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New equine antitoxins to botulinum neurotoxins serotypes A and B

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Abstract

Hyperimmune monovalent antitoxins to botulinum neurotoxin serotypes A and B have been produced by immunizing horses with newly developed formalin toxoids. After primary immunization horses were found to have developed acceptable prophylactic antibody titers (1–5 IU/mL). Three horses received additional toxoid booster injections to induce hyperimmune antibody titers with antitoxin-A and antitoxin-B titers reaching peaks of approximately 2000 IU/mL and 150–625 IU/mL, respectively. Titers were quantified throughout the process by antigen-capture ELISA and by in-vivo neutralization. ELISA titers and neutralization titers correlated ($R^2 \sim 0.62$ – 0.92), however, unique correlations between in-vitro and in-vivo titers were observed for each animal. Monovalent antitoxin pools were made by combining plasma that had been collected twice via plasmapheresis several months after primary immunization. Neutralizing units were established for each pool relative to the current US and WHO reference standards. Titers were determined at the L_{+10} and L_{+40} toxin dose for Toxin types A and B, respectively, and both U.S. and international units assigned to each monovalent antitoxin. Avidity of the new Anti-A pool was equivalent to the WHO Anti-A reference at the L_{+1} , L_{+10} and L_{+30} dose. Each monovalent plasma pool failed to cross-neutralize other botulinum neurotoxin serotypes indicating a high degree of specificity of each antitoxin for the toxin serotype used during immunization.

Introduction

Botulism is a life-threatening, paralytic disease caused by neurotoxins secreted by *C. botulinum* and related bacteria. Van Ermengem performed the first complete modern laboratory investigation of the disease by isolating and identifying the pathogenic bacteria that replicated disease in animal models. Eventual demonstration that cell-free culture filtrates caused a similar paralytic illness in a variety of animal species led to the conclusion that a soluble toxin was responsible for imparting disease [1]. Lammana concluded that the mode of illness was a toxemia usually absent infection [2]. Today in the US, combined incidents of food-borne and infant botulism number around 300 cases annually [3].

In 1910 and again in 1919, studies by Leuchs and Burke provided clear evidence that two separate strains of *C. botulinum* produce antigenically distinct toxins that induced similar forms of disease [4,5]. Since the soluble toxins were not cross-neutralized by antiserum prepared with the other toxin, each bacterial strain and its toxin were arbitrarily classified as either type A or type B. Bengtson found a third strain leading to the identification of type C toxin [6]. Currently, seven antigenically distinct botulinum neurotoxins (BoNT) are known,

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Types A through G, each named in the order of discovery after determining that existing type-specific antitoxins could not neutralize it.

Medical treatment of the disease has historically relied upon antibody therapy. Typically equine antitoxin preparations have been used for this purpose since horses can provide large volumes of high titer plasma to support large-scale commercial antitoxin manufacture. Clinical benefit from administering antitoxin is thought to be due to the elimination of circulating toxin which reduces the duration and/or severity of disease. Recent clinical evidence in support of antibody therapy is provided by a human immunoglobulin product derived from the plasma of volunteers immunized with an experimental formalin toxoid [7]. This antitoxin product has reduced hospital time for infant botulism cases by about 50% providing the clearest clinical evidence that antibody-mediated neutralization of circulating toxin is linked to reducing the duration of symptoms. Thus, antibodies to the botulinum toxins are used as diagnostic reagents to classify toxin serotypes, and as therapeutics to treat illness.

Immunization to induce humoral responses against BoNT has been studied using several botulinum antigens. Historically, formalin-detoxified toxins have been used and more recently recombinant or chemically-altered derivatives of the toxins have been reported [8–11]. Improved formalin-detoxified toxoids were prepared from BoNT/A and BoNT/B in this laboratory and were shown to induce higher protective antibody titers in mice and rabbits compared to commercially available formalin botulinum toxoids. These new toxoids appeared to resemble the native toxins more so than recombinant antigens when comparing antibody titers attained in several separate studies [10,12,31]. The present paper describes our efforts to immunize a small number of horses with our new formalin-treated antigens, Toxoid A and Toxoid B, to determine whether a simple immunization regimen might be employed to successfully elicit hyperimmune titers against BoNT/A and BoNT/B that could support future clinical or diagnostic needs [13].

