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Review

Botulinum neurotoxins

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1. Brief introduction

Few species of the anaerobic bacteria of the genus *Clostridium* produce the botulinum neurotoxins (BoNT), which cause a disease of vertebrates known as botulism (Johnson and Montecucco, 2008). The flaccid paralysis and all the other symptoms of botulism are due to the BoNT induced inhibition of skeletal and autonomic peripheral cholinergic nerve terminals. Owing to the nature of these symptoms, botulism was described only at the beginning of the nineteenth century (Erbguth, 2004). BoNT producing *Clostridia* spp. are widespread, mainly in the form of spores very resistant to atmospheric agents; under anaerobiosis and in the presence of water they can germinate with production of a single 150 kDa polypeptide chain neurotoxin which is released from the cytosol following bacterial autolysis. Usually, botulism follows the ingestion of spore-contaminated food preserved under anaerobiosis, conditions that favour the production of BoNT, which accumulates in the form of complexes with associated non-toxic proteins that help the BoNT to pass unaltered through the stomach (Gu et al., 2012). The BoNT complex rapidly dissociates in the intestine and is adsorbed across the intestinal barrier to reach the general fluid circulations (Johnson and Montecucco, 2008). A particular case of botulism is "infant botulism" which follows the ingestion of spores that germinate in the intestine where *Clostridia* can proliferate because of the limited competition exerted by the intestinal flora of infants, at variance from the very effective one exerted by the adult gut microbial community (Arnon et al., 1981). In this case, BoNT is continuously produced and adsorbed and this disease is to be considered a toxo-infection rather than an intoxication as food-borne botulism is. Nowadays, botulism is mainly a disease of animals in farms and in the wilderness where hundreds of thousand of individuals may be involved in outbreaks of botulism among birds or fish (Wobeser, 1997). But episodes of botulism originated by the injection of large doses of BoNT made illegally by non-medical personnel for "cosmetic purposes" cannot be forgotten (Chertow et al., 2006).

1.1. History of the toxin

The first analytical documentation of botulism was made in 1817 and infant botulism was described only in 1976 (Arnon et al., 1981; Erbguth, 2004). Botulism was shown to be caused by a bacterial toxin released from an

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anaerobic bacterium by van Ermengen (1897) and the toxin is presently known as botulinum neurotoxin (BoNT). With time, seven different serotypes of BoNT have been identified (Schiavo et al., 2000). In addition, a growing number of serotype isoforms is identified owing to the use of DNA sequencing and mass spectrometry (Arndt et al., 2006; Hill et al., 2007; Kalb et al., 2012a).

A breakthrough was made with the finding that BoNT/A blocked the release of acetylcholine at the neuromuscular junction (Burgen et al., 1949), and that all BoNT serotypes do the same though with different kinetics (Sellin, 1987; Schiavo et al., 2000; Humeau et al., 2000). Following the identification of the enzymatic activity of tetanus neurotoxin (Schiavo et al., 1992a), the BoNTs were shown to be metallopeptidases specific for the SNARE proteins (Schiavo et al., 1992b, 1992c; Blasi et al., 1993a, 1993b; Schiavo et al., 1993a, 1993b, 1993c, 1994, 1995; Binz et al., 1994; Yamasaki et al., 1994a, 1994b; Vaidyanathan et al., 1999), which are the core of the nanomachine that mediates the release of neurotransmitters (Jahn and Scheller, 2006; Montecucco et al., 2005).

1.2. Nomenclature

According to the established current nomenclature the bacterial toxins which cause botulism are termed botulinum neurotoxins and are abbreviated as BoNT/X where X stands for the serotype which is identified with a letter from A to G (Niemann, 1992), and serotype isoform is then identified by a number, for example: BoNT/A1 (Arndt et al., 2006).

1.3. Source(s)

BoNTs are made by few *Clostridia* spp. as single polypeptide chains of 150 kDa, which accumulate in the cytosol until release by bacterial autolysis (Johnson and Montecucco, 2008). BoNTs are released with a variable number of accessory, non-toxic proteins, which are believed to protect these neurotoxins in their passage through the acidic and proteolytic stomach juice, and which rapidly dissociate at the neutral pH of the intestine. The structure of BoNT/A complexed with its accessory protein has been recently determined (Gu et al., 2012).

