The Role of the Interchain Disulfide Bond in Governing the Pharmacological Actions of Botulinum Toxin

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ABSTRACT
All serotypes of botulinum toxin possess a disulfide bond that links the heavy chain and light chain components of the holo-toxin. Experiments were done to assess the functional significance of this covalent bond, and the work was facilitated by use of mercurial compounds that modify residues in the vicinity of the catalytic site. The data indicated that reduction of the interchain disulfide bond had two major effects: 1) changing conformation or orientation of the two chains, which diminished toxicity against intact cells, and 2) loosening or relocating a heavy chain belt segment that encircles the light chain and occludes the catalytic site. Interestingly, disulfide bond reduction of all serotypes produced conformational changes that diminished toxicity against intact cells, but it produced conformational changes that led to exposure of the catalytic site in only three serotypes. For the other serotypes, the catalytic site was accessible even before disulfide bond reduction. Neither of the major structural effects was dependent upon separation of the heavy chain and light chain components of the toxin, nor were they dependent on toxin substrate. Depending on the initial state of the toxin molecule, the combination of disulfide bond reduction and treatment with a mercurial compound could abolish toxicity. Therefore, this combination of treatments was used to convert active toxin into a parenteral vaccine. Administration of the modified toxin evoked a substantial IgG response, and it produced complete protection against a large dose of native toxin.

Botulinum neurotoxin (BoNT) is a 150,000-Da protein composed of a heavy chain (ca. 100 kDa) and a light chain (ca. 50 kDa) linked by a single disulfide bond (Herreros et al., 1999; Humeau et al., 2000). This dichain molecule acts preferentially on cholinergic nerve endings to block exocytosis. To produce this poisoning effect, the toxin progresses through a well described sequence of events that includes binding to receptors on the surface of cholinergic nerve endings, penetrating the plasma membrane by receptor-mediated endocytosis, penetrating the endosome membrane by pH-induced translocation, and then acting in the cytosol to block transmitter release (Schiavo et al., 2000; Simpson, 2004). BoNT is a zinc-dependent metalloendoprotease that acts on a family of substrates found in nerve endings [synaptosomal-associated protein of 25 kDa (SNAP-25), vesicle-associated membrane protein (VAMP; synaptobrevin), and syntaxin]. SNAP-25, VAMP, and syntaxin form a multimeric complex that is essential for exocytosis, and therefore, toxin-induced cleavage of these substrates impairs transmitter release (Herreros et al., 1999; Humeau et al., 2000).

BoNT exists in seven different serotypes, designated A, B, C, D, E, F, and G. Although immunologically distinct, these serotypes possess many of the same structural features and functional properties. Thus, all serotypes share the same structure-function relationships that govern blockade of transmission (Simpson et al., 1999; Schiavo et al., 2000; Simpson, 2004). The carboxy-terminal half of each heavy chain mediates binding, the amino-terminal half of each heavy chain plays a key role in productive internalization, and the light chains express zinc-dependent endoprotease activity.

Interestingly, the dissimilarities and individually unique properties of the seven serotypes are equally important to the mechanism of toxin action (Schiavo et al., 2000; Simpson, 2004). For example, the heavy chain component of each serotype displays binding properties, but each binds to its own unique receptor. As another example, the light chain components of all serotypes are endoproteases, but each has its own unique site of cleavage within its substrate.

ABBREVIATIONS: BoNT, botulinum neurotoxin; BoNT/A serotype A; DTT, dithiothreitol; E_n, serotype E nicked; E_un, serotype E unnicked; SNAP-25, synaptosomal protein of 25 kDa; VAMP, vesicle-associated membrane protein (synaptobrevin); ELISA, enzyme-linked immunosorbent assay.
One of the most obvious properties shared by the seven serotypes is an interchain disulfide bond that links the heavy chain and light chain components (DasGupta and Sugiyama, 1972). There is a body of research indicating that 1) this disulfide bond must be intact for the toxin to poison whole cells, but 2) the disulfide bond must be broken for the light chain to act catalytically in the cytosol (Schiavo et al., 1990; de Paiva et al., 1993). There does not exist a complete explanation at the molecular level for the former observation, but there is a presumptive explanation for the latter. In the intact, disulfide-linked holotoxin, the catalytic site is structurally hindered. When the disulfide bond is reduced, the catalytic site becomes exposed (Lacy et al., 1998).

