Delineating the susceptibility of botulinum neurotoxins to denaturation through thermal effects

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Abstract Botulinum neurotoxins (BoNT) are the etiological agents responsible for botulism and are acknowledged terrorist threat agents. Passive immunotherapy may provide one countermeasure. Importantly, in the virtually unlimited repertoire of antibody specificities, enzyme linked immunosorbent assays (ELISA) has become an indispensable method for antibody selection. We report that of the BoNTs, BoNT/E is highly susceptible to polystyrene induced denaturation. To further dissect this result and the potential susceptibility of other BoNTs to denaturation we selected a thermal platform, which could be readily quantified using surface plasmon resonance (SPR), a primary rat spinal cord cell-based assay and an animal lethality model.

Keywords: Toxin stability; Botulinum neurotoxin; Surface plasmon resonance; Antibody–toxin interaction

1. Introduction

Botulism can be a fatal disease caused by one of seven botulinum neurotoxins (BoNTs) (distinct antigenic serotypes A–G), that are produced by Clostridium botulinum [1]. The disease is characterized by progressive muscle weakness that can result in complete flaccid paralysis caused by blockage of acetylcholine release from nerve terminals at the neuromuscular junction [2]. All seven BoNTs have a similar structural organization and are synthesized first as a single polypeptide chain of 150 kDa, which is then proteolytically activated as dichain toxin composed of a heavy chain (~100 kDa) and a light chain (~50 kDa) covalently linked by at least one disulfide bond [3]. General concerns surrounding the BoNTs are related to their potency with BoNT/A having a 50% lethal dose (LD\textsubscript{50}) by parenteral administration of 0.001 \textmu g/kg [4]. Due to lethality issues, its ease of production and transport, and the need for prolonged intensive care among afflicted individuals, BoNTs are a real threat to be used as weapons of mass destruction (WMD) [5,6].

Human botulism is attributed to serotypes A, B and E and unfortunately there is no known cure for the disorder. Patients who contract the illness are typically provided with supportive therapy (ventilator), while prophylactic strategies utilize active vaccination with BoNT toxoid vaccines [7,8]. Although several new-generation vaccines are in clinical trials [9–13], passive immunotherapy still provides an attractive therapeutic approach against BoNTs intoxication, as it is host-independent and can provide a state of immediate immunity and protection [14]. Many research groups have sought to develop human protective monoclonal antibody (mAb) against BoNTs (/A/B/E); yet, no single human mAb has been disclosed that can effectively prevent BoNT intoxication. However, a recent in vivo study reported that a series of oligoclonal antibodies could effectively protect mice against BoNT/A intoxication [15].

In the process of developing human protective mAbs to BoNT/A, /B, and /E, and the mapping of their corresponding epitopes, we discovered an inconsistency in our data, especially with BoNT/E, wherein our defined mAbs could recognize neurotoxin, heavy chain binding domain (H\textsubscript{C}), and/or peptide fragments (derived from H\textsubscript{C} domain) via an enzyme linked immunosorbent assays (ELISA) assay; yet these same antibodies were unable to recognize neurotoxin using capture surface plasmon resonance (SPR). We hypothesized this discrepancy could be attributed to ELISA as systems that employ immobilization of proteins directly onto a solid plastic surface, suffer from some limitations, the most critical being a physiochemical tendency of immobilized proteins to undergo conformational changes and denaturation as a consequence of their binding to the polystyrene surface. While there is a wealth of data using ELISA as a means to discover antibodies with varied affinity and selectivity to BoNTs, there have been no reports of BoNT denaturation when using ELISA technology. However, several groups have reported that serum ELISA titers to the BoNTs do not correspond to neutralizing titers [16,17]. To examine BoNT stability and to better define these inconsistent binding dynamics, we initiated a study using temperature as a thermal

