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Clostridium Botulinum Toxins: Nature and Preparation for Clinical Use

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Summary
***C. botulinum* neurotoxins are acutely toxic materials and act by inhibiting release of the neurotransmitter acetylcholine. The specific nature of this inhibition is discussed and the preparation and purification of Type A toxin specifically for clinical use is described.**

There have been few drugs whose mode and site of action and detailed pharmacology have been so well understood prior to their clinical application as Type A botulinum toxin. Indeed, the nature of this family of neuroparalytic agents as the most acutely potent microbial toxins and the cause of the food-borne illness botulism has made regulatory authorities particularly cautious in approving their clinical application. Nevertheless the use of the toxin particularly to treat a variety of dystonic conditions represents a most elegant, precise and safe treatment which reflects considerable credit on the pioneering work of Scott[1] who first instituted the use of the toxin for human therapy.

The therapeutic, as well as the prophylactic (vaccine) use of microbial products is, of course, well established and as well as native products, especially antibiotics, also includes bacterial asparaginases used to treat acute lymphoid and lymphoblastic leukaemia in children.[2] Increasingly we are also seeing human gene products such as insulin, growth hormone and interferons produced in micro-organisms and used for human therapy. It is virtually certain that many other potent human regulatory proteins will also become available by this route.

Thus when it comes to pharmaceutical production *C. botulinum* toxin can be viewed as one of the first of a group of high activity protein products prepared from cultures of micro-organisms and the problems and solutions in handling such materials have general applications.

The purpose of this article is to review the nature of *C. botulinum* toxins and to describe the procedures involved in the production, purification and presentation of the type A toxin for clinical use.

The Nature of the Toxins

Structure

Complex toxins. There are eight serologically distinct toxins (A, B, C_1, C_2, D, E, F and G) produced by *C. botulinum* that are among the most powerful neuroparalytic agents known. The toxins so far studied all appear to occur both naturally and in *in vitro* culture complexed with non-toxic proteins which may co-purify with the toxin moiety (Fig. 1). Of these complexes the smallest, the M or medium complexes, comprise the neurotoxin moiety (ca. 150,000 daltons) associated with a similar sized non-toxic protein of undetermined biological activity and are the only forms found for the types C[3], E[4,5] and F[6] and

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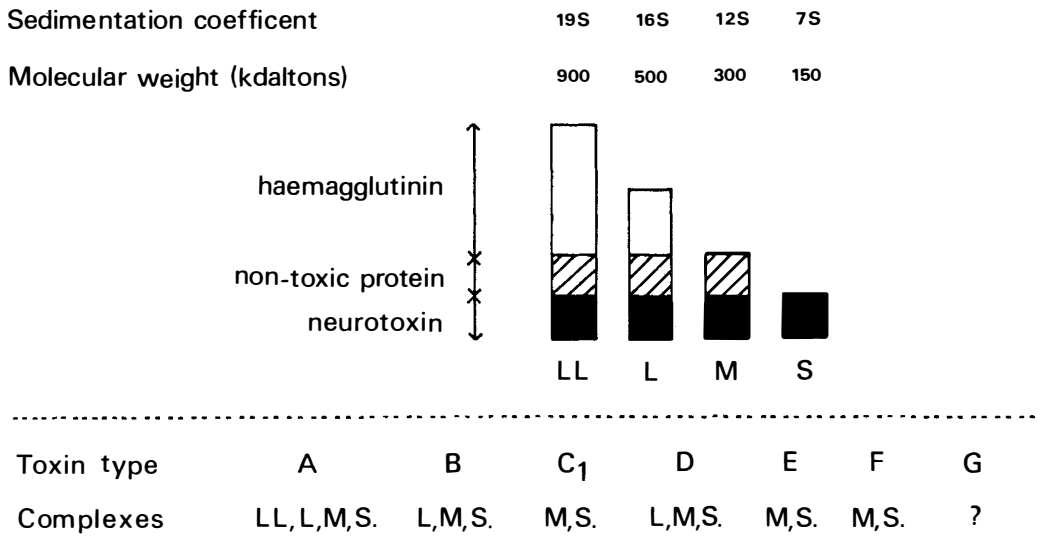