A careful examination of the literature reveals that almost the entire body of research dealing with the functional importance of the interchain disulfide bond has focused on only one serotype (i.e., serotype A). Thus, there is convincing evidence that reduction of the disulfide bond in BoNT/A diminishes toxicity on intact cells but increases catalytic activity on broken cells or on isolated substrate. There do not exist equally convincing data for other serotypes.

In the work that follows, an attempt has been made to assess the significance of the interchain disulfide bond for the pharmacological activity of several toxin serotypes. This goal was achieved by using two mercurial compounds (mercuric chloride and p-chloromercuribenzoic acid) as reporter groups. Ahmed and Smith (2000) have provided evidence that there is a protein thiold in the immediate vicinity of the catalytic site. Treatment of isolated light chain from BoNT/A with mercurial compounds modifies these groups and dramatically reduces enzymatic activity. Therefore, mercurial compounds were used to gauge the accessibility of the catalytic site before and after reduction of the interchain disulfide bond. The data that have emerged, when combined with existing reports on three-dimensional structure (Lacy et al., 1998; Swaminathan and Eswaramoorthy, 2000), reveal that there is considerable variability in the structural and functional role of the disulfide bond in the several toxin serotypes.

### Materials and Methods

#### Toxins. BoNT serotype A was isolated from cultures according to techniques described previously (Sakaguchi, 1982; DasGupta and Sathyamoorthy, 1984; Simpson et al., 1988). Serotypes B, C, D, E, and F were purchased from Wako Bioproducts (Richmond, VA). To facilitate comparisons of data, preliminary dose-response experiments were done to identify the concentration of each serotype that would paralyze neuromuscular transmission within 55 to 75 min. Unless otherwise noted, this standardized amount was used in all experiments.

Some experiments involved a comparison of the relatively inactive, unnicked form (single chain molecule) and fully active, nicked form (dichain molecule) of serotype E. Nicking was accomplished by using standard procedures. Briefly, trypsin that had been treated with t-1-tosylamido-2-phenylethyl chloromethyl ketone and was crosslinked to 4% beaded agarose was washed three times in 10 mM sodium phosphate buffer, pH 7.5. Single chain toxin was added to trypsin (10:1) in phosphate buffer and incubated at room temperature (ca. 23°C) for 1 h. After incubation, the reaction mixture was centrifuged at 10,000 rpm in an Eppendorf tabletop centrifuge for 5 min. The supernatant containing nicked toxin was collected and stored at −20°C until use.

#### Treatment of Toxins. The various serotypes were exposed to mercuric chloride, p-chloromercuribenzoic acid, or dithiothreitol (DTT). All reagents were obtained from Sigma-Aldrich (St. Louis, MO). Exposure was for 60 min at room temperature.

When toxins were exposed to single reagents for in vitro experiments, no subsequent effort was made to separate reactants. Instead, the volume of the reaction mixture (20–100 μl) compared with the volume of the tissue bath (40 ml) was sufficient to dilute the reagent to a concentration that was below concern. However, when toxins were exposed to sequential treatment with reactants (e.g., DTT, then a mercurial), an intervening step was used. Following the initial treatment, the reaction mixture was fractionated by centrifugation in a NANOSEP Microconcentrator (molecular weight cutoff, 10,000; Pall Filtron Corporation, Northborough, MA). The retentate was washed twice in physiological buffer (see below) and then resuspended in buffer with the second reactant. At the end of the second reaction, the mixture was added to tissue baths as described above.

When toxins were exposed to reagents for in vivo experiments, the reactants were separated. Thus, when toxin was exposed to reducing agents and to mercurials to generate putative vaccines (see below), DTT and mercurials were separated from toxin (see above). This means that animals in the vaccination experiments did not receive either DTT or mercuric chloride.

#### Neuronal preparations. Mouse phrenic nerve-hemidiaphragm preparations were excised and suspended in physiological buffer that was aerated with 95% O2 and 5% CO2 and maintained at 35°C. The physiological buffer had the following composition: 137 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1.0 mM MgSO4, 24 mM NaHCO3, 1 mM Na2HPO4, and 11 mM glucose. In an initial series of experiments, gelatin (0.01%) was added to diminish nonspecific inactivation of toxin, but this procedure was discontinued (see Results). Phrenic nerves were stimulated continuously (1.0 Hz; 0.3-ma duration), and muscle twitch was recorded. Toxin-induced paralysis was measured as a 90% reduction in muscle twitch response to neurogenic stimulation.