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Abbreviations: BoNT(s), botulinum neurotoxin(s); BoNT/A–G, botulinum neurotoxin type A–G; BoNT/He/E, heavy chain neuro-specific binding domain of botulinum neurotoxin type E; ELISA, enzyme linked immunosorbent assay; SPR, surface plasmon resonance; mAb, monoclonal antibody; pAb, polyclonal antibody; RSC, rat spinal cord; \textit{T}_{\text{Mdp}}, midpoint when 50% of an epitope(s) conformation is changed; \textit{T}_{\text{Epi}}, endpoint when 100% of an epitope(s) conformation is changed.
denaturant to probe the functional stability of BoNT/A, B/E, and /E. As readouts to address the biological/structural integrity of each neurotoxin, we employed three techniques/assays including: SPR, a rat spinal cord cell assay, and a mouse lethality bioassay. Our results indicate that BoNT/E is easily denatured, either thermal or polyethylene induced, and thus routine solid-phase-based antibody selection methods are not readily amenable for defining neutralizing antibodies and/or epitopes against BoNT/E.

2. Materials and methods

2.1. Botulinum neurotoxin, neurotoxin fragments, antibodies and SPR assays

Pure BoNT types A1, A2, B and E (150 kDa) were prepared from C. botulinum Hall A hyper, Kyoto A2, Okra B, and Beluga E as previously described [18,19]. Toxicity of the BoNT/A, B/E, and /E preparations were determined by a standard mouse bioassay (MBA) [20]. Pure BoNT type C, D, F and G were purchased from Metabiologics Inc. BoNT/C/E heavy chain neuro-specific binding domain (HcE, Beluga) was kindly provided by Dr. Joseph T. Barbieri (Medical college of Wisconsin, Milwaukee, WI, USA), and was prepared in HBS-EP ga) was kindly provided by Dr. Joseph T. Barbieri (Medical college of Wisconsin, Milwaukee, WI, USA), and was prepared in HBS-EP buffer (BR1001-88, Biacore Inc.) at a concentration of 10 µg/mL for SPR analysis. Toxin solutions (A-G) were individually prepared at a concentration of 2 µg/mL in HBS-EP buffer for all SPR assays, cell-based assays and the mouse bioassay. Heat-treated toxin refers to toxin solution that was incubated at a defined temperature for 30 min with gentle shaking, and then was allowed to cool to the ambient temperature of 25°C for 1 h. Control toxin refers to a toxin solution that was prepared and kept at 25°C. The SPR assays were conducted in triplicate on a Biacore 3000 instrument (Biacore Ab, Uppsala, Sweden), using HBS-EP buffer as a running buffer, and all were double referenced, i.e. with a blank reference flow cell and with blank buffer injections. A positive signal is defined as the SPR response being equal or greater than 10 standard deviations (10 × SD) of response for the control, a buffer injection. All ligands were immobilized onto a Biacore CM5 chip using NHS/EDC coupling chemistry. Mouse anti-BoNT/E monoclonal antibodies (mAbs) were uncovered and characterized by routine hybridoma technology using HcE as the antigen. Rabbit anti-BoNT type A, B and E protective polyvalent antibodies (pAbs) were developed and purified as previously described [21].

2.2. SPR investigations delineating the biophysical interactions of ELISA defined BoNT/E mAbs and heat-treated BoNT/E

Once a protein is immobilized onto a polystyrene surface, e.g., an ELISA plate or immunoassay, it is difficult to remove it from the solid surface without undermining its natural 3-dimensional structure. To address protein denaturation in an alternative well-controlled environment, we sought the use of temperature. Thus, a solution of BoNT/E was incubated under various temperatures ranging from 40°C to 90°C, with 10°C intervals and each heat-treated toxin sample was injected into a Biacore 3000 containing a CM5 chip immobilized with either our previously ELISA defined anti-BoNT/E mAbs or rabbit anti-BoNT/E pAb (control). Initially native BoNT/E and recombinant HcE were injected to commence the studies and this was followed by specificity analysis with heat-treated BoNT/E and native and heat-treated BoNTs (A–G, from 40°C to 90°C with intervals of 10°C). We noted that all samples were injected at a flow rate of 10 µL/min, and were allowed to bind for 5 min (association phase), followed by washing for 10 min (dissociation phase). The kinetic constants for mAbs 8F3 and 9E5 to the BoNTs, were determined using the software provided with the Biacore 3000 and has been described elsewhere [22].

