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Glycosphingolipids—Sweets for botulinum neurotoxin

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A number of viruses, bacteria, and bacterial toxins can only act on cells that express the appropriate glycosphingolipids (GSLs) on the outer surface of their plasma membranes. An example of this dependency is provided by botulinum neurotoxin (BoNT) which is synthesized by *Clostridium botulinum* and inhibits neurotransmission at the neuromuscular junction by catalyzing hydrolysis of a SNARE protein, thereby inducing a flaccid paralysis. Haemagglutinin components of progenitor forms of BoNT mediate its adherence to glycosphingolipids (GSLs) on intestinal epithelial cells while the cellular activity of most isolated serotypes requires the presence of certain gangliosides, especially those of the Gg1b family. This review discusses available information about the identity and the roles of GSLs in the activity of BoNT. Observations that serotypes A–F of BoNT require gangliosides for optimum activity (serotype G apparently does not), permits the hypothesis that it should be possible to develop an antagonist of this interaction thereby inhibiting/reducing its effect.
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Due to their high concentration in the central nervous system, the biological role of gangliosides has been extensively studied. A result of these studies was the discovery that various pathogens interact with the carbohydrate portion of cell-surface gangliosides and use them as receptors. One of these pathogens, botulinum neurotoxin progenitor complex, binds to a variety of GSLs, depending on the serotype and subunit of the complex. It is these interactions that have been the focus of several recent papers and are the subject of this review.

General description of botulinum neurotoxin

Each of the seven serotypes (A–G) of BoNT is produced by *Clostridium botulinum* as a single polypeptide chain about 150 kDa in mass that is proteolytically cleaved to yield a light (∼50 kDa) and a heavy chain (∼100 kDa) that are held together by a disulfide bond [1]. The carboxy terminal half of the heavy chain mediates cellular adherence and the amino terminal half is thought to mediate cytoplasmic entry of the light chain which is a zinc endoprotease [2]. When produced by the bacterium, the neurotoxin is found as part of a complex [3], called the progenitor toxin. Three different forms of progenitor toxins have been identified, a 12S (∼300 kDa), 16S (∼500 kDa), and 19S (∼900 kDa) [4]. BoNT/A exists in all three progenitor forms, B, C, and D exist as 12S and 16S complexes, G as a 16S and E and F in the 12S form [5]. 12S progenitor toxins consist of one molecule of neurotoxin (NTX) and one molecule of a non toxic nonhaemagglutinating protein (NTNH), 16S consist of NTX, NTNH, and 2 molecules of hemagglutinin (HA), and the 19S is a dimer of the 16S which is thought to be held together by 8 HA1 subunits of HA [5]. HA is made up of five subunits, two molecules of HA1, and one each of HA2, HA3a and HA3b. Binding of the progenitor complex and transport of the neurotoxin across the intestinal epithelium is mediated by the HA [6]. The associated proteins are also believed to protect orally ingested BoNT from degradation as it passes down the digestive tract. Upon absorption from the upper portion of the small intestine, the progenitor toxin enters the lymphatic system and then into the blood [4]. The increase in pH encountered upon leaving the digestive tract is believed to cause dissociation of the complex since free BoNT is found in both the lymphatic circulation and blood [7,8].

Free BoNT can then bind to its receptor(s) on the terminae of neurons at the neuromuscular junction and be internalized. Once in the cytosol, it catalyzes the cleavage of one

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of the SNARE proteins (SNAP-25-serotypes A, C, and E, synaptobrevin-serotypes B, D, F, and G, or syntaxin-serotype C) [for a review see 9] thereby inducing a flaccid paralysis. Because the toxin is active at extremely low concentrations, no one has quantified what constitutes a toxic dose [10], although it is estimated to be 100 billion times more toxic than cyanide [11]. Therefore, BoNT is considered to be a biological warfare threat by the military. In contrast, clinicians find it useful for the treatment of a number of problems induced by excessive activity of peripheral cholinergic neurons [12].