Materials and Methods

Materials

BoNT/A – G were purchased from Metabionics Inc. (Madison, WI). Toxins used to titrate antitoxin potency were high molecular weight toxin complexes (Mw ~300–900 kDa). Complex toxins for BoNT/A and BoNT/B were derived from Hall A and Okra B strains, additionally, where indicated, some tests examined BoNT/A and /B from *C. botulinum* strains, 62A and TJ980, respectively. Toxins used for toxoid synthesis and immunization were the highly purified 150 kDa proteins derived from the Hall A strain and Okra B strain (U. Wisc. Food Research Institute). Formalin toxoids derived from BoNT/A and BoNT/B were detoxified as described previously [12]. Briefly, each toxin was diluted to 1 µM in PBS, pH 7.2 and incubated with 20 mM formalin at 37° C for 4–5 weeks. At the end of the detoxification reaction, each toxoid was stored undiluted refrigerated. Goat anti horse antibody conjugated to alkaline phosphatase and Titermax Gold™ were from Sigma Aldrich (St. Louis, MO). Alhydrogel was purchased from Accurate Chemical and Scientific Corp. (Westbury NY). Male Balb/cAnNCr mice were purchased from the National Cancer Institute (Frederick, MD) and were 6 to 14 weeks old. Weights ranged from 20–24 grams. All animal experiments were performed according the NIH Guide for the Care and Use of Laboratory Animals [14] and had prior approval from the CBER institutional animal care and use committee.

Antitoxin preparation

Freeze-dried reference antitoxins from the WHO and the US NIH Division of Biologics were reconstituted using PBS followed by the addition of glycerol. The WHO stocks were labeled BTUSA and BTUSB each containing 500 IU (1 IU = 0.1360 mg for Anti-A and 0.1740 mg for Anti-B) [37]. Both WHO references were packaged by Statens Seruminstitut, Copenhagen S., Denmark. Each vial was a dried white material in the original sealed glass ampules. Each was opened by notching the glass neck, then 4 mL ice-cold PBS was added to each and solids were allowed to dissolve slowly for 30 min without agitation. Upon complete dissolution, 1.2 grams of glycerol was added to each. Aliquots were made in screw-capped vials then stored at -20°C . US NIH reference antitoxins were packaged in 1940 as whole serum. At the time of packaging each was freeze-dried in vials containing 1400 US units per vial for Anti-A or 700 US units for Anti-B. For this study, each reference vial was opened and the contents were dissolved overnight in the cold with PBS. The dissolved antiserum was adjusted to make a final solution containing 66% glycerol having 10 and 20 US units/mL for the US Anti-A and Anti-B references, respectively and were stored at -20°C .

Three Percheron (1 male and 2 females) and one Belgium horse (male) were housed at facilities maintained by the NIH (Poolesville, MD). Animal health was monitored daily under an approved animal-use protocol. Tooth growth patterns suggested the horses ranged in age from 15 to 27 yrs old. None of the horses had detectable protective antibodies to either BoNT/A or BoNT/B prior to immunization. One male & one female horse were immunized with either the Toxoid A or Toxoid B according to schedules shown in Tables 1 and 2. Antigen was formulated with adjuvant on the morning of the injection. Alhydrogel was diluted 10-fold to a final concentration of 0.3% $\text{Al}(\text{OH})_3$. Emulsions with Titermax Gold, a squalene-based adjuvant, were made using a dual-syringe pumping system which contained Titermax Gold in one chamber and antigen (0.12 mg/mL) in the other. Both solutions were pumped between each syringe until the combined solution became a homogeneous emulsion (~ 0.06 mg/mL). Prior to each injection, an area of about 150 cm^2 on the neck of each animal was shaved then sterilized with Betadine. Each immunization was delivered using a 20 gauge syringe containing 1 mL total liquid volume, and was delivered subcutaneously in 8 to 10 sites receiving approximately 0.1 mL each. Subsequent immunizations were delivered on alternating sides of the neck. Plasmaphoresis was performed at the NIH Animal Center (Poolesville, MD) where 3 L was collected from each horse on two separate occasions.

