On Botulinum Neurotoxin Variability

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ABSTRACT **The rapidly growing number of botulinum neurotoxin sequences poses the problem of the possible evolutionary significance of the variability of these superpotent neurotoxins for toxin-producing *Clostridium* species. To progress in the understanding of this remarkable phenomenon, we suggest that researchers should (i) abandon an anthropocentric view of these neurotoxins as human botulism-causing agents or as human therapeutics, (ii) begin to investigate in depth the role of botulinum neurotoxins in animal botulism in the wilderness, and (iii) devote large efforts to next-generation sequencing of soil samples to identify novel botulinum neurotoxins. In order to compare the fitness of the different toxins, we suggest that assays of all the steps from toxin production to animal death should be performed.**

An intrinsic aspect of science in general and of experimental sciences in particular is that a field or domain can remain unchanged or stagnant for many years. Then, it may change rapidly, following the introduction of a novel paradigm or, more frequently, a technical innovation (1). This has recently happened for the botulinum neurotoxins (BoNTs), whose number rose rapidly from a few BoNTs to several dozens in a few years, and many more are expected to be reported soon. These neurotoxins are the most potent bacterial toxins yet known; therefore, they are classified as category A select agents by the Centers for Disease Control and Prevention (2). At the same time, due to scientific and clinical research, the BoNTs have become human therapeutics, currently used in many millions of doses per year worldwide (3–6). In fact the injection of one of them (BoNT serotype A1) has become the therapy of choice for several human syndromes, and possible novel therapeutic applications are currently being investigated.

THE GROWING FAMILY OF BOTULINUM NEUROTOXINS

The first BoNT (serotype A) was discovered in 1896 and subsequently, one after the other, a total of seven serotypically distinct types were identified by 1970. They are classified on the basis of specific antibody recognition and are termed BoNT/A to BoNT/G (7, 8). An additional BoNT serotype (H) has recently been proposed (9); however, this finding awaits confirmation. These neurotoxins are produced by anaerobic bacteria of the *Clostridium* genus and specifically affect vertebrates (7, 10). Serotypes A, B, and E are those more frequently associated with human botulism, with fewer cases being caused by BoNT/F (8). Almost exclusively associated with botulism among birds is BoNT/C, while BoNT/D causes botulism in different animal species but not in humans, with very few exceptions. BoNT/E is more frequently associated with botulism of marine vertebrates and fish eating birds (7). BoNT/G is produced by a bacterium first isolated from a cornfield (11) and classified as *Clostridium argentinensis*, while the other BoNTs are produced by *C. botulinum*, *C. baratii*, or *C. butyricum*. Such a range of target organisms results from the mode of interaction of each type of BoNT with the different animals and/or the ecology, spore germination requirements, and bacterial cell growth properties of the neurotoxigenic bacteria. Despite these variations, all BoNTs, when injected into sensitive vertebrate species, cause a flaccid paralysis with the same pathogenetic mechanism. This mechanism is a BoNT-induced blockade of skeletal and autonomic cholinergic nerve terminals after specific binding and entry into the cytosol of nerve terminals (8, 10). Indeed, all BoNTs are structurally and functionally very similar and consist of three domains termed L, HN, and HC, with L linked to HN by a single interchain disulfide bond (10, 12–14). BoNTs bind the axon terminals of skeletal and autonomic peripheral neurons via a unique double receptor binding mode, with the two receptors binding sites located in HC (10). However, additional binding interactions with the external surface of the presynaptic membrane are possible (15, 16). BoNTs are then internalized inside synaptic vesicles, where the pH becomes acidic, causing a concerted BoNT-and-membrane change of structure. HN forms a transmembrane chaperone that assists the translocation of the L domain from the lumen to the cytosolic side of the vesicle membrane (10, 14). Here, L refolds and is released upon the reduction of the L-SS-HN bridge by a thioredoxin reductase-thioredoxin protein disulfide-reducing system localized on the cytosolic face of the synaptic vesicle membrane (17–19). L is a metalloprotease that specifically cleaves VAMP/synaptobrevin, SNAP-25, or syntaxin, which are three proteins essential for neuroexocytosis. This cleaving causes a prolonged inactivation of neurotransmitter release (20, 21) with ensuing peripheral neuroparalysis and death. In the case of humans and caged mice, death follows the paralysis of respiratory muscles. However, if respiration is mechanically assisted, botulism patients recover completely from the neuroparalysis, though with different time courses depending on the BoNT type involved (7, 8, 10, 22).