Fig. 1.

haemagglutinin-negative strains of type D[7]. Types A[8], B[9] and haemagglutinin—positive D[7] also occur as trimolecular or L (large) complexes comprising the M complex in association with a non-toxic protein having haemagglutinin activity. The L complexes (Ca, 500,000 daltons) are the largest complex states of these toxins with the exception of type A toxin which also occurs as a complex of about 900,000 daltons. This latter complex, which is thought to consist of dimers of the L complex, is the molecular size of the type A toxin in the crystalline state.

Neurotoxins. The various *C. botulinum* neurotoxins, although serologically distinct, appear to be structurally very similar to each other; the purified neurotoxins having similar molecular sizes and sharing a common subunit structure (Table II). These single chain progenitor toxins may be cleaved by proteolytic enzymes to give biologically active dichain toxins (Fig. 2).

The dichain toxins consist of one large (heavy, H) and one small (light, L) subunit linked by at least one disulphide bridge; the H subunits are about double the molecular size of the L subunits[4,6,10–19] (Table I).

Fig. 2.

The amino acid composition of several toxin types (A, B, C, D, E and F) have been evaluated[16,18,20–22] and similarities are apparent. In particular all have high contents of hydrophobic amino acids residues which may be relevant to the membrane binding/internalisation of the toxin as discussed later. The similarities in amino acid composition together with their shared pharmacological action imply that, despite their lack of antigenic cross-reactivity, there may well be regions of structural homology within the neurotoxins.

Neurotoxin subunits. Reduction of the disulphide link(s) between H and L subunits generally leads to a loss of toxicity and a reduction in the solubility of the toxin. The subunits of reduced neurotoxin remain associated by covalent forces but these can be dissociated and solubilised by urea and subsequently separated chromatographically.[16–18,23–25]

Purified H and L subunits of the neurotoxins are of greatly reduced specific toxicity (<0.5 per cent) compared to the intact toxin; the residual toxicity probably being due to trace contamination with intact toxin.[16] Separated neurotoxin subunits appear to maintain their structural integrity and can be recombined into intact neurotoxin by mixing equimolar portions of each subunit, in reducing conditions, and slowly removing the

Table I *Molecular size and subunit structure of* C. botulinum *neurotoxins*

	Molecular size (k daltons)			
Toxin type	*Holotoxin*	*H subunit*	*L subunit*	*Reference*
A	145	95– 97	55	10, 11
B	155–170	101–104	51–59	12–14
C_1	141–144	92– 98	52–53	15–17
D	140	85	55	18
E	147–150	102	50	4
F	155	105	56	6, 19
G	Ca.150?	Not yet chracterised		

reducing agent. Some 30 per cent of the potential toxicities of reconstituted types A[23] and B[24] toxins were regained and up to 70 per cent for type C, toxin.[16]

Toxicity. When administered by the parenteral route the non-toxic proteins of the complexes appear not to play a role in toxicity, indeed, the highest specific toxicities are observed with the purified neurotoxin component (Table II). For example, the purified 145,000 dalton type A neurotoxin (S-form) has a specific toxicity about 3 fold higher than that of its L complex. In contrast, the complex toxins are more toxic than purified neurotoxins when administered orally (Table II). This difference is even more pronounced with the type B toxin where oral toxicities of the S, M and L forms are in the ratio of 1/2/1,600 respectively.[26] Differences in the oral toxicities of toxins from two different type B strains of *C. botulinum* were attributed entirely to differences in the properties of the non-toxic proteins of the respective complex toxins. The enhanced oral toxicity of the complex toxins probably reflects the ability of the non-toxic proteins to protect the neurotoxin moiety from the hostile environment of the digestive tract prior to its uptake into the blood and lymphatic systems.[27,28]

Table II *Enteral and parenteral toxicities of different molecular forms of* C. botulinum *Type A toxin*