It has been a universal finding that dose-response curves that describe toxin action on isolated neuromuscular preparations are logarithmic. More precisely, a 2-fold increase in paralysis times is typically associated with a 10-fold decrease in toxin concentration or toxin potency. Therefore, whenever the paralysis times of experimental tissues exceeded those of control tissues by 2-fold or more, an experiment was terminated.

#### Toxin Electrophoresis. Neurotoxin samples were separated in 10% polyacrylamide gels according to the method of Laemmli (1970), using nonreducing conditions. Gels were stained with Coomassie Blue for 30 min, destained, and photographed.

#### Substrate Cleavage Assay. BoNT/A was assayed for endoprotease activity using mouse brain synaptosomes as the source of substrate. Native or reduced toxin was incubated with 10 to 50 μg of synaptosomal membranes in reaction buffer containing 50 mM Hepes, pH 7.1, 20 mM ZnCl2, and 1% N-octyl-β-D-glucopyranoside. Reduced toxin was prepared by incubation with DTT (20 mM; 1 h; room temperature) in phosphate-buffered saline. The cleavage reaction was initiated by addition of toxin (200 nM final concentration) to substrate, and the reaction was allowed to proceed for 3 h at 37°C. Recombinant light chain was assayed for enzymatic activity in the same manner, except that recombinant SNAP-25 was used as substrate. When samples were incubated with mercuric chloride, or with a combination of DTT and mercuric chloride, the reactants were handled as described above.

Toxin endoprotease activity was assayed using Western blot analysis and anti-C-terminal SNAP-25 antibody (StressGen Biotechnologies, Victoria, BC, Canada) for immunodetection of substrate (Kiyatkin et al., 1997; Poirier et al., 1998). For visualization of SNAP-25, samples were separated on 16.5% Tris-Tricine gels (Schägger and Von Jagow, 1987). After separation, proteins were transferred to NitroPure membranes (Osmonics Inc., Minnetonka, MN) in Tris-glycine transfer buffer at 50 V for 1 h. Blotted membranes were rinsed in distilled water and stained for 1 min with 0.2% Ponceau S in 1% acetic acid. After a brief rinse with distilled water,
molecular weight markers and transferred proteins were identified. Membranes were destained in phosphate-buffered saline-Tween (pH 7.5; 0.1% Tween 20) and then blocked with 5% nonfat powdered milk in phosphate-buffered saline-Tween for 1 h at room temperature. Subsequently, membranes were incubated in 0.5% milk with a 1:5000 dilution of anti-SNAP-25 polyclonal antibody. Secondary antibody was used at 1:20,000 dilution. Membranes were washed again (five times) and visualized using enhanced chemiluminescence (SuperSignal West Pico; Pierce Chemical, Rockford, IL) according to the manufacturer’s instructions. Membranes were exposed to film (Hyperfilm ECL; Amersham Biosciences Inc., Piscataway, NJ) for times adequate to visualize chemiluminescence bands. Peptides were identified by comparison with known standards.

Vaccination and Challenge of Animals. Modified and nontoxic variants of BoNT/E were administered to specific pathogen-free female Swiss-Webster mice. Antigen (2 µg per mouse) was administered intraperitoneally in phosphate-buffered saline at 0, 14, 28, 42, and 56 days. Mice were bled approximately 1 week after the third, fourth, and fifth injections, and specimens were analyzed by ELISA for immunoreactivity.

ELISA was performed as described by Siegel (1988) with only minor modifications. BoNT/E was diluted to 5 µg/ml in phosphate-buffered saline, pH 7.4, and then added to microtiter plates (100 µl/well) that were incubated at 4°C overnight in a sealed container. One percent bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 was used to block nonspecific binding. Serum samples were initially diluted 1:30 and then serially diluted 4-fold for a total of seven dilutions (1:30 to 1:122,880). Diluted sera were added in duplicate to toxin-coated wells (100 µl/well). The secondary antibody was alkaline-phosphate-conjugated goat anti-mouse IgG diluted 1:1000. The primary and secondary antibodies were incubated for 60 min at 37°C. p-Nitrophenyl phosphate (100 µl/well) was added as a substrate. Plates were incubated at room temperature for 30 min, and absorbance was measured with a microplate reader at 405 nm. ELISA titers were defined as the reciprocal of the highest serum dilution giving an absorbance of 0.2 (units) above background.