To summarize, from a functional point of view, BoNTs are very sophisticated nanomachines (14), finely tuned around the following four unique physiological features of vertebrate nerve terminals: (i) binding to polysialogangliosides (glycolipids of vertebrates which are highly enriched in presynaptic terminals) and to the luminal domain of integral membrane proteins of synaptic vesicles (SV) (subcellular organelles unique to animals endowed with a nervous system); (ii) low-pH-driven membrane translocation across the SV membrane, which is physiologically necessary

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for neurotransmitter refilling of SV after release by neuroexocytosis; (iii) reduction of the interchain SS bond by an SV redox system; and (iv) metalloproteolytic activity specific for VAMP, SNAP-25, or syntaxin (10, 14, 20, 21). It should be noted that these three proteins (i) contain unique sites of proteolysis by the L chains of BoNTs and (ii) are specifically recognized by the BoNT L chains through extended enzyme-substrate interactions, which include several exosites in addition to the active site (20, 21, 23, 24).

Until a few years ago, the field of the study of BoNTs in general seemed to have reached a kind of steady state, with a small number of basic science papers and an overflow of thousands of clinical papers per year. Then, faster and cheaper DNA sequencing became accessible to microbiology laboratories and national botulism reference centers. Thus, clinical isolates of *Clostridium* species, accumulated over decades, were and are being sequenced. Only very recently have microbiologists begun to sequence isolates from the environment, where the large majority of BoNT-producing clostridia reside. Within a short period of time, the sequences of more than forty different BoNTs, recognizable as variations of known serotypes, were deposited (10). But many more are about to be released. At this time, the BoNTs are classified in serotypes (capital letters from A to G) and subtypes (Arabic numbers); for example, BoNT/A1, A2, A3, etc. However, there is no consensus on what defines a subtype. Some authors have proposed that >2.5% of the primary sequence must be different to define a novel subtype (see reference 25 for a recent review). Clearly, this is an arbitrary figure, as a change in even 1 amino acid residue within the active site of the L chain, in the binding sites of the HC domain, or elsewhere can bring about a profound change in biological properties. It was recently shown that the replacement of only 3 of a total of about 1,300 residues was sufficient to change the substrate cleavage specificity of BoNT/C (26) and the toxicity of BoNT/B1 (27). The increasing number of BoNT variants and the expectation that the soil isolates will provide a potentially unlimited source of novel sequences calls for an international agreement of the botulism research community for a shared categorization. More importantly, the discovery of novel BoNTs poses major problems to current programs aimed at developing BoNT-neutralizing antibodies and vaccines. In fact, some of the presently known novel BoNT subtypes are poorly neutralized by available antibodies developed against subtype 1 of the seven serotypes. This is well illustrated by the proposed definition of a novel serotype H, based on the evidence that the corresponding protein toxin is not neutralized by any of the available anti-BoNT antibodies (9). At the same time, this multitude of novel BoNTs provides a real goldmine of potential novel therapeutics endowed with improved properties with respect to those of existing BoNTs.

The existence of many BoNTs endowed with different immunogenic properties but very similar cellular and molecular mechanisms of intoxication calls for an intensification of the current studies aimed at identifying inhibitors of specific steps of BoNT neuron intoxication (28). At the same time, from a biological point of view, the variability of these neurotoxins raises questions about the processes leading to the generation of such a large number of BoNTs and their possible adaptive significance.