Antibody Titer determination

Antibody titers were measured using a capture ELISA. Microtiter plates were coated with 150 kDa toxin in PBS (1 $\mu\text{g/mL}$) overnight. Wells were washed three times with PBS containing 0.3% BSA then blocked in the same buffer. Serial 10-fold dilutions of immune serum in PBS/BSA buffer were applied to wells for 1 hr at $20\text{--}25^{\circ}\text{C}$. Wells were washed then treated with goat anti-horse antibody coupled to alkaline phosphates (1:1000) for 1 hr. Color development with *p*-nitrophenyl phosphate substrate was measured at 405 nm. Toxin-specific titers are reported as the dilution that produces an OD reading that is twice the baseline signal.

Neutralizing antibody titers were measured using two variations of the same method as previously described by Fulthorpe [13] and Bowmer [15]. Data shown in Fig. 1 were derived using the Fulthorpe method where one or two mice per group were used to establish a dose range for the neutralizing titer. This was iteratively re-tested using new mice with antitoxin dilutions of 30–40% to identify the neutralizing titer. Data in Fig. 3 were acquired by using a modified version of the Bowmer method where data were compiled from

multiple identical tests using three to five mice per group. Whereas Bowmer's approach reports antitoxin units per *mouse* and LD₅₀ per *mL*, we normalized the units for both reactants (antibody and toxin) and report the quantity of each per milliliter. Since reference antitoxins (WHO or US) were diluted equivalently and included in all side-by-side neutralization assay runs, the final assigned units are not affected by the change in reaction mixtures (i.e. Antitoxin/*mouse* versus toxin LD₅₀/*mL*). In this assay, standard curves using each reference antitoxin were prepared using four to five dilutions of each antitoxin at 10% – 20% increments. A fixed concentration of challenge toxin was used. A four-point standard curve, for example, was prepared using the WHO Anti-BoNT/A reference at 0.08, 0.09, 0.11 and 0.12 IU/mL. A five-point standard curve was used for the WHO Anti-BoNT/B reference using 0.018, 0.022, 0.025, 0.028 and 0.033 IU/mL. The toxin dose of BoNT/A was the quantity of toxin when mixed with 0.1 IU/mL of the WHO antitoxin that produced 50% lethality in mice. According to Ehrlich's terminology, this constitutes the L₊/10 dose and was 770 to 850 LD₅₀/mL for the BoNT/A preparation used in this study. This same level of challenge required 0.03 US units/mL to produce 50% lethality, meaning that 770 to 850 LD₅₀/mL of toxin is equivalent to the L₊/33 dose for the US anti-BoNT/A reference standard. For BoNT/B, 350–400 LD₅₀/mL, which produces 50% lethality when mixed with 0.03 IU/mL or 0.008 US units/mL, respectively. The method of Reed and Meunch [16] was used to calculate the antitoxin volume that protected 50% of the mice.

Cross-neutralization was assessed by diluting each of the seven toxin serotypes into PBS then adding anti-BoNT/A or anti-BoNT/B antitoxin to generate a final antibody concentration of 100 or 75 IU/mL in a mixture containing about 10 LD₅₀/mL of each individual toxin serotype. Two mice received each mixture and were monitored for up to 120 hr. Two additional mice received the toxin-only control. Avidity for the US anti-A and WHO anti-A reference antitoxins was measured by diluting each antitoxin to 1 IU/mL, 0.1 IU/mL or 0.033 IU/mL then adding measured volumes of BoNT/A to each mixture to determine the quantity of toxin that is neutralized by each level of antitoxin.

RESULTS

None of the horses used in this study had pre-existing neutralizing antibody titers to BoNT/A or BoNT/B prior to beginning the immunization (< 0.02 IU/mL). Details of the immunization regimen are shown in Tables 1 and 2. Upon immunization using Titermax as adjuvant, horses showed significant local reactogenicity, tissue swelling at the injection sites and mild fever persisting for several days following immunization. When aluminum hydroxide was used as adjuvant, animals did not present noticeable local or systemic adverse reactions. One to two weeks after the second immunization, all four horses had developed sizeable neutralizing IgG titers exceeding 1 IU/mL (Table 3). Neither of the horses immunized with Toxoid A had measurable neutralizing titers until after the Day 35 immunization. On the other hand, horses immunized with Toxoid B developed low but measurable neutralizing titers just above the level of detection (>0.02 IU/mL) four weeks after the first immunization. Anti-A and Anti-B titers rose following the second immunization by 100 to 500-fold over the limit of detection. In total, each horse received about 0.1 mg of toxoid protein during this primary immunization series, which elicited peak anti-BoNT/A and anti-BoNT/B titers between 7.9 and 26.7 IU/mL eight to ten weeks after the first injection (Fig. 1).