Results

Structure-Function Relationships in BoNT/A and BoNT/B. Structural analyses of serotypes A (Lacy et al., 1998) and B (Swaminathan and Eswaramoorthy, 2000) have revealed that both holotoxins have a “belt segment” that arises in the heavy chain and encircles the light chain. In serotype A, the belt domain covers the orifice that leads to the catalytic site, whereas in serotype B the belt domain is slightly removed from the orifice and provides no obstruction. These structural features suggest that mercurial compounds should have little or no ability to modify the catalytic site in serotype A, but substantial ability to alter the catalytic site in serotype B. These predictions were tested by measuring the ability of mercuric chloride (Fig. 1) and p-chloromercuribenzoic acid (Fig. 2) to alter the neuromuscular blocking activity of native BoNT/A and native BoNT/B. Interestingly, even when used at rather high concentrations, neither mercuric compound abolished the neuromuscular blocking activity of BoNT/A. However, both compounds produced >99% inactivation of BoNT/B.

In related experiments, mercuric chloride was tested for its ability to alter the catalytic activity of BoNT/A in two types of preparations: 1) recombinant light chain that was expressed in the absence of heavy chain, and 2) native holotoxin that was treated with DTT to break the interchain disulfide bond, which is the only covalent bond between the light and heavy chains of activated toxin. As shown in Fig. 3, the mercuric compound virtually abolished the ability of BoNT/A light chain to cleave SNAP-25. This observation was true both for recombinant light chain and for native light chain that was no longer covalently associated with heavy chain.

When taken in the aggregate, these data demonstrate three things. First, when the belt domain does not cover the orifice that leads to the catalytic site (e.g., native BoNT/B),
mercurial compounds can modify the site and dramatically reduce enzymatic activity. Second, when the belt domain covers the opening to the catalytic site (e.g., native BoNT/A), mercurial compounds are excluded and enzymatic activity is little affected. Third, when techniques are used to remove the belt domain from the orifice to the catalytic site (i.e., expression of light chain in the absence of heavy chain, or disulfide bond reduction of holotoxin), the catalytic site becomes accessible and can be modified.

The Effects of Disulfide Bond Reduction on BoNT/A and BoNT/B Activity and on Dichain Structure. There is a prevailing belief that reduction of the interchain disulfide bond in botulinum toxin will greatly diminish toxicity when measured in vivo (i.e., mouse lethality assay) or in vitro on intact cell preparations (e.g., phrenic nerve-hemidiaphragm preparation). However, almost the entire literature in this area has focused on serotype A. Therefore, experiments were done to determine the generalizability of the premise that an interchain disulfide is needed for toxicity.

BoNT/A, which cleaves SNAP-25, and BoNT/B, which cleaves VAMP, were incubated in various concentrations of DTT for 60 min, after which the solutions were assayed for toxicity on neuromuscular preparations. The results with BoNT/A were in keeping with expectations (Fig. 4A). Thus, incubation with DTT produced concentration-dependent losses in neuromuscular blocking activity. At the highest concentration of reducing agent tested, the apparent toxicity of BoNT/A was reduced by more than an order of magnitude. By contrast, the same concentrations of DTT produced only a small decrease in the potency of BoNT/B (i.e., ca. 20%). For unknown reasons, the failure of DTT to produce a more striking effect was due to the presence of protein (i.e., gelatin) that was added to stabilize toxin in physiological solution. When the experiments with DTT were redone in the absence of protective protein, the loss of neuromuscular blocking activity by BoNT/B was more pronounced (Fig. 4A).

In parallel experiments, samples of DTT-treated toxin were run under nonreducing conditions in denaturing gels. As shown in Fig. 4B, pretreatment with 20 mM DTT reduced the interchain disulfide bond in both serotypes, yielding heavy chain and light chain components in denaturing gels.