An ecological role of the BoNTs? To date, the number of studies on the biology and ecology of toxigenic clostridia is very limited, particularly in comparison with the extremely large cohort dedicated to the therapeutic uses of BoNTs or to the comprehension of how BoNTs cause neuroparalysis. In particular, there are no reports on possible roles of the BoNTs in the life cycles of clostridia in the various ecological niches where they reside (communication, signaling, fighting competing species, etc.). Such roles cannot be excluded, and it is possible that the pathogenic action of BoNTs on vertebrates is accidental, as is the case for many toxins produced by plants and fungi (29).

The currently predominant explanation concerning the role played by BoNTs in the clostridial life cycle is that they serve the goals of bacterial multiplication and diffusion by means of killing vertebrate animals. Causing host death is seen as a proactive strategy to expand the anaerobic ecological niche that is supportive of clostridial growth with respect to the passive strategy of proliferating on anaerobic decomposing biological materials or on animals that die for any reason. In fact, an oxygenated animal is transformed by death into a large anaerobic fermenter capable of hosting the proliferation of toxigenic and nontoxigenic clostridia in numbers of billions and billions. Clostridia may derive internally from limited anaerobic portions of the intestine (7) or be acquired by spore contamination from the environment. When the nutrients are consumed and/or the bacteria are exposed to O_2, the clostridia sporulate and the spores are diffused in the environment by physical forces. When a niche permitting spore germination is found, vegetative cells arise, and they may produce and release BoNTs. However, given that the cellular pathogenesis of botulism caused by the known BoNT isoforms is very similar if not identical, how can one reconcile the existence of so many variants? It appears to us that, in order to decipher the evolutionary significance of BoNT variability, an effective approach would be to put greater efforts into the study of animal botulism occurring in the wild, where the disease affects many different vertebrates in numbers that are orders of magnitude larger than the numbers of cases in humans or pets and, thus, has a much higher evolutionary impact (7, 8, 30).

Neurotoxigenic clostridia are present in the environment mainly in the form of spores, which can be found in several terrestrial and aquatic environments and in the intestine of some animals (7, 30). Clostridial spores can germinate where appropriate conditions of anaerobiosis, pH, and nutrients are met, giving rise to the vegetative cells. These conditions occur in decaying biological materials in soil and mud at the bottom of lakes, ponds and wells and in cadavers and carcasses of animals that have died for any reason and that are contaminated by clostridia present in their gut before death, by spores of clostridia acquired from the environment, or by anaerobic aliments and forages. Given the diversity of ecological habitats, of species involved, and of feeding habits, animal botulism is actually extremely variable in terms of ecology and epidemiology, as well as in the mode of entry of the BoNTs into an animal's body, though not in the molecular and cellular pathogenesis processes leading to neuroparalysis. In addition, outbreaks of animal botulism frequently take the appearance of a toxin infection rather than that of an intoxication, due to cannibalism of cadavers or coprophagy of contaminated feces or feeding on invertebrates acting as toxin-insensitive vectors of the BoNTs, rapidly leading to the involvement of many individuals (7, 30). There is evidence, based on accurate observations in the field and on farms, that animal botulism can follow different sequences of events. It begins with the germination of spores within anaerobic vegetable or animal materials and is continued by the proliferation of neurotoxigenic bacteria, producing one or more BoNT