Three months after the initial immunization, a Toxoid A-immunized horse died of intestinal inflammation and colic, which was deemed unrelated to the antigen or immunization regimen by a post-mortem investigation. Subsequent hyperimmunization of the remaining horses proceeded on different schedules to accommodate delays during post-mortem analysis. Hyperimmunization of each horse entailed a series of up to seven injections of

about 50 µg of Toxoid A or Toxoid B. Total antigen used was 0.36 mg of Toxoid A or 0.39 mg of Toxoid B over the course of the entire schedule. On Day 132, a single dose of 0.1 mg of Toxoid B was given to both horses without adjuvant in hopes of mimicking hyperimmunization using unmodified toxin [20]. This dose, however, did not elicit a rise in neutralizing titers indicating that the sum total of the Toxoid B, 0.29 mg with adjuvant was responsible for reaching peak Anti-B titers (Table 2 and Fig. 1B). Antibody titers in all three horses showed a typical booster effect with neutralizing titers peaking at approximately 2000 IU/mL for the single Toxoid A-immunized horse and 150–625 IU/mL for the Toxoid B-immunized horses (Fig. 1).

ELISA titer analysis was conducted on all bleed samples. Titers often overlapped with neutralizing titers measured in-vivo (Fig. 1). Both methods generate comparable data to quantify changes in antibody level over time (Fig 2). The correlation between the two methods varied from an R^2 of 0.70 for Anti-A titers and an R^2 of 0.92 and 0.62 for antibodies from each Toxoid B horse. Combining all Anti-BoNT/B data into a single regression fit produces a correlation coefficient of 0.66. Different slopes were observed for each horse ranging from 1.84 ± 0.31 for Anti-A titers to 1.67 ± 0.16 and 1.23 ± 0.32 for Anti-B titers, implying that direct correlation between ELISA and neutralizing titer assays cannot be assumed and that the linear relationships between ELISA and protective titers are unique for each animal.

Bulk antitoxin pools collected via plasmaphoresis were analyzed relative to US and WHO reference antitoxins (Figs. 3 and 4). All reference antisera and the new Anti-A and Anti-B bulk pools produced steep survival curves where a 15–20% change in antitoxin volume resulted in a transition between complete protection and no protection. Each of the new antitoxins was diluted similarly and were found to have anti-BoNT/A and anti-BoNT/B neutralizing titers of about 1158 and 373 IU/mL, respectively. (Table 4). One US unit was found to be equivalent to approximately 2.9 IU for both WHO Anti-A and Anti-B standards.

Neither of the antitoxin bulk pools produced cross-protection against other BoNTs (Table 5). Each antitoxin was diluted to either 100 or 75 IU/mL then mixed with 10 LD₅₀/mL of each toxin serotype A-G. Mice were protected against homologous toxin (i.e. Anti-A protected against BoNT/A) but protection against other toxin serotypes was not observed. Anti-B antitoxin at 75 IU/mL delayed death from BoNT/G challenge by about 24-h but the survival outcome at the end of the observational period was not altered. Anti-A antitoxin produced a slight delay in the onset of symptoms for BoNT/F but survival was not altered (data not shown). The magnitude of cross-protection was similar regardless of the toxin strain used, for example BoNT/A from Hall and 62A strains were both neutralized with similar efficiencies.