Disulfide Bond Reduction and Neuromuscular Blocking Activity of Serotypes C, D, E, and F. An effort was made to ensure that all toxin serotypes, regardless of substrate affinity, were vulnerable to DTT. Therefore, experiments were done with BoNT/C (substrate preference, syntaxin), BoNT/D (VAMP), BoNT/E (SNAP-25), and BoNT/F (VAMP). Each serotype was incubated for 60 min with 20 mM DTT and then assayed for neuromuscular blocking activity. All incubations were performed in the absence of added gelatin.

As shown in Table 1, BoNT/C, D, E, and F all had diminished activity following exposure to DTT. Hence, there was no evidence that vulnerability to DTT is a function of substrate preference.

Pretreatment with Mercurials and Neuromuscular Blocking Activity of Serotypes C, D, E, and F. The underlying rationale for these experiments was the same as that just described for experiments with DTT. Each serotype was incubated with 50 μM mercuric chloride for 60 min, after
which toxicity was assayed on phrenic nerve-hemidiaphragm preparations. As shown in Table 2, the pretreatment did not produce a consistent effect. When tested on holotoxins that cleave SNAP-25 (BoNT/A, see Figs. 1 and 2; BoNT/E, see Table 2), mercuric chloride had a variable effect. It had little ability to diminish the toxicity of BoNT/A, but it had substantial ability to diminish the toxicity of BoNT/E. Mercuric chloride also had a variable effect on serotypes that attack VAMP. Thus, the compound greatly diminished the toxicity of BoNT/B (Figs. 1 and 2) and BoNT/F (Table 2), but it had little effect on BoNT/D (Table 2). Finally, mercuric chloride had little ability to diminish the toxicity of BoNT/C, which is the only serotype that acts preferentially on syntaxin. Thus, the data show that vulnerability to modification by mercurial compounds, like vulnerability to disulfide bond reduction, is not simply a function of substrate preference.

The Effect of Disulfide Bond Reduction on Subsequent Vulnerability to Treatment with Mercurials. The data indicate that native holotoxins of BoNT/A, BoNT/C, and BoNT/D are the least susceptible to the detoxifying effects of mercuric chloride. One possible explanation for this phenomenon is that the interchain disulfide bond maintains the holotoxin in a conformation that obstructs access to the catalytic site. This possibility was strongly supported by the finding that recombinant BoNT/A light chain, which lacks both a disulfide bond and an associated heavy chain, was susceptible to inactivation by mercuric compounds (see above).

A different approach was used with BoNT/C and BoNT/D. Experiments were done in which these serotypes were incubated in 20 mM DTT and then separated from the reagents by gel filtration. For serotypes B, C, E, and F, protein thiol modification produced a statistically significant reduction in toxicity ($P < 0.01$).

### Table 1

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Control</th>
<th>DTT (20 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>73 ± 6</td>
<td>&gt;150</td>
</tr>
<tr>
<td>B</td>
<td>68 ± 5</td>
<td>&gt;122 ± 9</td>
</tr>
<tr>
<td>C</td>
<td>73 ± 9</td>
<td>121 ± 12</td>
</tr>
<tr>
<td>D</td>
<td>55 ± 4</td>
<td>125 ± 9</td>
</tr>
<tr>
<td>E</td>
<td>61 ± 8</td>
<td>&gt;150</td>
</tr>
<tr>
<td>F</td>
<td>56 ± 5</td>
<td>130 ± 11</td>
</tr>
</tbody>
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Effect of DTT on neuromuscular blocking activity

Native or DTT-treated toxin was added to mouse phrenic nerve-hemidiaphragm preparations (group $n = 4$ or more), and paralysis times (minutes ± S.E.M.) were monitored. The values for serotypes A and B were extracted from the experiments illustrated in Fig. 4A. For all serotypes, DTT treatment produced a statistically significant ($P < 0.01$) increase in paralysis times.