Molecular form	*Intraperitoneal route mouse LD_{50}/mg protein*	*Oral Route mouse LD_{50}/mg protein*
LL	3.8×10^7	3.2×10^5
L	4.8×10^7	2.2×10^4
M	8.0×10^7	2.2×10^4
S	1.6×10^8	3.7×10^3

Data calculated from Sakaguchi *et al.*[29]

Mode of Action

The botulinum neurotoxins act primarily on the peripheral cholinergic synapses where, by inhibiting the release of the neurotransmitter acetylcholine, they cause the widespread muscular paralysis characteristic of the fatal syndrome botulism. Studies with isolated neuromuscular junction preparations show that the action of the toxin is not mediated merely by its binding to the nerve membrane; since binding is rapid, whereas paralysis has a slower onset.[30] Furthermore, in contrast to the rapid binding, stimulation of the nerve is required to induce paralysis which is temperature sensitive and requires the presence of calcium ions in the fluid bathing the neuromuscular junction.[31–33]

It is now believed that at least three steps are involved in the inhibitory action of botulinum toxins. Firstly, there is a binding step, whereby the toxin attaches rapidly and avidly to the presynaptic nerve membrane. Secondly, an internalisation step in which toxin crosses the presynaptic membrane and a final step, or steps, whereby toxin inhibits the release of the neurotransmitter substance, acetylcholine.

Toxin binding. Selective, saturable binding of botulinum neurotoxins to presynaptic nerve membranes of both peripheral and central nerves has been shown using labelled toxin molecules.[34–36] Neurotoxin visualised with ferritin-labelled antibody was observed to bind in discrete patches of varying size rather than uniformly over the nerve surface.[36]

Studies using synaptosomes from rat or mouse brain have shown that the binding of

radio-labelled toxin is inhibited by an excess of unlabelled toxin, indicating that a limited number of binding sites are available.[37] Two types of acceptor component appear to be involved; a small proportion of sites bind neurotoxin with high affinity (Kd<1 nM) and a larger pool of acceptor sites bind toxin with lower affinity (Kd>20 nM) (Table III). If one assumes that each neurotoxin contains but one binding (active) site then it seems most likely that the toxin forms a 'good fit' with the high affinity sites but a 'poor fit' with the low affinity ones.

Table III *Binding constants for high and low affinity synaptosomal acceptor sites for botulinum toxins*

	Kd (nM)		
Toxin type	*High*	*Low*	*Reference*
A	0.6	25	37
B	0.3–0.5	20	14
C	0.08	35	38,39
D	0.023	35	18

Although the similar structural and pharmacological properties of the various botulinum neurotoxin types suggest they may have similar modes of action, it would appear that the synaptosomal acceptor sites may differ from some toxin types. There may, indeed, be at least two groups of synaptosomal acceptors, one recognised by the A and E toxins and one by the Type B toxin, since there is little or no competition between Types A and B or B and E for binding to synaptosomes whereas Types A and E appear to compete for the same acceptor sites.[14,37,40] Similarly, binding of Type D toxin is completely inhibited by C_1 toxin, suggesting common acceptor components.[18] Whether these are similar to the acceptors for either A and E or B toxins is not yet known.

The role of subunits in binding to acceptors. In those botulinum neurotoxins so far studied the H subunit appears to contain the active site which binds to synaptosomal acceptor. For both A and B toxins homologous unlabelled H-subunit reduces binding of radio-labelled toxin, whereas the L chain is without effect. Similarly Type D toxin binding is inhibited by Types C, and D H subunits. The single chain forms of B and E toxins bind to synaptosomes with similar affinities to nicked, active toxin[14,42] implying that the relative non-toxicity of the single chain molecule does not reflect an inability to bind to the nerve terminal but rather to some subsequent step in the toxins' action.