Avidity was examined over a thirty-fold range by diluting WHO Anti-A reference and the new bulk Anti-BoNT/A antitoxin to 1.0, 0.1 and 0.033 IU/mL. The minimum quantity of toxin neutralized by each level of antitoxin was determined by mixing each with various amounts of toxin in vitro, followed by injection into mice. BoNT/A from Hall and 62A strains were used for this analysis. Both antisera produced comparable degrees of protection. For example, 1.0 IU/mL of the WHO Anti-A reference neutralized BoNT/A in the range of 18,000–19,000 LD₅₀/mL, whereas the same quantity of the new bulk Anti-A serum (e.g. 1.0 IU/mL) demonstrated a range slightly higher: 19,000–19,500 LD₅₀/mL (Table 6). The difference in avidity was not statistically significant with slopes ranging from -1.62 ± 0.12 and -1.62 ± 0.11 for the WHO Anti-A reference and the new Anti-A antitoxin (Fig. 4). The avidity profiles for both antitoxins overlap with previous avidity results for the WHO Anti-A reference determined in 1963, which was converted to IU/mL from IU/mouse and is presented for comparison purposes. The resulting slope of -1.51 ± 0.14 was obtained (Fig.

4) [15, 30]. Correlation coefficients (R^2) for all three determinations were between 0.97 and 0.98.

DISCUSSION

This study provides a clear demonstration that immunization and hyperimmunization with the new formalin toxoids can rapidly elicit high neutralizing titers against BoNT in horses. The toxoids used in this study were made from purified toxins (150 kDa) treated with 20 mM formaldehyde for several weeks at 37°C in PBS, pH 7.2 [12]. This approach for the BoNT/A was described previously by our group and similar data exist for the BoNT/B. Inactivation of the toxin using formalin has several critical parameters, including formalin and protein concentrations, temperature and duration of inactivation, which if not optimized can negatively impact the immunogenicity of the antigen. The initial goal of this study was to determine if primary immunization with no more than 0.1 mg of antigen would elicit protective titers of about 1 IU/mL. All four horses developed titers that met or exceeded this level by the seventh week after the first injection. Subsequent hyperimmunization elicited neutralizing peak titers of around 2000 IU/mL for the remaining Anti-A horse and about 400 IU/mL for both Anti-B horses on average. It is likely that changes in dose, adjuvant and schedule will produce different outcomes, but in principle the results clearly demonstrate the feasibility of this approach to establish hyperimmune status in horses within 240 days using Toxoid A and 150 days with Toxoid B.

In general, equine antibody products are manufactured by commercial entities who rarely publish precise details regarding antigen and immunization regimen. The current study relied substantially on work by A.T. Glenn who pioneered formalin toxoid synthesis, toxoid antigenicity assays and horse hyperimmunization methods using many bacterial toxins such as diphtheria and tetanus toxins [17–19]. Based on these historical references, we established an initial set of conditions for dosing and schedule to explore and potentially replicate Glenn's findings using our new formalin toxoids to BoNT/A and /B. We attempted to implement the longest practical rest period between the end of primary immunization and the start of hyperimmunization, eventually settling on a 3-month rest interval prior to hyperimmunization. Glenn's findings demonstrate that initiating hyperimmunization immediately after completing prophylactic immunization, yields low titer and poor avidity antitoxin that is inadequate for commercial manufacture of tetanus and diphtheria antitoxin products due to the poor ability of such antitoxins to clear circulating toxins from the body [20,21].

Another parameter that affects antitoxin quality is the adjuvant. Limited information on the role of adjuvant during hyperimmunization presented a dilemma. Ramon first provided information indicating that inflammation caused by co-administration of various chemicals such as oils and extracts of tapioca facilitated elevated antibody production in horses [22]. Glenn was the first to co-administer aluminum salts with antigen and Freund later developed oil emulsions containing inactivated mycobacterium tuberculosis to enhance the immune response [19,23,24]. More recently, studies have examined the effect of combinations of $Al(OH)_3$ and oil-emulsion adjuvants when hyperimmunizing sheep [25–27]. Yu et. al. (2010) used incomplete Freund's adjuvant to immunize horses with a recombinant botulinum antigen [35]. We adopted an approach to combine each botulinum toxoid with $Al(OH)_3$ followed by emulsification with an oil-based adjuvant, Titermax Gold™. The appropriateness of adjuvant within every dose of antigen during hyperimmunization remains unclear and we are at a loss as to whether antigen suspended in oil-water emulsions in every immunization leads to superior titers compared to aluminum salts alone especially in later stages of hyperimmunization. Additionally it is unclear whether alternating or mixing adjuvants can facilitate better results. In a small follow-up