types depending on the clostridia present. Different sequences of events may then follow depending on the particular situation and environment. In the muddy bottom of aquatic environments, a variety of invertebrates can eat the BoNT-containing biological material and, thus, become vectors of the toxins, spores, and bacteria, which are delivered to any animal in the food chain, including vertebrates, which will then be affected by botulism (7, 30). The mixture of BoNTs, spores, and bacteria can also be ingested directly by birds and fish feeding within the muddy bottom of ponds and rivers. A very relevant point to be noted is that the amount of BoNT necessary to cause death is much lower in the environment than in the laboratory. In fact, even the very small amount of BoNT required to cause the first signs of botulism, such as impairment of vision or decreased mobility, are lethal for an animal in its environment, e.g., a duck that has to fly, a fish that has to swim well to feed or to escape predators, etc. Much larger amounts of BoNTs are necessary to kill a caged animal in the laboratory, as death comes by respiratory failure. One extreme example illustrating the difference between environment and laboratory is that of frogs, who can survive the blockade of the respiratory function by exchanging oxygen through the skin. BoNT-injected frogs were observed to remain immobile but alive in the laboratory for months (J. Molgo, personal communication), while they would have been rapidly predated in the environment.

Any cadaver, whatever the cause of death, if exposed to air will be soon colonized by flies. They will deposit their eggs, causing the cadaver to become full of larvae, which grow by eating the decaying flesh and become very toxic if neurotoxigenic *Clostridium* is present. Many birds and fish consume maggots and will consequently die of botulism, perpetuating a cycle that can, in a few days, involve very large numbers of individuals (up to millions have been recorded) (7, 30). Alternatively, there are animals that may feed on cadavers, and hence, dead vertebrates can act as direct BoNT sources. An additional potentially toxic food is constituted by feces. Moreover, BoNT-containing decaying biological materials can contaminate food such as hay or water, thus transmitting the BoNT to large animals, including cows and horses, during feeding or drinking (7, 30). In addition, intoxicated animals may travel some distance from the site of ingestion, thus diffusing an animal botulism outbreak over a large geographic area. It is also possible that some toxin-resistant vertebrate species (cats, pigs, etc.) act as carriers of clostridia and/or BoNTs (7). Such chains can be interrupted only by removing all cadavers, which is rarely feasible in the wilderness. As animal botulism develops among carnivorous and omnivorous animals or in species feeding in the anaerobic muddy bottoms of lakes, ponds, and rivers, many vertebrates species may be potentially involved. Additionally, insectivorous birds are prone to die of botulism for the reasons mentioned above (7, 30). The emerging picture of animal botulism in the wilderness is a very complex one.

The production of BoNT appears to be a quality distinct from bacterial survival and growth, as clostridia may survive and replicate without the requirement for residence in the vertebrate host. Therefore, the origin of the extremely toxic BoNTs is a matter of speculation, which includes the possibility of accidental or cryptic pathogenesis (29). Regardless of the origin, once acquired, the BoNTs would have been maintained only if the trade-off between metabolic/genetic costs and the advantages conferred in terms of clostridial growth and diffusion was relevant for an increase of bacterial fitness. From this point of view, the quality of the BoNT variants should be evaluated solely for their performance in increasing the diffusion of clostridia. It is difficult to escape the conclusion that, while it is true that vegetative cells may survive and replicate without BoNTs, the action of the neurotoxin greatly expands the dimensions of the environments favorable to clostridial growth and multiplication. Neurotoxigenic clostrida would act as ecological molecular engineers that use BoNTs to expand the physical and geographical extension of anaerobic environments rich in nutrients.

Large botulism outbreaks, with their enormous proliferation and diffusion of clostridia, provide the opportunity for the generation of BoNT gene and BoNT-associated gene variants. These variants are produced by mutations and by gene or gene fragment exchanges by various means, including phages and plasmids and other mobile genetic elements. In other words, each botulism outbreak is an occasion for the occurrence of novel BoNT variants that may be selected by (i) any of the factors that characterize BoNT survival within the different biological materials and fluids outside and within the invertebrates and vertebrates that are involved in animal botulism and (ii) by the parameters determining the various steps of the pathogenesis of the disease in the different species of vertebrates. In this respect, the BoNT/E-producing *Clostridium* spp. and the BoNT/E neurotoxins, which are associated with botulism of fish and other aquatic animals (31), appear to have evolved under the pressure imposed by the need to be capable of growth and production of the neurotoxins at low temperatures.

