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Botulinus Toxin "Botox"

[Botulinum Toksini "Botoks"]

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ABSTRACT

The toxin from Clostridium botulinum is one of the most deadly toxins known. The structure of the type A toxin protein has been solved by x-ray crystallography and this reveals how the toxin acts on nervous transmission. The 150 kDa molecule has three parts: a receptor-binding domain that enables it to bind to nerve cells, a translocation domain that enables the toxin to enter cells, and a protease domain that specifically digests proteins concerned with the delivery of the neurotransmitter acetylcholine at the neuromuscular junction leading to paralysis of the muscles required for breathing. Despite its highly lethal nature type A toxin, as Botox®, is used medically in tiny doses to treat a number of clinical conditions, and more recently, cosmetically, to remove wrinkles and frown lines.

Key Words: Botulinus toxin, BoNT, neurotransmission, neuromuscular junction, acetylcholine

ÖZET

Clostridium botulinum toksini, bilinen en öldürücü toksinlerden biridir. Tip A toksinin yapısı X-ışını kristallografisi yöntemi ile aydınlatılmış ve toksinin sinir iletimi üzerindeki etkisi açıklanmıştır. 150 kDa molekül ağırlığına sahip olan molekülde 3 bölge vardır: sinir hücrelerine bağlanabilme özelliğindeki reseptör-bağlama bölgesi; toksinin hücrelere girişini sağlayan translokasyon bölgesi ve sinir-kas kavşağında kas kasılmasını sağlayan asetilkolin nörotransmitterinin salıverilişinden sorumlu proteini özgül olarak sindiren proteaz bölgesi. Botox® olarak bilinen tip A toksini son derece öldürücü bir özelliğe sahip olmasına rağmen birçok klinik olguda tedavi amacıyla ve son yıllarda kozmetik alanında kırışıkları gidermek ve çizgileri düzeltmek amacıyla kullanılmaktadır.

Anahtar Kelimeler: Botulinum toksini, BoNT, sinir iletimi, sinir-kas kavşağı, asetilkolin

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INTRODUCTION

Many of the bacteria that cause common diseases produce protein toxins that are responsible for the clinical effects of infection. Although the diseases (e.g. cholera, typhoid, tetanus, pertussis) have been around for a long time, and are less common these days because of the use of antibiotics, the basis for their clinical effects have only recently been elucidated. Before antibiotics the treatment was to inject horse antisera to the toxins which neutralised their effects, but this often gave rise to anaphylactic reactions in sensitive patients. Subsequently it was found that chemical treatment of the toxin proteins could produce a so-called 'toxoid' that was harmless (i.e. non-toxic) but was still antigenic. Such toxoids produce active immunity and are widely used today.

As molecular biology progressed, the structures of many of these protein toxins were elucidated by x-ray crystallography, and in parallel studies, were carried out to find out exactly how they achieved their biological effects – usually at very low concentrations. It was shown for example that the toxin of Bordetella pertussis, or pertussis toxin, which causes whooping cough, consisted of two subunits. The B subunits served to bind the toxin to specific cells and the A subunit has enzyme activity. After crossing the cell membrane the A subunit hydrolyses NAD+ to nicotinamide and ADP-ribose and this ADP-ribose is transferred to several G proteins involved in the control of adenylate cyclase, phospholipase C and ion channels. Cholera toxin, produced by Vibrio cholerae, binds to the epithelial layer of the intestine upsetting the secretion of Cl- and inhibiting the absorption of Na+. The result is that there is copious fluid loss causing severe diahorroea. The patient loses large amounts of water and also suffers electrolyte imbalance. In summary, for many bacterial toxins their structure and their precise mode of action at the cellular level is now well understood. Moreover, biochemists and others are now using these toxins as tools to specifically interfere with and study aspects of cell physiology.

In what follows we discuss a different toxin, that from Clostridium botulinum, one of the most lethal toxins known. Not only is its structure and mode of action now well understood, but also it is being widely used as a cosmetic treatment to reduce facial wrinkles. However, the basis for this is comes from earlier uses to treat a number of serious medical conditions.