study, five new horses were acquired and were immunized with the A Toxoid. All horses were immunized using 50 µg antigen combined with an adjuvant mixture of Al(OH)₃ and Titermax. Five weeks later, two horses were boosted with the same adjuvant mixture and three horses were boosted with antigen and Al(OH)₃. Seven weeks after the first immunization, two horses immunized with the adjuvant mixtures developed titers of 30–40 IU/mL and three horses boosted with only antigen and Al(OH)₃ had titers of about 10–20 IU/mL (data not shown) comparable to the rise in titers for the single Toxoid A-immunized horse described in this study. These results indicate that the use of a combination adjuvant of Al(OH)₃ and oil-water emulsion for both immunizations yields about a two-fold improvement in neutralizing titer upon completing primary immunization.

Comparing the magnitude of the equine titers elicited by our antigen and regimen to equine titers elicited in horses immunized with a recombinant antigen, we see that our Anti-A pool of about 1000 IU/mL obtained by using 0.36 mg of formalin toxoid was much lower than the 27,000 IU/mL obtained by immunizing with 32 mg of recombinant antigen using incomplete Freund's adjuvant [35]. Although schedules, adjuvants and dosages were different between the two approaches, the final ratio of IU:antigen shows that the formalin toxoid had an efficiency of 1000 IU/0.36 mg $\approx$ 2,700 IU/mg whereas the recombinant antigen efficiency was 27,000 IU/32 mg $\approx$ 840 IU/mg. Therefore, the formalin toxoid used in the current study was approximately three-fold stronger than the recombinant antigen regimen. Avidity of the immune plasma elicited with the recombinant protein has not been reported by other groups, making direct comparisons regarding the quality of the two antibody preparations not possible. Glenny and Barr (1945) indicated that establishing strong protective titers with toxoid followed by hyperimmunization with active toxin yields the best quality immune serum for further manufacture into pharmaceutical-grade product. For safety and security reasons, we did not attempt immunization with active toxins.

Analysis of blood samples relied upon two methods: a toxin-based ELISA to quantify total toxin-specific antibody titers and a mouse-based toxin neutralization assay. We found that ELISA titer data generally overlapped with functional antibody titers in this study. The usefulness of the correlation, however, should be carefully considered. It is established in the botulinum toxin field that ELISA titers have poor correlation with protective titers when serum samples have been collected at a single time point from a diverse group of immunized individuals or when serum samples are screened for ELISA binding capacity to antigen or native toxin [28, 9]. In some cases, ELISA titers provide entirely misleading data regarding the magnitude of protective titers [36]. Our data do not dispute these previous observations. In the present study, correlation between the two titer methods is accomplished by collecting timed samples from single animals. This is not analogous to comparing titer data obtained from many individuals at a single time point. Our results allow us to conclude that once a slope relationship between ELISA and protective titers is established for an individual, ELISA titers can provide information for estimating changes in the magnitude of protective titers during subsequent boosting or during stability testing of bulk antitoxin to reduce the reliance on mouse lethality titrations.

The strength of vaccine or antitoxin products used to prevent or treat botulism is often measured relative to antitoxin reference standards. Bengtson facilitated the initial acquisition and use of antitoxin references by the US Public Health Service in the early 1920s where 0.1 US unit was defined as that amount of antitoxin needed to neutralize about 100 guinea pig minimum lethal doses such that guinea pig death is delayed to 96 hr post-challenge [29]. She later contributed to the production of a reference antitoxin to serotype E in the 1930s. The current lots of reference antitoxins maintained by FDA/CBER were prepared by the NIH in 1940 as freeze-dried plasma samples. Independently, the WHO established international reference standards to serotypes A-E in 1963 [15]. US antitoxin units and IUs are not