Evidence that the progenitor toxin adheres to GSLs

Observations indicating that certain viruses, bacteria and bacterial toxins require the presence of specific cell surface GSLs in order to infect or act on target cells [for a recent review see 13] appear to apply to both the 16S and 19S forms of progenitor botulinum neurotoxin as well as to the free neurotoxin (BoNT) with the exception of serotype G [14].

In regards to the progenitor toxin, a series of studies indicated that the haemagglutinin component found in the 16S and 19S complexes can bind to specific GSLs. Initial evidence was provided by the observation that the 16S form of BoNT/C adhered to the microvilli of epithelial cells of the small intestine while free BoNT/C and the 12S form of the neurotoxin did not [6]. In contrast, free BoNT/A and /B were found to bind human intestinal cell lines and to be transcytosed to the basolateral compartment [15]. When the 16S type C progenitor toxin was injected into the upper small intestine of guinea pigs, severe symptoms were seen within eight hours while no symptoms or transfer of the neurotoxin into the blood stream was observed in animals injected with free BoNT/C or the 12S progenitor [6]. Analysis of the binding of HA1 from type C progenitor toxin indicated that it bound to sialoparagloboside, ganglioside GM3, and paragloboside, and did not bind in detectable amounts to sialidase-treated human erythrocytes or porcine intestinal epithelial cells. Composition of and abbreviations for the saccharide portion of GSLs referred to are shown in Table 1. The later observation indicated that type C progenitor toxin binding is dependent upon the presence of sialic acid [16]. X-ray crystallographic studies of type C HA1 indicated that it has two β-trefoil domains and structural similarity to the B chain of ricin. In addition, type C HA3b was found to adhere to the same sialylated GSLs as HA1 but not to paragloboside [16]. Protease treatment of erythrocytes was found to reduce binding by the type C progenitor toxin which suggests that the toxin may also adhere to glycoproteins on the cell surface [16].

In contrast to type C, analysis of the binding of type A progenitor toxin HA1 to lipids separated by thin layer chromatography indicated that it adhered to GSLs having terminal galactosyl moieties. Paragloboside appeared to be the best ligand followed by asialo-GM1, Gb3, Gb4, and LacCer [17]. While type A HA3b was found to bind sialylated carbohydrate components [18], it was found that the HA1 actually mediated binding to cells by binding to Galβ1–4GlcNAc residues [17]. Structural comparison of type A HA1 with the B chain of ricin bound to lactose indicated the potential location of the HA1 carbohydrate binding site, while isothermal titration calorimetry indicated which mutations resulted in failure of the HA1 to bind lactose, a proven ligand. The results of the later studies indicated that type A HA1 has only one carbohydrate binding site [19].

Evidence that the neurotoxin binds to gangliosides

Numerous reports indicate that most BoNT serotypes require the presence of gangliosides of the 1b series to act most effectively on target cells. The only exception to this appears to be BoNT/G which has been reported to bind target cells in the absence of gangliosides [14]. The requirement of gangliosides for the activity of a Clostridial neurotoxin was first

Table 1. Structure of the saccharide portion of glycolipids discussed in this review

Glycolipid	*Saccharide structure*
Lactosylceramide	Galβ1–4Glcβ1–
Trihexosylceramide (Gb3)	Galα1–4Galβ1–4Glcβ1–
Paragloboside (nLc4)	Galß1–4GlcNAcß1–3Galβ1–4Glcβ1–
Sialosylparagloboside (SnLc4)	NeuAcα2–3 Galß1–4GlcNAcß1–3Galβ1–4Glcβ1–
Globoside (Gb4)	GalNAcß1–3Galα1–4Galß1–4Glcß1–
GM3	NeuAc/NeuGcα2–3Galβ1–4Glcβ1–
GM1	Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ1–
Asialo-GM1	Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–
GD1a	NeuAcα2–3Galβ1–3GalNAcβ1–4(NeuAcα2–3)Galβ1–4Glcβ1–
GD1b	Galβ1–3GalNAcβ1–4(NeuAcα2–8NeuAcα2–3)Galβ1–4Glcβ1–
GT1b	NeuAcα2–3Galβ1–3GalNAcβ1–4(NeuAcα2–8NeuAcα2–3)Galβ1–4Glcβ1–
GQ1b	Galβ1–3GalNAcβ1–4(NeuAcα2–8NeuAcα2–3)Galβ1–4Glcβ1– \|α2–3 NeuAcα2–8 NeuAc