2.3. SPR analysis elucidating the binding interactions of rabbit protective pAbs with heat-treated BoNTs

To study how rabbit anti-BoNT (A/B/E) protective pAbs as selected by ELISA would interact with thermal induced changes to these neurotoxins but now using SPR analysis we immobilized each rabbit anti-BoNT (A/B/E) pAb directly onto a CM5 chip (vide supra). BoNT (A/B/E) solutions were prepared and an aliquot of each toxin was incubated at a defined temperature for 30 min; the temperatures ranged from 37°C to 60°C with 1°C increment and the assay conditions were identical to that described in Section 2.2. To check the specificity of rabbit anti-BoNT (A/B/E) pAbs towards heat-treated toxins, native and heat-treated BoNTs (A1, A2, B at 54°C and C-G at 50°C) were injected over a rabbit pAb immobilized chip surface as described in Section 2.2.

2.4. Validation of the biological activity of heat-denatured BoNT by an in vitro cell-based assay

To study the influence of thermal effects on the biological integrity of the BoNTs (A/B/E), we employed a primary rat spinal cord (RSC) cell assay [23]. Here, toxin solution was incubated for 30 min at 49°C and 54°C for BoNT/A and /B and 45°C and 50°C for BoNT/E. The primary rat spinal cord cells were prepared in 96-well plates [23], and the cells were exposed to 20, 2, 0.2 and 0.02 ng of heat-treated BoNT (or native BoNT as control) in a volume of 50 µl, respectively. Each dilution was tested in triplicate. Cell exposure was for 24 h for BoNT/A and /E, and 48 h for BoNT/B. Cells were harvested in 75 µl of 1 x LDS buffer (Invitrogen), and cell lysates were analyzed by Western blot as described previously [23].

2.5. Confirmation of the biological activity of heat-denatured BoNT by an in vivo mouse lethality bioassay

To further evaluate the impact of heat treatment on biological activity of the BoNTs (A/B/E), we also conducted a standard in vivo mouse lethality bioassay [20]. In brief, heat-treated BoNTs and control toxin solution were prepared, as previously described (see Sections 2.1 and 2.4). Pairs of mice (ICR) were injected intraperitoneally with several dilutions of the toxin as specified in Table 2, and mice were observed for signs of botulism for up to 4 days before they were killed.

3. Results

3.1. SPR analysis of ELISA selected mAbs and heat-treated BoNT/E

As shown in Fig. 1, none of the previously selected mAbs, 18 total by ELISA to BoNT/E, could bind to native BoNT/E using capture SPR, but all mAbs could strongly bind to HcE; while seven of the mAbs (2A3, 3F11, 8C6, 8F3, 9A11, 9E5, and 16A8) displayed significant affinity to heat-treated BoNT/E (at 50°C), albeit to different extents. Finally, any observed binding affinity decreased gradually to a basal level when the toxin was treated at 60°C or higher.

The outcome of our specificity analysis using native and heat-inactivated BoNT serotypes revealed that none of these seven defined mAbs could bind to the native toxins (A–G), but they could cross-react with heat-treated BoNT/A2 (at 54°C), /B (at 54°C), and /F (at 50°C), again to different extents; in addition these antibodies were able to bind heat-treated BoNT/E (at 50°C). Amongst these seven mAbs, only 8F3 could cross-react with heat-treated BoNT/F (50°C), whereas 9E5 cross-reacted with heat-treated BoNT/A2 (at 54°C). The association/dissociation rate constants and affinities (k_on, k_off, and K_D) for these unique antibodies are summarized in Table 1.

3.2. SPR investigations into the ability of rabbit protective pAbs to recognize heat-treated BoNTs

The ability of rabbit protective pAbs to bind to BoNTs (/A/B/E) that have heat induced conformational changes is illustrated in Fig. 2. The data demonstrate that the T_{50%} (midpoint when 50% of the epitope(s) conformation is changed) was 49°C for BoNT/A and /B, and 45°C for BoNT/E, and T_{50%} (endpoint when 100% of the epitope(s) conformation is changed) occurred at 54°C for BoNT/A and /B, and 50°C for...
Fig. 1. Use of capture SPR to determine the relationship between anti-BoNT/E mAbs and thermal induced BoNT/E conformational changes.