identical, however. An in-house evaluation performed in 1967–1968 established that 1 Anti-A US antitoxin unit was equivalent to 2.5–3.2 IU and 1 Anti-B US antitoxin unit was 2.8–3.4 IU (unpublished data). The current study replicated this ratio (Table 4) and demonstrated that the neutralizing capacities of the new Anti-A antitoxin and the WHO reference are comparable from dilutions as low as 0.03 to 1.0 IU. By demonstrating the reproducibility of these ratios in the current study we were able to confirm (1) that all the reference antitoxins used in this study have retained undiminished potency since the 1960s and (2) that the parameters we adopted for measuring neutralizing titers (mouse strain, buffers, injection volume, etc.) generate data equivalent to original results. Although US standard antitoxins to botulism are still in use today, most studies tend to report the strength anti-botulinum toxin antibodies using IUs. The current study was designed to use both US units and IUs to show the relative comparability between both references. This type of comparison proved to be a useful quality control step for ensuring antitoxin standardization and antitoxin stability without requiring the need to retain standard reference toxins.

Our successful effort to test whether new formalin toxoids would efficiently generate equine antitoxin have validated separate findings advocated by Glenny and Ramon in the early 20th century: successful hyperimmunization of horses requires a method to reliably produce antigen and a separate method to quantify its antigenic integrity [32,33]. We used tools previously developed in this laboratory to synthesize several batches of formalin toxoid and characterized these *in vitro* prior to administration to horses. The resulting antitoxins have performed comparably to the WHO reference antitoxins. Avidity of the new Anti-A antitoxin was measured side-by-side with the WHO Anti-A antitoxin reference and both results were compared to avidity reported by Bowmer [15,30]. Bowmer's original data show that one IU neutralized about 10,000 LD₅₀ of BoNT/A strain 4587 [30]. Avidity measurements in our laboratory show that the WHO anti-A reference antitoxin and our new anti-BoNT/A bulk antitoxin both have similar protective capacities: each was able to neutralize about 19,000 LD₅₀/mL of BoNT/A Hall strain and about 6,000 LD₅₀/mL of BoNT/A 62A strain at 1 IU/mL. Bowmer's avidity data is reported as LD₅₀/mL and antitoxin (i.e. IU)/mouse not antitoxin/mL [15], which is not directly comparable with our approach where we normalized units for both reactants (antibody/mL and toxin/mL). We retrieved antitoxin and LD₅₀ data from Bowmer's dissertation [30], and replotted his published L₊ avidity data in LD₅₀/mL and antitoxin/mL, (Fig. 4). Results are within the same range as avidity data generated using the same WHO standard in this study regardless of whether the BoNT/A complex toxin was produced by Hall-A or 62A strains (Fig. 4). This demonstrates that the magnitude of protection afforded by 1 IU varies depending on the toxin source, yet, regardless of the toxin source the old and new antitoxins performed equivalently in all side-by-side experiments. We therefore, conclude that fairly simple technology is needed to assure good antitoxin quality and that properly balancing dosage, adjuvant and rest period prior to hyperimmunization will produce a high quality antitoxin using small quantities of antigen.

Acknowledgments

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy

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1A

1B

Fig. 1. Time-course of antibody induction. Frame A. Duplicate determinations for Anti-BoNT/A neutralization titers from Horse 1 (●). Frame B. Anti-BoNT/B neutralization titers from Horses 3 (●) and 4 (■). Solid arrows indicate the time of immunization, while dashed arrows represent bulk plasma collection.

Fig. 2.

Correlation of antibody titers determined by ELISA and in vivo neutralization. Frame A. Correlation between Anti-BoNT/A titers from Horse 1. Frame B. Correlation between Anti-BoNT/B titers from Horse 3 (blue line) and 4 (red line) determined by both methods. Dashed lines in both frames represent 95% confidence intervals.

3A

Fig. 3.

Antitoxin titration traces depicting incremental protection from varying amounts of different antitoxin preparations. Frame A. Challenge with BoNT/A. Frame B. Challenge with BoNT/B. The y-axis shows survival, while the x-axis represents μL of antitoxin per mL within each toxin-antitoxin mixture. Antitoxin units were calculated as described in the Material and Methods. Antitoxins used in each assay determination are: WHO reference antitoxin at 0.1 IU/mL (●, ○), US1940 at 0.030 IU/mL (□) and US 2010 (Δ).

Fig. 4.