Table 2. GSLs bound by different serotypes of BoNT or components of the progenitor toxin

Component	*GSL bound*	*Reference*
A	GD1a, GD1b, GT1b, GQ1b	[22,51]
B	GD1a, GD1b, GT1b	[23,25]
C1	GD1a, GD1b, GT1b	[25,52]
D	GT1b	[23]
E	GD1a, GT1b, GQ1b	[23,32]
F	GD1a, GD1b, GT1b	[25]
G	Not needed	[14]
A-HA1	nLc4, Asialo-GM1, Lac-Cer, Gb3, Gb4	[17]
C-HA3, HA3b	SnLc4, GM3	[16]
C-HA1	SnLc4, GM3, nLc4	[16]

observed in studies of tetanus toxin (TTx) [20]. Information learned about TTx, which has ∼65% sequence homology and ∼35% sequence identity and an even greater conservation in its predicted secondary structure with BoNT/A [1,21], has often served as a model for subsequent studies of BoNT. In this instance ten years elapsed before studies of the interaction of BoNT with gangliosides were done. The results of this work indicated that preincubation of either BoNT/A, /B, /D, or /E with gangliosides, especially GT1b resulted in detoxification [22,23]. Subsequently it was found that when BoNT/A, /B, /C, or /F was overlaid on gangliosides separated by thin layer chromatography (TLC) each serotype was able to bind to specific gangliosides [*e.g.* 24,25]. Table 2 provides a summary of ganglioside ligands identified for different serotypes of BoNT as well as for HA components of the progenitor toxin. Furthermore, studies in which BoNT/A or /B was injected into GM2/GD3 synthase (β1,4GalNAc transferase) knockout mice indicated that those animals survived for a longer period of time than control mice expressing the gene [26]. Subsequent studies indicated that BoNT/A was unable to block acetylcholine release in hemi-diaphragm preparations prepared from the knockout animals [27].

Results of studies in which the activity of BoNT/A on neuroblastoma cells expressing gangliosides of the 1b series were compared to its activity on cells in which ganglioside synthesis had been inhibited by PPMP (d,l-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-propanol·HCl) also indicated that gangliosides were needed for BoNT/A activity. Only intact SNAP-25 was found in PPMP-treated cells exposed to BoNT/A, while predominantly cleaved SNAP-25 was found in untreated, control, cells exposed to the neurotoxin (Figure 1, lanes A–D). The converse experiment gave the same results (Figure 1, lanes E–G). In that study, neuroblastoma cells lacking polysialylated 1b gangliosides were not acted upon by BoNT/A. However, when the cells were grown in the presence of added GT1b, they were susceptible to BoNT/A, as indicated by the cleavage of SNAP-25 [28].

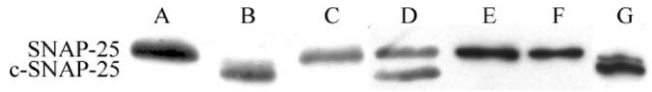

Figure 1. Botulinum neurotoxin A activity in cultured cells, as monitored by SNAP-25 cleavage using Western blot analysis. Lanes A–D show results obtained when N2a murine neuroblastoma cells were (A) untreated, (B) exposed to BoNT/A (C) treated with PPMP for 48 hrs prior to and while exposed to BoNT/A, and (D) treated with PPMP as in C and also with GT1b for 24 hr prior to exposure to BoNT/A. Lanes E–G show results obtained for SK-N-SH human neuroblastoma cells treated with (E) no BoNT/A, (F) BoNT/A, or (G) GT1b for 24 hr prior to exposure to BoNT/A. The BoNT/A concentration used was 6 nM and the time of exposure was 48 hr. Proteins were separated by SDS-PAGE on a 5% stacking, 13% running gel. After transfer to a PVDF membrane SNAP-25 was visualized using an anti-SNAP-25 monoclonal antibody followed by an HRP-conjugated goat anti-mouse antibody. Bands were visualized using Super-Signal West Pico Chemiluminescent Substrate and exposure to film. (Adapted from [28]).