Toxin was then exposed to mercuric chloride (50 μM; 60 min; room temperature) and subsequently tested for neuromuscular blocking activity. Interestingly, preincubation with DTT had a striking effect on susceptibility to mercuric chloride. For serotype C, the paralysis time for control tissues ($n = 5$) was 79 ± 6 min; the paralysis times for tissues treated with only DTT ($n = 6$) was 111 ± 8 min; the paralysis times for tissues treated sequentially with DTT and with mercuric chloride ($n = 6$) was >200 min. For serotype D, the paralysis time for control tissues ($n = 3$) was 61 ± 5 min; the paralysis time for tissues treated with only DTT ($n = 6$) was 81 ± 8 min; the paralysis times for tissues treated sequentially with DTT and with mercuric chloride ($n = 6$) was >150 min. Thus, BoNT/A, BoNT/C, and BoNT/D appear to have two properties in common: 1) prior to disulfide reduction, toxicity and catalytic activity are little affected by a mercurial compound (e.g., Table 2), and 2) after disulfide bond reduction, activity is substantially diminished by the mercurial (e.g., data in this section).

A close examination of these data reveals another finding. The fact that disulfide bond reduction due to pretreatment with a reducing agent made serotypes A, C, and D susceptible to mercurials means that the mercurials by themselves were not able to break the interchain disulfide bonds.

The Relationship between Chain Structure and Susceptibility to DTT plus a Mercu- rial Compound. All serotypes of BoNT are synthesized as single chain polypeptides of low toxicity. The single chain molecule undergoes post-translational modification in which an endoprotease cleaves ("nicks") the polypeptide to yield the activated dichain structure. In most cases, clostridia possess an endogenous protease that nicks the toxin. However, in certain cases, such as BoNT/E, the toxin is released by bacteria in an un nicked form. Therefore, experiments were done to assess whether the conformation of the single chain species of BoNT/E would hinder the actions of DTT or mercuric chloride.

In the initial series of experiments, various concentrations of unnicked BoNT/E (BoNT/E$_{un}$) were assayed for toxicity (viz., neuromuscular blocking activity) to establish functional equivalence with nicked BoNT/E (BoNT/E$_{n}$). In keeping with earlier work, the results indicated that a molar ratio of ca. 39:1 (BoNT/E$_{un}$:BoNT/E$_{n}$) produced equivalent paralysis times (Simpson and DasGupta, 1983).

BoNT/E$_{un}$ was incubated with either DTT (20 mM; 60 min; room temperature) or mercuric chloride (50 μM; 60 min; room temperature) and then tested for its ability to paralyze phrenic nerve-hemidiaphragm preparations. In both cases the reagents had a striking effect. Thus, the paralysis times were as follows (group $n = 5$ or more): control, 68 ± 9 min; DTT-treated, >150 min; mercuric chloride-treated, >150 min. Combined treatment with DTT and mercuric chloride caused complete loss of neuromuscular blocking activity (and see below).

Combined Use of Disulfide Bond Reduction and Mercu- rial Chloride Treatment to Generate a Vaccine against Botulism. A number of techniques have been used to generate potential vaccines against botulism, most of which fall into two broad categories: either native toxin is modified to render it nontoxic, or a fragment of the toxin is isolated that is nontoxic. As an example of the former, native toxin has been treated with formaldehyde to induce so many structural changes that the molecule is no longer toxic. As an
example of the latter, the carboxy-terminal half of the heavy chain has been generated by the techniques of protein chemistry or molecular biology, and this polypeptide lacks the ability to cause neuromuscular blockade.

An obvious drawback to the two traditional approaches is that they involve putative vaccines that do not contain all the epitopes found in native toxin. An alternative was recently described in which site-directed mutagenesis was used to modify only two amino acids in the entire toxin structure (ca. 1200 amino acids), and this nontoxic mutant was an efficient vaccine (Kiyatkin et al., 1997). The data presented in the preceding section suggest that there could be an even simpler approach.

BoNT/Eun was treated sequentially with DTT (20 mM; 60 min; room temperature) and with mercuric chloride (200 μM; 60 min; room temperature). The resulting product was administered i.p. to mice (group n = 5) at a dose of 2 μg/animal. This modified version of BoNT/Eun did not cause death, nor did it evoke any of the characteristic signs of poisoning (e.g., flaccid paralysis). By comparison, administration of an equivalent amount of untreated BoNT/Eun to mice (n = 5) caused death in 119 ± 21 min.

The potential vaccine utility of BoNT/Eun treated as described above was tested by administering the protein (2 μg/mouse) at times 0, 14, 28, 42, and 56 days. On days 35, 49, and 63, aliquots of blood from vaccinated animals (n = 5) were assayed for the presence of IgG. As shown in Fig. 5, there was a progressive increase in the circulating titer of antibody. Subtyping on day 63 revealed a ratio of 1.0 IgG1:0.01 IgG2a:0.08 IgG2b.