THE ORGANISM AND BOTULISM

Clostridium botulinum is a Gram-positive, spore-forming bacterium whose toxin (actually a group of related proteins) is one of the most toxic substances known. The lethal dose for a human is of the order of 1 µg. Like all the Clostridia, the bacterial cells are relatively large rods and are metabolically unable to use oxygen as the final electron acceptor. Therefore they can only grow in anaerobic conditions. In general, Clostridia are not invasive organisms and their action in producing disease

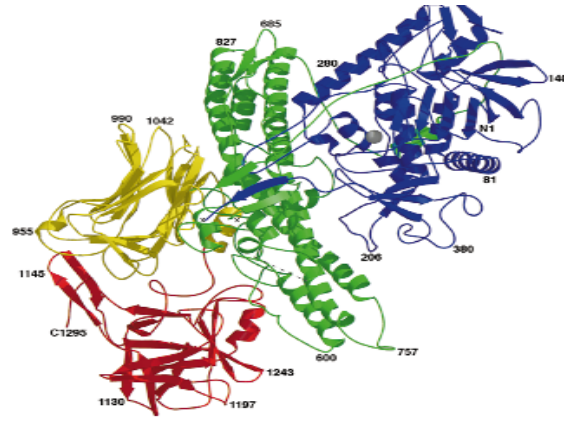

Figure 1: Structure of botulinus toype A toxin. Backbone trace for the BoNT/A molecule generated using MOLSCRIPT. The catalytic domain is at the top right (with Zn atom), the translocation domain with several long α-helices is in the centre, and the binding domains are at the bottom left (see ref. 5).

("botulism") comes about as a result of the formation of the secreted exotoxins. The toxins cause flaccid muscle paralysis, and at present no antidote is known. Polyvalent antisera are available but need to be injected rapidly and their effectiveness is uncertain. Although most cases of botulism are food-borne, there is an increase in wound botulism in individuals abusing drugs (injection with contaminated needles can produce depots of bacteria deep in the skin where the conditions can become anaerobic). Increasing knowledge of the structure and precise mode of action of the toxin may help the development of antidotes in the future.

C. botulinum is widely distributed in soil, lake bottoms and decaying vegetation, and consequently many foods, both vegetables and meat, can become contaminated with the bacteria, and numerous animals die each year after ingestion of fermenting grains for example [1]. The bacterial spores are widespread in dust and soil and are amazingly resistant to heat for example being able to withstand boiling water for several hours. Botulism in humans tends to occur as a result of eating food that has undergone inadequate sterilisation and has then been placed in an anaerobic environment where the spores can germinate and produce the toxins. Food-borne botulism probably evolved through the ages with man but little was known about the disease before the 19th century. Botulism resulting from commercially produced foods is now rare because manufacturers take proper precautions to autoclave food materials at the appropriate temperatures and pressures. The rare cases typically now result from the consumption of smoked, salted or spiced meats or fish, or home-canned or bottled fruit or vegetables.

BOTULINUS TOXINS

C. botulinum bacteria produce a group of protein toxins. Seven immunologically distinct forms are known, produced by different strains of the bacterium, and in contrast to the spores, the toxin proteins themselves are heat

labile. Toxins types A, B, E and F are responsible for the majority of cases of human botulism, and type A (and sometimes B) is the material used clinically and cosmetically. Type C is most common in ducks and chickens, and type D in cattle and horses. These endotoxins had been used clinically for a number of conditions many years before their cosmetic use was developed. Botulinum toxin type A is sold commercially as 'Botox'®, but molecular biologists use the general term 'BoNTs' for botulinum neurotoxins [2].

The toxins produce paralysis by binding to receptors at nerve synapses, entering the nerve cell and blocking the release of the neurotransmitter, acetylcholine (see below). Symptoms usually begin after an incubation period of 18–38h and include nausea and vomiting, double vision, difficulty in swallowing and some muscle paralysis [3]. This may be followed by muscle weakness, blurred vision, and ultimately death from respiratory failure as the nerves activating the muscles of the chest are blocked.