Although available data and hints indicate that the BoNTs are the result of adaptive evolution, we cannot exclude the possibility that nonadaptive processes have influenced their variability. Indeed, one should always keep in mind that "Variations neither useful nor injurious would not be affected by natural selection, and would be left either a fluctuating element, as perhaps we see in certain polymorphic species, or would ultimately become fixed, owing to the nature of the organism and the nature of the conditions." (32). A formalization of this prediction by Darwin is provided by the neutral theory of molecular evolution. This theory predicts that most variation at the molecular level does not affect fitness and is best explained by stochastic processes. This prediction has been widely confirmed by the analysis of genomic sequences (33). Thus, to investigate the occurrence and maintenance of BoNT variability, both nonadaptive and adaptive processes should be taken into account. To evaluate whether or which BoNT isoforms were the result of nonadaptive processes, such as a combination of neutral mutations and genetic drift, one could take advantage of predictions of the neutral theory that can be experimentally tested (33). According to these predictions, by comparing BoNT amino acid sequences, we should expect to find that (i) conservative changes occur much more frequently than radical changes, (ii) synonymous base substitutions, which do not cause amino acid changes, almost always occur at a much higher rate than nonsynonymous substitutions, and (iii) pseudogenes evolve at a high rate and this rate is the same in the three-codon positions. When large numbers of *bont* sequences, including those derived from environmental samples, are made available, the testing of these predictions will shed light on the contribution of nonadaptive evolutionary processes to the maintenance of BoNT variability.

At the same time, to highlight the adaptive value, it is necessary to evaluate the overall performance of each BoNT subtype with

TABLE 1 Proposed steps from toxin production release to neuroparalysis to be considered in order to evaluate the adaptive value of the different BoNTs[a]

Step	Role	Possible method of evaluation	Reference(s)
1	Preservation of the integrity of the BoNT molecule, either alone or in complex with NTNHA or in complex with NTNHA and HA proteins or HA-like proteins in decaying biological materials	MS techniques using isotopically labeled BoNTs, assay of SNARE-specific metalloprotease activity	10, 25, 39–41
2	Preservation of the integrity of the BoNT molecule, either alone or in complex with NTNHA or in complex with NTNHA and HA proteins or HA-like proteins inside invertebrates	MS techniques using isotopically labeled BoNTs, assay of SNARE-specific metalloprotease activity	7, 10, 30
3	Preservation of the integrity of the BoNT molecule, either alone or in complex with NTNHA or in complex with NTNHA and HA proteins or HA-like proteins facing the activity of the proteolytic gastric juices	MS techniques using isotopically labeled BoNTs	7, 39, 40
4	Capability of BoNT to cross the intestinal wall using intestinal loop animal models (BoNT/C)	Intestine models and sensitive methods of detection of the biologically active BoNTs	10, 39, 40
5	Preservation of the neurotoxic active form of BoNT within the body fluids (lymph, blood)	Isotopically enriched BoNTs and assays of neuroparalysis, such as the DAS assay	46
6	Binding to the presynaptic membrane	Biochemical assays	47
7	Endocytosis inside nerve terminals	Quantitative methods to be established	9
8	Membrane translocation	Biophysical and cell biology methods	48–50
9	SNARE proteolysis	Biochemical assays	20, 37, 38, 48–50
10	Duration of action of the L metalloprotease activity inside nerve terminals	DAS assay, rotating wheel assay, electrophysiological methods	51–54

[a] NTNHA, nontoxic nonhemagglutinin protein; HA, hemagglutinin; MS, mass spectrometry; DAS, digit abduction score.