Animals that had been vaccinated with modified BoNT/Eun were challenged i.p. with 1 × 10^8 LD₅₀ of native, activated toxin (BoNT/Eₐ). None of the test animals (n = 5) died or developed signs of botulism over a period of 4 days, whereas all animals in a control, nonimmunized group (n = 5) died within less than 2 h.

Discussion

There is a single disulfide bond that links the heavy chain and light chain of BoNT. For serotype A, there are a number of studies that describe the relationship between this bond and pharmacological activity. Thus, the existence of an intact disulfide bond is known to be necessary for expression of activity against intact cells and animals. It has been reported that this bond is essential to maintain the correct conformation of the heavy chain during the process of productive internalization (de Paiva et al., 1993). However, the disulfide bond must be reduced for the light chain to be enzymatically active: 1) when the toxin reaches the cytosol of intact cells, or 2) when toxin is added to broken cells or to isolated substrate.

The apparent explanation is that the catalytic site is hindered in the native toxin molecule but accessible in the reduced molecule (Lacy et al., 1998).

Assuming that these observations and conclusions for serotype A are correct, they give rise to two important questions: 1) is an interchain disulfide bond necessary for all toxin serotypes to poison intact cells, and 2) what is the relationship between the disulfide bond and the accessibility of the catalytic site?

The Role of an Interchain Disulfide Bond in Toxin-Induced Neuromuscular Blockade. In keeping with previous reports (see above), disulfide bond reduction of BoNT/A substantially reduced toxicity as assayed on the murine phrenic nerve-hemidiaphragm preparation (Fig. 4). Essentially the same observation was made with five other serotypes, although there was one unanticipated finding. It is customary to add a protective protein such as gelatin to physiological solutions of BoNT. This is done to diminish phenomena such as surface denaturation and nonspecific adsorption. The presence of gelatin did not prevent DTT from substantially diminishing the activity of BoNT/A, but it did have an effect on experiments with BoNT/B. Simply removing gelatin allowed DTT to inactivate BoNT/B. The underlying basis for the effect of gelatin was not pursued. However, to eliminate the possibility that added protein could have a variable effect on other serotypes, gelatin was not used in subsequent experiments.

The structural consequences of disulfide bond reduction were examined in two ways. Initially, reduced preparations of BoNT/A and BoNT/B were examined to ensure that the covalent linkage between heavy chains and light chains had been broken (Fig. 4). Polyacrylamide gel electrophoresis under nonreducing but denaturing conditions confirmed that the interchain bond had been broken. In the second, disulfide bond reduction was examined in a preparation (BoNT/Eun) in which the chains could not separate. The data indicated that DTT caused a marked reduction in the potency of BoNT/Eun. The fact that DTT had an inactivating effect on both nicked (BoNT/A and BoNT/B) and unnicked (BoNT/E) preparations means that the underlying basis for DTT action cannot be that it allows the heavy and light chains to separate.

Taken in the aggregate, the results show that all serotypes of botulinum toxin have a diminished ability to poison intact cells when exposed to reducing agents. This is true both for nicked and for unnicked preparations. It is intuitively obvious that disulfide bond reduction alters the structure of the toxin molecule. However, the structural change that accounts for loss of toxicity on intact cells is not the separation of heavy and light chain components in the holotoxin.

The Role of the Interchain Disulfide Bond in Governing Accessibility to the Catalytic Site. As indicated earlier, BoNT/A is the serotype for which there is the greatest

![Fig. 5. The ability of modified BoNT/Eun to evoke an antibody response. BoNT/Eun was treated with DTT (20 mM; 60 min), filtered, and then treated with mercuric chloride (200 μM; 60 min). Mice were injected with the modified toxin (2 μg/animal) at 0, 14, 28, 42, and 56 days. Aliquots of blood were assayed for total IgG at 35, 49, and 63 days. As illustrated above, the injection paradigm evoked a strong antibody response.](image-url)
amount of information on the functional consequences of disulfide bond reduction (de Paiva et al., 1993; Simpson, 2004). It is also the serotype for which there is the greatest amount of information on structure (e.g., Lacy et al., 1998). When one places the data in the present article into the context of past reports on disulfide bond reduction and tertiary structure, they appear to support a simple model. In its native conformation, the heavy chain of BoNT/A light chain possesses a belt segment that encircles the light chain. This belt covers the orifice to a cavity that has been implicated in enzymatic activity (Lacy et al., 1998). Thus, one can hypothesize that disulfide bond reduction alters the position of the belt in serotype A, and as a result, it promotes access to the catalytic site.