Identification of the GSL-binding site of botulinum neurotoxin

Tetanus toxin and botulinum neurotoxin are AB toxins, in other words, they both have a B-binding peptide and an A-activating peptide. Studies of the binding of fragments of TTx with gangliosides provided the first indication that the binding site was in the carboxy-terminal portion of the heavy chain [29]. Subsequently it was found that deletion of the carboxy-terminal ten amino acids resulted in a loss of its ability to bind to gangliosides [30]. Analysis of the crystal structure of the carboxy-terminal half of the heavy chain of TTx indicated that it contained a structure similar to that found in certain lectins that contain carbohydrate binding sites. Because of the similarities between tetanus toxin and BoNT/A the prediction was made that the carbohydrate binding site(s) of BoNT would be similar [31]. Experiments to identify the GSL binding site of BoNT followed those done with TTx. Initial studies with BoNT/E indicated that the GSL-binding site was associated with the C-terminal half of the heavy chain [32]. Further fractionation of the heavy chain indicated that loss of the C-terminal 42 kDa portion from either BoNT/A, /B, or /E inhibited the neurotoxin from forming homodimers or other aggregates while loss of the C-terminal 18 kDa fragment from BoNT/A resulted in loss of binding to cells [33]. X-ray crystallographic analysis of the structure of BoNT/A [34] and /B [35] indicated that the binding domains of each were similar to that of TTx. Subsequent studies indicated that in contrast to TTx which has two binding sites on the carboxy-terminal half of its heavy chain [36], there is only one carbohydrate binding site on that of both BoNT/A and /B [37]. Mutagenesis studies also indicate that the ganglioside binding site for both of these serotypes involves amino acids in the carboxy terminal half of the heavy chain [37].

While crystallographic information is available for BoNT/B bound to sialyllactose [35], its structure in association with the oligosaccharide portion of a ganglioside ligand such as GT1b has not been determined. Such a study would indicate the number and location of binding sites as well as whether binding of the neurotoxin to GT1b induced changes in its structure that might affect subsequent steps in its action.

Characterization of the interaction of BoNT with gangliosides

Evidence indicates that it is gangliosides of the Gg1b series that are adhered to by BoNT. Work in this lab has centered on characterizing that interaction. Studies of the binding of BoNT/A, /B, and /E to gangliosides indicated that each of the three serotypes adhered to GT1b better than to GD1a, or GM1, and that as the ionic strength increased, less binding was observed [38]. It was also found that BoNT/A appeared to bind to the gangliosides more effectively than did BoNT/B or E [38]. Studies by Ochanda *et al.* [25] indicated that binding of BoNT/B and /F was stonger at low ionic strength while BoNT/C1 bound gangliosides at both low and high (physiological) ionic strengths. Interestingly, the binding of BoNT/C1 was biphasic at high ionic strengths. This raises the question of whether this serotype has two carbohydrate binding sites. Differences between the serotypes of BoNT may reflect the significant amino acid sequence divergence between them [1].