Table 1
A list of association/dissociation rate constants and affinities ($k_a$, $k_d$, and $K_D$) as determined for mAb 8F3 and 9E5

<table>
<thead>
<tr>
<th>mAb</th>
<th>Analyte (50 °C)</th>
<th>$k_a$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_D$ (M)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8F3</td>
<td>BoNT/E</td>
<td>$9.76 \times 10^5$</td>
<td>$1.99 \times 10^{-7}$</td>
<td>$2.04 \times 10^{-13}$</td>
<td>0.184</td>
</tr>
<tr>
<td>8F3</td>
<td>BoNT/F</td>
<td>$1.84 \times 10^6$</td>
<td>$1.43 \times 10^{-3}$</td>
<td>$7.76 \times 10^{-10}$</td>
<td>0.147</td>
</tr>
<tr>
<td>9E5</td>
<td>BoNT/E</td>
<td>$2.26 \times 10^5$</td>
<td>$1.29 \times 10^{-3}$</td>
<td>$5.72 \times 10^{-9}$</td>
<td>0.133</td>
</tr>
</tbody>
</table>

Fig. 2. Relative binding efficiency of rabbit anti-BoNT/E pAbs to heat-treated BoNT/A, /B, and /E toxins as detected by capture SPR.
BoNT/E. The specificity analysis using native and heat-treated BoNTs serotypes (A–G) illustrated that rabbit anti-BoNT pIg-Gs only recognized the corresponding native toxin, as no cross-reactivity was observed with the other native BoNT serotypes and/or with any of the heat-treated BoNTs.

3.3. Investigations into the biological activity of heat-treated BoNT by an in vitro cell-based assay

How thermal induced changes impact the BoNT/C213 biochemical activity in a RSC assay is shown in Fig. 3. After heat treatment at the determined T_Eepi, no significant target protein cleavage (SNAP25 for BoNT/A and E and VAMP2 for BoNT/B) was observed. In contrast after heat treatment at the T_Mepi, some SNAP25 or VAMP2 cleavage was observed with all toxin dilutions tested; however, at a 2–3–fold lower efficiency than the untreated controls for BoNT/A and B, and 1.5–2–fold lower efficiency for BoNT/E.

3.4. Confirmation of the biological activity of heat-inactivated BoNTs by an in vivo mouse bioassay

Findings for the mouse bioassay are summarized in Table 2. As shown, an LD_{50} for BoNT/A was determined to be approximately 5 pg. For BoNT/B, the LD_{50} was found to be less than 5 pg; while for BoNT/E was 20 pg. In our thermal studies an LD_{50} for BoNT/A treated at 49 °C was estimated to be between 10 and 20 pg; BoNT/B at 49 °C was estimated to be 10 pg, and for BoNT/E treated at 45 °C this was established to be 20 pg. No biological activity was detected with BoNT/A or/B treated at 54 °C and BoNT/E treated at 50 °C; the latter was true for BoNTs even when 20 ng of the toxin (≥ 4000 mouse LD_{50} for/A and /B or ~1000 LD_{50} for/E) was injected.

4. Discussion

There is an impressive body of evidence in the literature using ELISA to select for neutralizing antibodies to epitopes of protein antigens. However, ELISA systems may also pose problems since proteins can denature upon contact with a polystyrene surface [24,25]. The binding of a protein to a polystyrene surface depends on the characteristics of the surface and on the structure of the protein; binding can induce conformational changes in the adsorbed molecules [26,27]. At the onset of our studies we selected 18 anti-BoNT/E mAbs, which were developed by immunizing mice with the BoNT/HCE fragment. Using this strategy we obtained high titers to HCE and BoNT/E neurotoxin as judged by ELISA, but these results were not recapitulated using either “direct”-SPR (BoNT/E immobilized onto a chips surface) or “capture”-SPR assays (mAb immobilized onto a chip’s surface, unpublished data). Seeking to solve this quandary, we conducted extensive studies using SPR technology with defined monoclonal or polyclonal antibodies and native or thermally denatured BoNTs. The challenge of delineating polystyrene surface effects and neurotoxin toxin inactivation was undertaken using temperature as opposed to pH or a chemical denaturant as we required a method that would be common [26,28,29] and need no additional manipulations before SPR analysis could be conducted on the sample. In sum, none of the 18 ELISA selected anti-BoNT/E mAbs had possessed any binding activity to native BoNT/E in the SPR capture study, Fig. 1.