This hypothesis was tested by using mercurial compounds as a form of reporter group. Ahmed and Smith (2000) have found that exposure of isolated BoNT/A light chain to mercurials causes dramatic loss of enzymatic activity. They suggested that this loss could be attributed to modification of a protein thiol in or near the catalytic site. Given the nature of mercurial compounds, one might also suggest that loss of protein thiol in or near the catalytic site. Whether thiols or histidines (or both) are implicated, the important fact is that mercurials cannot modify the catalytic site unless they can gain access to it.

When mercuric chloride and p-chloromercuribenzoic acid were added to preparations of BoNT/A holotoxin, they had little effect on toxicity. However, when these compounds were added to reduced preparations of holotoxin or to isolated light chain, they virtually abolished catalytic activity. These findings are fully compatible with the hypothesis that the belt domain hinders accessibility to the catalytic site in native toxin, but the belt does not hinder access when the interchain disulfide is reduced (viz., the position of the belt is altered), or when the light chain is physically separated from heavy chain (viz., the belt is missing).

Five other toxin serotypes were examined to determine whether they would adhere to the simple model exemplified by serotype A. The results indicated that only serotypes C and D were similar. The addition of a mercurial had little effect on the activity of the native holotoxin, but it sharply diminished the activity of reduced toxin. On the other hand, serotypes B, E, and F did not fit the simple model. In their native states, these serotypes were susceptible to the actions of mercurials, leading to diminished toxicity. However, disulfide bond reduction must have the ability to alter structure, because reduction caused losses of toxicity against intact cells. In these serotypes, disulfide bond reduction must govern something other than, or in addition to, location of the belt segment.

One possible way to explain and reconcile the data is as follows. Every serotype has an interchain disulfide bond. Depending on serotype, reduction of the interchain disulfide bond can have two possible consequences: 1) a change in structure or orientation of the heavy chain and light chain, and 2) a loosening and/or relocating of the belt segment. Serotypes A, C, and D displayed both of these structural consequences. The addition of DTT diminished toxicity against intact cells, reflecting that the heavy chain could no longer orient the light chain to produce cell poisoning (de Paiva et al., 1993). Reduction of the disulfide bond also rendered these serotypes sensitive to reagents that act in the vicinity of the catalytic domain. The latter finding was a reflection that there had been a loosening or relocation of the belt segment (Lacy et al., 1998). Serotypes B, E, and F appeared to display only one of the structural consequences. Thus, disulfide bond reduction reduced toxicity against intact cells, but it was not necessary to expose the catalytic domain or to make it susceptible to mercurials.

It should be noted that neither of the two proposed structural consequences of disulfide bond reduction requires that the heavy and light chain components of the holotoxin become separated. This conclusion is supported by the observation that DTT and mercurials exerted their characteristic effects on BoNT/Ehu, which is a single chain molecule. It should also be noted that neither of the two actions is related to or dependent upon substrate specificity. Thus, serotypes A and E, which both act on SNAP-25, have different requirements for rendering the catalytic domain susceptible to mercurials. The same is true for serotypes B and F versus serotype D, all of which act on VAMP.

**Combined Effects of Disulfide Bond Reduction and Treatment with a Mercurial.** Some serotypes were affected by DTT alone and by mercurials alone, and the combination of DTT and mercurial had an even more striking effect. As an example, the combination abolished the in vivo toxicity BoNT/Ehu. The effect of combined treatment was so great that modified toxin was tested as a parenteral vaccine against native, nicked BoNT/E. The results indicated that the modified toxin evoked a substantial IgG response and immunity. In molecular terms, these data make clear that the structural changes produced by reducing agents and by mercurials are sufficient to alter productive internalization and enzymatic activity by the toxin, but they are not sufficient to eliminate antigenicity. To the contrary, the modified toxin is a highly efficient parenteral vaccine.

References


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