1.4. Isolation method(s)

As the BoNTs are the most potent poisons known to humans, their isolation from the bacterial culture supernatants must be performed in class III microbiological safety cabinets by trained personnel. A good description of the preparation has been given by Shone and Tranter (1995). Alternatively, the BoNTs may be produced in *E. coli* by recombinant DNA in appropriate laboratories (Binz et al., 1990).

2. Structure

2.1. Chemistry

The 150 kDa BoNTs chains are inactive and are activated by proteolytic cleavage at an exposed loop; this generates two polypeptide chains: a light chain of 50 kDa termed L chain and a heavy chain of 100 kDa termed H, which remain covalently linked via a single disulfide bridge. The complete crystallographic structures of three BoNTs (A, B and E) are available (Lacy et al., 1998; Lacy and Stevens, 1999; Swaminathan and Eswaramoorthy, 2000; Kumaran et al., 2009; Montal, 2010; Swaminathan, 2011) and reveal four domains, which are functionally linked to the different steps of the mechanism of neuron intoxication by BoNTs. The N-terminal domain is the L chain and it is a metalloprotease (MEROPS Data Base: M27.002) of a unique family, which includes tetanus neurotoxin and the anthrax lethal factor (Rao et al., 2005; Tonello and Montecucco, 2009). These proteases bind zinc in a unique mode which implies five ligands: the two His and the Glu residue of the classic zinc binding motif HExxH, an additional Glu and a Tyr which are located at a distance along the sequence (Rigoni et al., 2001; Binz et al., 2002; Tonello and Montecucco, 2009). Another peculiar feature of these metalloproteases is that they recognize their specific substrates via multiple interactions with different superficial parts of the substrate (see below).

The L chain is disulfide-linked to the domain H_N (50 kDa), which is characterised by the presence of two 11 nm long helices and by additional short helices arranged in a bundle. This domain is then implicated in the transmembrane translocation of the L chain from the lumen of endosomal vesicles into the neuronal cytosol (Montal, 2010).

The third domain (H_C-N, 25 kDa) is linked to H_N and its function is presently unknown, though the H_C-N of BoNT/A has been suggested to interact with the head group of phosphatidylinositides and assists the membrane interaction of H_N (Muraro et al., 2009). H_C-N is linked to the C-terminal domain (H_C-C, 25 kDa), which is responsible for the neurospecific binding of the BoNTs via a double interaction mediated by two binding sites. One site is very similar among serotypes and isoforms and binds the oligosaccharide portion of a polysialoganglioside. The second one varies among serotypes and binds different receptors (Binz and Rummel, 2009): BoNT/A and/E bind two different loops of the lumenal domain of the synaptic vesicle protein SV2 (Dong et al., 2006, 2008; Mahrhold et al., 2006); BoNT/B and/G bind a short alpha-helical segment of the lumenal domain of the synaptic vesicle protein synaptotagmin (Nishiki et al., 1994; Chai et al., 2006; Jin et al., 2006; Rummel et al., 2007). The mode of binding of BoNT/C and BoNT/D is less well understood. A coreceptor for BoNT/C has yet to be identified, although Rummel et al. (2009) demonstrated an increase in toxicity upon stimulation, indicating that entry may be synaptic vesicle-specific. It has been proposed that BoNT/C and/D use dual carbohydrates as receptors, analogous to tetanus toxin (Karalewitz et al., 2010; Strotmeier et al., 2010; Kroken et al., 2011) although Peng et al. (2011) reported the entry of BoNT/D by binding SV2. Therefore it is not clear at the present time how the second binding may drive these two serotypes of BoNT into the lumen of synaptic vesicles. It is possible that the second site of the H_C-C of BoNT/C and/D bind the oligosaccharide group of a glycoprotein present on the lumen of a synaptic vesicle.