When analyzing the results of studies of the binding of BoNT to GSLs, consideration needs to be given to the type of assay used to monitor it. ELISAs generally require multiple washes to remove unbound neurotoxin, antibody, and possibly a secondary antibody. This can be a potential problem when the protein binds with low affinity to its GSL ligand, a condition which may permit it to dissociate from its ligand during the washes. In order to obtain real-time measurements of the binding of BoNT/A to gangliosides, we used surface plasmon resonance. We observed that BoNT/A bound to GT1b when the ionic strength was increased from 0.06 to 0.16 and that the K_D was similar ($\sim 10^{-7}$ M) for each ionic strength tested. The observation that the longer the BoNT/A was allowed to adhere to GT1b in a GT1b/phosphatidylcholine monolayer the more slowly it dissociated (Figure 2A) indicated that it might undergo a change in conformation over time. This was confirmed by determining the CD spectra at different time intervals after exposure of the neurotoxin to GT1b. The results indicated that there was a significant increase in alpha-helical content and a concomitant decrease in the proportion of beta-sheet. No change in spectra was seen when the toxin was incubated with GM1 [39, Figure 2B]. The ganglioside-induced change in conformation may enable the toxin to (1) bind a protein that is essential for its uptake or (2) facilitate a subsequent step in its trip from outside the neuron into the cytosol.

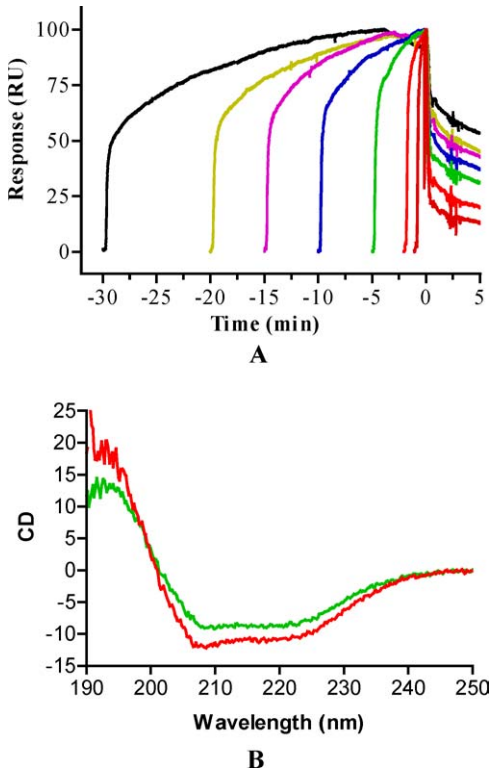

Figure 2. SPR and CD data indicate that BoNT/A changes conformation upon binding to GT1b. (A) Sensorgrams of the injection of BoNT/A (100 nM) for various times (1–30 min) over a 2%GT1b/dimyristoyl phosphatidylcholine monolayer. Each sensorgram was normalized to 100 response units (RUs) and aligned at the injection stop point, in order to compare the dissociation phases. (B) CD Spectra obtained for BoNT/A alone (upper line at 210 nm) or incubated with GT1b (137 μM) (lower line at 210 nm) for 180 min. (Adapted from [39]).

Portion of the GSL needed for binding by the progenitor complex and the neurotoxin

Studies of the progenitor toxin, as stated above, showed that the haemagglutinin of the type A progenitor toxin bound to paragloboside and asialo-GM1. In contrast, the HA of type C1 progenitor toxin preferred the sialylated lipids, sialylparagloboside and hematoside (GM3). At this point it is not known whether the oligosaccharide portion of these lipids can function as effective ligands for the progenitor toxins.

Experiments to determine components of GT1b needed for adherence by BoNT/A indicated that conversion of the sialosyl residues to the C(7) analog or the C(7) aldehyde had little effect on adherence, while formation of the methyl ester of sialic acid resulted in just a two-fold increase in its IC_{50} [38]. These observations indicate that C(8) and C(9) and the free carboxyl group are not essential components for binding by the toxin. The fact that GT1b is a better ligand than GD1a or GM1 for the neurotoxin indicates that the presence of multiple sialic acids is necessary for optimum binding. The observation that the oligosaccharide portion of GT1b was a much poorer

ligand for BoNT/A than the intact GSL [38] permits the hypothesis that the ceramide portion is necessary either as part of the lipid recognized by the neurotoxin or to present a cluster of oligosaccharide residues for binding by the neurotoxin. The similarity between the ceramide portions of GM1, GD1a, GD1b, and GT1b, and the fact that the ceramide portion is part of the lipid bilayer of the cell membrane, supports the hypothesis that instead of functioning as part of a binding site the ceramide serves to present a cluster of oligosaccharide residues. This question is currently being investigated.