Avidity of antitoxins to serotype A. The new anti-BoNT/A bulk antitoxin (□) was tested at various toxin levels in comparison to the WHO anti-A reference (▲) using Hall-A toxin (black solid line). Similarly, avidity was measured using a second toxin strain (A62) for WHO (△) and the new antitoxin (x), (solid red line). Previously published avidity data for the WHO antitoxin is presented here for comparison (○) where BoNT/A complex toxin was from strain 4587 (dashed blue line) [15, Table 4 in ref. 30].

Table 1

Dose per horse and adjuvant schedule for immunization with Toxoid A.

Toxoid A		
Day	Dose (µg)	Adjuvant
0	55	Titermax & Al(OH) ₃
35	36	Al(OH) ₃
139	53	Al(OH) ₃
155	63	Titermax
169	57	Titermax
190	60	Titermax
201	plasmaphoresis	3 L
225	34	Al(OH) ₃
238	Plasmaphoresis	3 L

* Total A antigen administered was 0.36 mg.

Table 2

Dose per horse and adjuvant schedule for immunization with formalin Toxoid B.

Toxoid B		
Day	Dose (µg)	Adjuvant
0	63	Titermax & Al(OH) ₃
36	55	Titermax
99	69	Titermax
113	66	Titermax
132	106	saline
147	Plasmaphoresis	3 L
218	34	Al(OH) ₃
231	Plasmaphoresis	3 L

* Total B antigen administered was 0.39 mg.

Table 3

Neutralizing antitoxin titers before and after immunization on Day 0 and Day 35–36.

	Pre-bleed	Day 14	Day 28	Day 35–36	Day 42	Day 56
Toxoid A	<0.014	<0.014	<0.014	boost	0.5–5.0	13.3–26.7
Horse 1	<0.014	<0.014	<0.014	boost	0.5–5.0	5.0–8.3
Horse 2	<0.014	<0.014	0.01–0.02	boost	NT *	49 days
Toxoid B	<0.014	<0.014	0.01–0.02	boost	NT *	7.9–13.8
Horse 3						15.4–26.0
Horse 4						

* NT = Not Tested

Table 4

Tabulated US units/mL and IU/mL.

	US units	IU	Ratio [IU/ US unit]
US-1940 Anti-A (working vials)	10 20	29 ± 2.1 (n = 6)	2.9 (2.7–3.0) *
US-1940 Anti-B (working vials)	424 ± 13 (n = 3)	58 ± 2 (n = 3)	2.9 (2.7–3.1) *
US-2010 Anti-A (bulk pool)	128 ± 7 (n = 3)	1158 ± 70 (n = 4)	2.7
US-2010 Anti-B (bulk pool)		373 ± 11 (n = 5)	2.9

* 95% confidence intervals in parentheses.

Table 5

Cross neutralization was not observed for any toxin serotypes.

Antitoxin type	IU/mL	Protected against	Failed to protect against [*]
US-2010 Anti-A	100	Toxin A	Toxin B, C, D, E, F, G
US-2010 Anti-B	75	Toxin B	Toxin A, C, D, E, F, G

* Both antitoxins were tested against 10 LD₅₀/mL of each toxin. BoNT/A complex from Hall and 62A strains, and BoNT/B from Okra B and TJ980 strains were similarly neutralized by homologous antitoxin.

Table 6

Comparison of avidity using Toxin A (Hall strain) doses were L_+ , $L_+/10$ and $L_+/30$.

IU/mL	US-2010 (LD ₅₀ /mL)	WHO-1963 (LD ₅₀ /mL)	WHO-1963* (LD ₅₀ /mL)
2.0	--	--	20,000
1.0	19,000–19,500	17,000–19,000	--
0.2	--	--	1375
0.1	770–880	770–880	--
0.033	60–70	55–65	--
0.010	--	--	15

* Toxin A strain 4587. Taken from Bowmer [Table 4 in ref. 30].

Table 7

Comparison of avidity using BoNT/A (62A strain).

IU/mL	US-2010 (LD ₅₀ /mL)	WHO-1963 (LD ₅₀ /mL)
1.0	5,900–7,100	5,300–6,500
0.1	120–300	165–300
0.033	12–35	12–30
0.010	--	--