From the selected pool of mAbs, 7 of the original 18 mAbs were found to bind to heat-denatured BoNT/E as judged by “capture”-SPR with excellent affinity (Table 1) that is consistent with their high titers as seen using the ELISA selection protocol. Interestingly, antibody specificity was also broadened to the other serotypes upon heating implying a common epitope, which might be conformationally linked between the other serotypes. Montal and co-workers [30] have elegantly shown while BoNT/A and/E have sequence similarity, their global structures between these neurotoxins are different, especially at their translocation domain. Translating our finding to Montal’s work suggests that common antibody binding we have seen between serotypes, which has been introduced ther-
mally, are restricted to the C-terminal receptor binding domain (H3E). Finally, the 11 mAbs that failed to recognize heat-denatured holotoxin may recognize epitope(s) that are exposed in the H3E, but are not readily accessible in native or partially denatured holotoxin; such as epitope(s) located within or in close proximity to the hydrophobic patch, where the translocation domain (H3E) packs tightly with the (H3E) binding domain.

To examine additional thermal effects on BoNT/E as well as BoNT/A and /B, rabbit anti-BoNT/A/B/E pAbs were used as a probe. The results, Fig. 2, illustrated that the affinity of the pAbs to the heat-treated BoNTs dropped precipitously at 49 °C for BoNT/A/B, and at approximately 45 °C for BoNT/ E. Furthermore, a total lack of antibody binding was observed for BoNT/A/B at 54 °C and 50 °C for BoNT/E. From these studies we can conclude that all properly folded conformational epitopes of the neurotoxins recognized by rabbit pAbs were ablated by heating at T_Eepi.

The biological function of a protein is related to its 3-dimensional structure. To define how thermal effects can impact BoNTs biological activity at their respective T_Mepi and T_Eepi we investigated two separate but complementary assays the first being a RSC assay (Fig. 3), and the second a mouse lethality assay (Table 2). Heat treatment of BoNTs at T_Eepi for 30 min caused at least 1000-fold decrease in toxin activity, signifying complete or near complete inactivation, whereas heat treatment of BoNTs at T_Mepi only slightly reduced toxin activity (2–3-fold for A and B, and 1.5–2-fold for E), see Fig. 2. In trying to interpret these results we propose that there is a general disruption of accessible epitopes around T_Mepi which contributes to the diminished binding seen with the pAbs but not the overall biochemical activity of the toxin. In contrast upon reaching the T_Eepi disruption of additional epitope(s) appears catastrophic to both biological activity and pAb antibody binding.

The results we have presented demonstrated that BoNT/E is both thermally unstable and highly susceptible to conformational changes upon adsorption to plastic surfaces. Furthermore, previous mAb binding ELISAs (unpublished data) and our thermal denaturation studies suggest that BoNT/A, /B are also susceptible to some form of conformational changes, but to a lesser extent than BoNT/E. As we have shown when constructing a strategy for neutralizing antibody selection for BoNT/E, the pursuit of antibody high titer/affinity as judged using ELISA could provide false leads of mAbs that recognize shifted or distinct epitopes. Interestingly, the groups of both Barbieri and Tekeda have observed that serum ELISA titers produced to BoNTs heavy chain did not correspond to neutralizing titers, which based on our findings maybe due to plastic surface denaturation of the BoNTs [16,17]. This is important as the Barbieri group used the same H3E in their immunization study and obtained serum that can protect 10,000 LD50 of BoNT/E challenge [16]; this result coupled to our data would preclude the possibility that the H3E itself is the culprit limiting our search for neutralizing mAbs. In light of these facts, the selection of protective antibodies from any library pool should be predicated upon ways to select directly against the BoNT in the solution phase. Resolutions to this problem would include any technique using solution phase, such as fluorescence sorting [31] or in the case of solid phase selection, preadsorbed onto an alternative surface such as aluminum hydroxide [25], and/or a sandwich capture assay could be engaged using non-protective mAbs with distinct epitopes, preferably located in light chain or the translocation domain.

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References