Direct evidence of toxin internalisation was provided by experiments using hemidiaphragms incubated with radio-labelled Type A neurotoxin.[34] Electron micro-autoradiographs showed that following binding to the nerve terminal membrane transfer of labelled toxin across the plasma membrane was evident after about 20 minutes and was maximal after 90 minutes. The translocation process which did not require the presence of Ca^{2+} ions nor indirect stimulation of the nerve was abolished at low temperatures or by inhibitors of energy production. The latter did not, however, prevent binding to the membrane surface indicating that energy is required only for internalisation. From electron micrographs of nerve preparations in which toxin internalisation was prevented it was estimated that there were between 150–500 acceptor sites per μm^2 on the nerve surface.[34] Detailed quantitation of EM autoradiographs revealed that not all bound material was internalised and that some 60 per cent remained associated with the membrane.

Neurotransmitter release. Since botulinum toxins appear to inhibit neither neurotransmitter synthesis nor storage disruption of the Ca^{2+}-mediated acetylcholine release mechanism seems probable. Three possibilities exist; toxin could inhibit Ca^{2+} influx during nerve stimulation or stimulate Ca^{2+} efflux, or it could act directly on some stage in the release mechanism itself. No hypothesis adequately accounts for all the available data although most evidence argues against botulinum toxin acting by inhibiting Ca^{2+} influx.

In the early stages of botulinum toxin poisoning of the neuromuscular junction the effects can be reversed by increasing the flow of Ca^{2+} into the nerve terminal.[43] However, as the poisoning progresses so the ability of Ca^{2+} to reverse the toxic effects is diminished. It is conceivable then, that the botulinum toxin may act by reducing the sensitivity of the neurotransmitter release mechanism to Ca^{2+} such

that the normal intracellular Ca^{2+} levels are insufficient to promote release.

Similar conclusions are indicated by studies with substances such as tetraethylammonium[43] and 4-aminopyridine[44–46] which, in prolonging the depolarisation/repolarisation cycle of the nerve, allow higher concentrations of Ca^{2+} to build up in the nerve terminal. In these conditions the end plate potentials of mildly botulinum-poisoned neuromuscular junction preparations can be restored to normal.

Many key aspects of the action of the botulinum neurotoxins still await clarification, including the appearance of minature end plate potentials shortly after the onset of poisoning and particularly the long duration of the toxic effects. One possibility is that the toxin evokes a permanent change in an enzyme, or other vital system, having a long half life.[47,79] Indeed it has been proposed that, in common with some other microbial toxins, the toxin itself may be an enzyme; the protracted action and high specificity of the toxin appears to support this view.

Preparation of Type A Toxin for Clinical Use

For a product to be acceptable and licensable for clinical use appropriate standards of quality, safety and efficacy must be achieved. Thus material must be reproducible in its composition and potency from batch to batch and stable during a particular period under defined and achievable storage conditions. Safety and efficacy need to be assessed both by animal experimentation and in human trials and to be verifiable on subsequent batches by the application of relevant quality control tests. All of the above requires the application of Good Manufacturing Practice (GMP) techniques. GMP builds on laboratory biochemistry and microbiology and translates a Research and Development process into an effective pharmaceutical operation. The importance of standardising conditions for production and purification is clearly apparent given the effect of proteolytic 'nicking' on toxic activity and the further susceptibility of the protein toxin to inactivation by proteolytic enzymes.

Culture. Cultures of *C. botulinum* from a verified seed stock are grown up and inoculated into a 30 l fermenter operated under anaerobic conditions and toxin production and other culture parameters are monitored (Fig. 3). When the maximum yield of toxin has been attained (2×10^6 mouse LD_{50}/ml) usually after 72 hours the toxin is harvested by centrifugation after acidifying the culture. In this form the toxin can be stored prior to purification. Cultures are extensively checked for toxin activitiy, identity and absence of contamination.

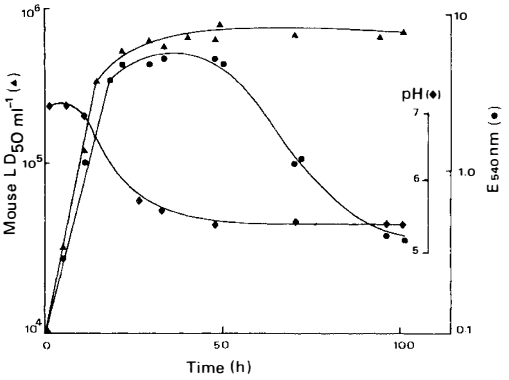

Fig. 3.

