repeated cases in USA developed, rich countries are also exposed to outbreaks of dangerous pathogens. In developed countries, the spread of antibiotic use results in bacteria becoming resistant. This, in turn, leads to a huge problem with treating bacterial infections. Although they have toxic functions, AB$_2$ enterotoxins can be used to treat other diseases. The unique structure and ability of the non-toxic subunit B to enter via the cell membrane allows its use as part of an anti-cancer treatment. Through the ability to induce potent mucosal immune responses, subunit B of cholera toxin can be used to improve vaccines against other diseases.

LITERATURE


Figure 4. Visualization of GM, and cholesterol rich regions in cells by the fluorescent subunit B of cholera toxin. Primary fibroblasts (C688) were fixed and permeabilized. Next, cells were stained with DAPI (4',6-diamidino-2-phenylindole, Sigma) and CTB conjugated with either (A) FITC (Sigma) or (B) Alexa Fluor® 594 (Invitrogen). Stained cells were visualized using Fluorescent microscope Axio Observer.Z1, Zeiss. Images by M. Komiazyk.
Enterotoksyny bakteryjne typu AB₅ — struktura, funkcja i mechanizm działania

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STRESZCZENIE
Biegunki pochodzenia bakteryjnego powstają na skutek działania AB₅ enterotoksyn na komórki nabłonkowe jelita cienkiego. Do grupy tej zaliczamy cztery rodziny, w tym toksyny Shiga i toksyny cholery. Toksyny AB₅ zdolne są do powstawania w komórkach hosta w trudnych warunkach. Odpowiedzią komórki hosta na toksyny AB₅ jest ciągłe produkcja cAMP, w wyniku czego dochodzi do otwarcia kanałów chlorkowych i uwolnienia jonów chlorkowych do światła jelita cienkiego. Do grupy tej zaliczamy cztery rodziny, w tym toksyny Shiga i toksyny cholery. Toksyny AB₅ zdolne są do powstawania w komórkach hosta w trudnych warunkach. Odpowiedzią komórki hosta na toksyny AB₅ jest ciągłe produkcja cAMP, w wyniku czego dochodzi do otwarcia kanałów chlorkowych i uwolnienia jonów chlorkowych do światła jelita cienkiego. Z kolei toksyny z rodziny Shiga pozwalają na wizualizację TRACE lithesses oraz badań na sorbentach.

1.1. Enterotoksyny bakteryjne typu AB₅ — struktura, funkcja i mechanizm działania

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