CLINICAL AND COSMETIC USES OF BOTULINUM TOXINS

Botulinum toxin A ("Botox") has for a number of years been used clinically to treat neurological disorders such as strabismus, blepharospasm, hemifacial spasm and hyperhidrosis. It has also been useful in the treatment of cerebral palsy, weakness due to stroke, cervical dystonias, and many other medical conditions. Subsequently it was discovered, while treating a patient for strabismus, that after the injections of toxin into the forehead, the facial 'frown lines' did not appear as pronounced as previously [4]. Soon Botox became a widely sought-after cosmetic therapy because of its apparent beneficial effects and relative lack of side effects compared to invasive (i.e. surgical 'face-lift') treatments. Botox injections are now a fast-growing cosmetic procedure and millions of people have been treated

Injection of small amounts of the potentially deadly botulinum toxin type A into muscles that underlie wrinkles and frown lines reduce the visibility of these lines. The areas that are commonly treated with Botox are mainly facial areas and expression lines. For example, it may be injected directly into the muscles that underlie the vertical lines between the eyebrows and on the bridge of the nose to erase the frown lines. Other popular areas for Botox therapy are the orbicularis oculi muscles around the eye that underlie 'crows feet'. To improve the appearance of glabellar lines that give the continued appearance of worry or frowning, Botox is injected into the corrugator and procerus muscles to block their hyperactivity. The frontalis muscle, which pulls the forehead skin in the vertical direction to give horizontal lines across the forehead can also be relaxed by Botox treatment. Lines along the lateral dorsum of the nose from smiling may be improved by injection of Botox into the nasofacial groove. The treatment is not permanent and typically needs to be repeated after about 6–9 months. Perhaps surprisingly there have been few reports of patients developing antibodies to the injected toxin.

THE CHEMISTRY OF BOTULINUM NEUROTOXINS

Botulinum neurotoxins are synthesized as single, high-mol-wt (~150 kDa) polypeptides called progenitor toxins which are cleaved by bacterial proteases to yield a heavy, ~100 kDa chain, and a light, ~50 kDa chain which remain linked by disulphide bonds [5]. The complete toxin protein is actually composed of three functionally distinct domains (Fig 1). The ~50 kDa light chain domain has catalytic activity, and is a highly specific zinc-dependent protease, but needs to be separated from the heavy chain for its activity to be revealed. The carboxy- and amino-terminal halves of the ~100 kDa heavy chain are involved with receptor binding and with the translocation of the protein across the cell membrane, respectively [6].

HOW THE TOXIN GETS TO THE NERVE CELLS

Although botulinus toxin can enter the body by several different routes, most cases of botulism result from the ingestion of food contaminated with preformed toxin or from ingestion of bacteria that can produce toxin in the gut The initially synthesised progenitor complex of the toxin seems to have other proteins associated with it, and these proteins may protect the toxin protein from the action of the digestive enzymes of the intestine. In fact, the preformed toxin needs to escape the gastric acidity as well as the proteolytic enzymes in the stomach and small intestine. It then needs to pass through the epithelial lining of the intestine in order to reach the blood and find its way to the nerve cells. In contrast to the events at the nerve terminals, comparatively little is known about how the toxin gets from the gut lumen into the blood. Since the toxin is lethal at exceptionally low concentrations the process must be extremely efficient. However, it should be remembered that the effective toxin is an enzyme. Therefore one molecule can catalyse a large number of events once it is inside the nerve cell.

There do not seem to be any studies on the actual mechanism by which the toxin penetrates gut cells, but presumably the mechanism is different from the one by which it enters nerve cells. The toxin protein must not only enter the gut epithelia (the luminal side) but must also exit from the opposite side (the serosal side) into the lymph or blood. Alternatively it may pass between the epithelial cells but this is thought unlikely.