Purification. The precipitated crude toxin is re-dissolved and purified by a series of procedures involving ammonium sulphate precipitation and ion-exchange chromatography (Table IV). During these procedures the process is monitored for the absence of contamination with extraneous micro-organisms as well as the toxicity and protein content of the extracts.

Formulation and Freeze Drying. The potency of the purified toxin is assessed and an appropriate quantity of the purified toxin solution is added to a diluent containing lactose and human serum albumin. The diluent is designed to provide protection for the toxin during freeze-drying and to act as a bulking agent for the freeze dried product. Prior to freeze drying, the diluted toxin is dispensed into vials and at the completion of the drying process vials are checked for integrity, sterility, moisture content and potency.

Clinical Use and Dosage

C. botulinum toxin has now been used to treat both experimentally and routinely a range of

Table IV *Purification of* C. botulinum *Type A Hemagglutinin-neurotoxin Complex*[48]

Stage procedure	*Protein (mg)*	*Toxicity*			
		Total mouse LD_{50}	*MLD_{50}/mg protein*	*Recovery %*	
				Stage	*Overall*
1 Whole culture (20.1)	—	10^{10}	—	100	100
2 Precipitation at pH 3.5, and adjusted with 3N H_2SO_4	4,600	7.5×10^9	1.6×10^6	75	75
3 Extraction with 0.2M phosphate buffer at pH 6.0	1,300	6.8×10^9	5.2×10^6	91	68
4 Ribonuclease treatment (100 ug/ml, 34°, 3 h)	1,300	6.8×10^9	5.2×10^6	100	68
5 Precipitation at 60 per cent saturation (at 25°) of ammonium sulphate	—	6.5×10^9	—	96	65
6 DEAE-Sephadex A50 batch preabsorption	860	5.0×10^9	5.8×10^6	77	50
7 DEAE-Sephacel ion-exchange chromatography at pH 5.5	195	4.8×10^9	2.5×10^7	96	48
8 Precipitation at 60 per cent saturation (at 25°) if ammonium sulphate	195	4.8×10^9	2.5×10^7	100	48

conditions where deliberate paralysis of a particular muscle may be of benefit. The conditions include strabismus,[1] blepharospasm,[49] lateral rectus paresis,[50] hemifacialspasm,[51] Meige syndrome,[52] facial synkinesis after facial nerve palsy and spasmodic torticollis.[53]

Clearly the muscle sizes, and most critically the number of cholinergic synapses, vary considerably among these conditions as it is clear does the dose of toxin required for effective action. It is likely that for the effective treatment of patients and the convenience of clinicians toxin will need to be made available in a range of doses appropriate to the particular condition and even for one condition individual patient responses may dictate varying doses. Accordingly we decided to present the toxin which has been used for blepharospasm and strabismus treatment as a weight of active protein determined by mouse potency; the use of units of activity based on human physiological activity may follow as more clinical data become available.

The stability of the toxin preparation over time is clearly critical to building up an effective clinical data base and we are able to confirm that our freeze-dried toxin retains stability even when kept at 4° C for not less than 3 years. Thus the potency of the preparation is a constant factor even when used in different clinics, countries and over a long time period and should enable a consistent dose regime to be established for a range of dystonia conditions.

As the data presented above show, not only is the mode and site of action of the toxin highly specific but it also possesses a further important characteristic which is its avidity for the site of action. Taken together these characteristics allow local injection into a muscle with little or no general dissemination. It will eventually be interesting to investigate the clinical use of the pure 150,000 MW neurotoxin as this molecule which does not have the haemagglutinin moiety of the complex toxin may give even better precision and reproducibility of action.

Further development may be required if there is evidence of production of antibody by patients receiving toxin. Should this become a problem it would be necessary to make available other of the serologically distinct toxin Types B through G and the information currently being pursued on the structure and activity of these other toxins should prove invaluable in assessing their suitability for clinical use.

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