respect to the steps defining the long road leading from toxin production to neuroparalysis. To date, the limited number of reports available show that the BoNTs vary in terms of toxicity, entry into neurons, enzymatic activity, site of SNARE cleavage, and duration of action (34–38). These studies, as relevant as they might be for the therapeutic use of the BoNTs, only partially reflect the relevance of the various BoNTs for clostridial growth and diffusion in the wilderness. Indeed, a specific BoNT's performance may be seen as crucial because of its medical application but, at the same, it may be insignificant to natural selection if it does not affect the toxin's ability to favor the spread of clostridia. For a full evaluation of a BoNT's performance, one has to consider all the steps a BoNT goes through from its synthesis to animal death and attempt to make a quantitative evaluation. Table 1 lists these steps and indicates possible assays. Step 1 refers to the decaying biological materials or foods where BoNTs are released, which contain proteases and protein-modifying chemicals that can inactivate the toxin protein molecule. This factor could vary greatly among the different *Clostridium* media. It should be evaluated in the BoNT complexes that are comprised of BoNT plus the additional proteins expressed by the various *bont* loci (10, 25, 39, 40). The most important one appears to be the nontoxic nonhemagglutinating protein, which folds very similarly to the BoNT molecule and forms a heterodimer with it that shields a large part of the BoNT surface (41). Similar considerations can be taken into account for steps 2 and 3. The crossing of the intestinal wall from the lumen to the tissue (step 4) is a particularly critical step, and it is expected to vary greatly in different animals. In addition, it should be considered that many strains produce more than one BoNT subtype, and so, there is the possibility that two BoNTs synergize, similarly to the two anthrax toxins and some snake toxins (42–44). Moreover, some strains of BoNT/C-producing *C. botulinum* also release a C2 toxin that modifies actin and therefore is very likely to affect the passage of BoNT/C across the polarized intestinal epithelial monolayer (45). Step 5 refers to the survival of the active form of a BoNT within the lymphatic and blood circulatory systems, which contain antibodies and other proteins endowed with potential BoNT-binding activities, proteases, chemicals, radicals, etc. (46). Steps 6 to 9 have been discussed previously (9, 20, 37, 38, 47–50). The last step of Table 1 refers to the duration of action inside nerve terminals. This factor can be inferred using animal tests, such as the DAS assay (51–54). *In vivo* methods have to be used because the duration of action is the result of biochemical factors acting inside neurons, as well as the activities of other cells and factors only present *in vivo* (perisynaptic Schwan cells, muscle fibers, other cells, etc.)

The evaluation of each of these steps for the various BoNT subtypes has to be made in order to compare the overall performance of different BoNTs. Currently, only a few of these steps are being considered, and this does not permit appropriate evolutionary considerations to be taken into account. As an example, one can envisage the case of a BoNT isoform that is highly resistant to proteases and well adsorbed into the circulation simultaneously having a low catalytic activity. This would be considered an unfavorable evolutionary product if one considers only the metalloprotease enzymatic activity in neurons in culture or even in a toxicity test. Other examples that one could encounter are those of a BoNT isoform that is highly toxic when injected into mice but is highly sensitive to proteases or a BoNT isoform that is poorly adsorbed into the general circulation but that compensates with a high neurotoxicity and a long duration of action. Overall, the latter isoform may be evolutionarily as fit as the first one, though it would clearly be a better candidate for therapeutic use. It should perhaps be emphasized that by performing these tests for the novel BoNT subtypes, one may well discover toxins with more useful therapeutic properties

Here, we have only touched upon the possible role(s) that BoNTs may have in clostridia within their environments. How-

ever, there is sufficient rationale to justify *ad hoc* investigations, and one can predict that such studies will also throw light on the fundamental question of the origin of such marvelous nanomachines. At the same time, the considerations put forth in this paper call for large efforts in next-generation sequencing of soil samples from all over the world because novel toxins endowed with useful therapeutic properties can be identified. In addition, it is possible that invertebrate-specific neurotoxins will be discovered and that these novel neurotoxins will help in tracing the evolutionary history of botulinum neurotoxins.

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