It has been shown in vitro using a monolayer of Caco-2 cells, that specific binding to the cell membrane followed by transcytosis occurs [7]. [Caco-2 cells are a cell line that was originally derived from a human colonic adenocarcinoma: the cells form a confluent monolayer that behaves in very similar ways to gut epithelial cells.]

The toxin remained in its di-chain structure during transit through the cells and when released from the opposite side was toxic in vivo to mice. Interestingly, botulinum toxin type C, which it toxic to fowl but not to humans, did not bind to human gut epithelial cells in culture and was not transcytosed. This may explain why certain toxin serotypes are toxic to humans while others are not.

ACTION AT NERVE CELLS

A nervous impulse is transmitted between nerve cells, or from a nerve cell at the neuromuscular junction, by acetylcholine. This compound is released from vesicles at the presynaptic membrane of one nerve cell into the synapse and it then reacts with receptors on the post-synaptic membrane before being hydrolysed and inactivated by cholinesterase. The acetylcholine is brought to the presynpatic membrane in vesicles from the Golgi apparatus which fuse with the neuronal cell membrane, thus releasing the chemical into the synapse. Botulinus toxin interferes with the fusion of the vesicles with the cell membrane with the result that acetylcholine is not released [8]. Hence, no nerve impulse is received at the neuromuscular junction by the muscle cell.

The change in pH upon leaving the acidic environment of the intestine is thought to trigger the dissociation of the progenitor complex into free toxin, which then binds to its receptor molecules on the terminal endings of neurones at the neuromuscular junctions and is internalised by receptor-mediated endocytosis. In the endosome, pore formation by the translocation domain is thought to be induced by the acidic environment, thus the catalytic domain of the toxin is allowed to be translocated into the cytosol of the nerve cell [8].

In the mechanism of action of the neurotoxins, the first step is the binding event that occurs between the binding domain of the complex (part of the toxin's heavy chain) and the presynaptic ending of the target nerve cell, followed by receptor-mediated endocytosis. Once inside the endosome it is believed that a conformational change occurs due to the acidic pH and that this initiates the formation of a pore through the endosomal membrane by the translocation domain of the heavy chain of the toxin [8]. This then facilitates the release and passage of the catalytic domain of the toxin (i.e. the light chain) out of the endosome and into the cytoplasm.

The neurotoxins have a zinc protease catalytic site that is specific for the C-terminal end of a protein called SNAP-25 which it cleaves. (SNAPs are "synaptosome-associated proteins".) SNAP-25 is necessary for synaptic vesicle fusion thus making the synapse incapable of releasing acetylcholine resulting in paralysis of the muscle supplied by the affected neuronal synapse.

SNAP-25 is one of the SNARE proteins that are cleaved by the botulinum neurotoxins, particularly types A, C and E. Other SNAREs that are affected by other botulinum neurotoxins are synaptobrevin, which is inactivated by toxin types B, D, F and G, and syntaxin, which is acted on by toxin type C. SNARE proteins are receptors on the post-synaptic membrane of a neurone and are essential for the docking and binding of the acetylcholine-filled vesicles that are released from the pre-synaptic membrane.

CONCLUSIONS

Botulinus toxin is one of the most toxic proteins known – it is about 100 billion times more toxic than cyanide, although surprisingly it has found extensive uses both medically and cosmetically. (Probably when the pages of 'women's magazines' speak of "spa treatments to remove toxins", they do not mean botulinus, tetanus or other such toxins!) IThe use of botulinus toxin for cosmetic purposes involves the injection of very tiny amounts of the type A toxin, as the commercial preparation "Botox", under the skin at several sites, usually on the face. There is now good understanding of the mechanism of action of the toxin at nerve endings which involves the inhibition of the secretion of the neurotransmitter acetyl choline. There is also reasonably good knowledge of how the toxin protein gets into nerve cells in order to exert its action. The three-dimensional structure of the toxin protein has been solved. It has three domains, a catalytic domain, a channel forming domain and a translocation domain. The channel-forming ability of one of the domains of the heavy chain might open new opportunities for targeted drug delivery

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