What is the function of GSLs in the action of botulinum neurotoxin?

Evidence available thus far indicates that binding of the HA component of the progenitor toxin to GSLs on the epithelial cells lining the intestine is the first step in its uptake by and subsequent release from those cells into the lymphatic circulation. The fact that orally administered BoNT/C associated with the 16S progenitor complex was found to be released into the circulation more readily than the free neurotoxin or the 12S progenitor toxin [6] supports this hypothesis. If after release of the complex and then of free neurotoxin into the lymphatic circulation, it enters the circulation via the thoracic duct, the neurotoxin would be carried to peripheral organs prior to the liver. This may result in a greater amount of the neurotoxin being available for binding to the terminae of cholinergic neurons that innervate muscle.

Studies of tetanus toxin indicated that its binding to GT1b induced a change that enabled it to bind to two proteins present in a mixture of synaptosomal proteins that had been separated by SDS-PAGE and then transferred to blots. The proteins were identified as synapsin I and adducin [40,41, respectively]. The change in binding induced by exposure of TTx to GT1b may reflect the conformational change that it was found to undergo when exposed to GT1b [42,43]. While BoNT/A bound to the same proteins in the absence of ganglioside, its binding appeared to increase significantly upon exposure to ganglioside. Binding to these intracellular components *in vivo* could serve to hold the neurotoxin at sites that would facilitate its ability to act on a SNARE protein.

In addition to the requirement for gangliosides, it has also been shown that disruption of lipid rafts by the removal of cholesterol using methyl β-cyclodextrin resulted in failure of NGF-differentiated PC12 cells to internalize TTx appropriately [44]. When cholesterol was present, TTx was found in detergent insoluble lipid domains present in the membranes of the PC12 cells. Because glycosphingolipids are enriched in lipid rafts [45], it is possible that one function binding to gangliosides serves is to bring the TTx into association with lipid rafts. Another is that the conformational change induced by binding to gangliosides permits the toxin to bind to a raft associated protein. Studies with BoNT/B indicated that gangliosides were needed in order for it to bind to synaptotagmin [46], and that mice lacking gangliosides of the Gg1b series had a longer survival time when injected with BoNT/B than animals expressing Gg1b gangliosides [47]. Based on the finding that adherence of BoNT/A to GT1b induced a change in conformation of the toxin, it can be hypothesized that the change (1) permits it to bind to a raft-associated protein that functions in its internalization by the cell, or (2) to bring it into juxtaposition with other, possibly protein, components present in the raft. These are two testable hypotheses.

Approaches for the development of potential antagonists of BoNT binding to GSLs

The experimental observations discussed provide a clear indication of the need for GSLs for the activity of at least most serotypes of BoNT. The observation that the oligosaccharide isolated from GT1b was a poor inhibitor of the binding of BoNT/A to GT1b, indicates that a "monovalent" oligosaccharide might not be appropriate. This may reflect the observation made in the study of lectins—that the inherently weak binding of a single saccharide unit by the lectin is strengthened by the ability of the protein to adhere to multiple carbohydrates [48]. While BoNT/A may have but one binding site, the fact that the toxin runs on a native gel with an apparent mass equal to a dimer [33] permits the hypothesis that the dimer may mediate binding to the cell surface. Therefore, experiments such as those done to determine the substrate requirements for binding of cholera toxin to GM1 [49,50] should be done to determine whether BoNT requires a cluster of oligosaccharides as a ligand. Similar experiments could be done to identify inhibitors of the binding of the progenitor toxins to their saccharide ligands. Identification of an effective antagonist of the binding of the progenitor toxin to receptors on the intestinal epithelial cells might provide a rational basis for the development of a drug that could be used prophylactically to protect against orally ingested toxin. Once bound to the clustered ligand on a nondigestible carrier, the toxin would eventually be excreted.

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