Monoclonal antibodies and reagents for botulinum research

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Abstract: Botulinum neurotoxin (BoNT) is produced by *Clostridium botulinum* as a dichain protein of ~ 150 kDa that blocks acetylcholine release resulting in muscular paralysis. Diagnosis of BoNT often relies on the mouse bioassay that has a detection limit of 10–20 pg/mL and can take up to four days to complete. Rapid *in vitro* methods for toxin detection are needed and to date, most rely on either immunoassay or endopeptidase activity. In the latter, many also use specific antibodies to concentrate the toxin. We have developed panels of monoclonal antibodies (mAbs) to purified toxin, serotypes A, B and E, as well as mAbs to the non-toxic associated proteins. Application of these mAbs in sandwich ELISAs and assay performance in milk and other foods is discussed. The assays described here are able to detect toxin in a few hours, at levels lower than the mouse bioassay.

Keywords: monoclonal antibodies to botulinum neurotoxin; immunoassays; toxin detection in food.

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Biographical notes: Larry H. Stanker is a Research Biologist at the USDA – Agricultural Research Service (ARS) since 1991. He has been working on the development of monoclonal antibodies and sensitive diagnostic assays for the past 31 years, and with detection of botulinum neurotoxins for the past seven years. He received his PhD in Botany from the University of Illinois Champaign-Urbana in 1980. He worked as a Research Scientist at M.D. Anderson Hospital and Tumor Institute, Houston, TX (1980–1982), and at the Lawrence Livermore National Laboratory (1982–1991). He served as the Lead Scientist and Research Leader for the ARS Food and Feed Safety Research Unit in College Station, TX (1991–2000) before relocating to the ARS Laboratory in Albany, CA where he served as the Research Leader and presently as a Research Biologist in the Foodborne Contaminants Unit.

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1 Introduction

Clostridium botulinum, an anaerobic spore-forming bacterium, produces a family of botulinum neurotoxins (BoNT, EC 3.4.24.69) consisting of seven serotypes, A-G (Gill, 1982). BoNT is synthesised as a single polypeptide, ~150 kDa, that is cleaved endogenously or exogenously forming a dichain molecule comprised of an ~100 kDa heavy chain (HC) and an ~50 kDa light chain (LC) linked by a single disulfide bond to form the holotoxin (Montecucco and Schiavo, 1995). Toxin entry into neurons is facilitated by the receptor binding domain of the HC. The LC functions as a zinc-dependent endopeptidase, cleaving SNARE proteins (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) blocking acetylcholine release resulting in muscular paralysis (Simpson et al., 2004; Turton et al., 2002). BoNT is secreted as a large molecular weight complex containing neurotoxin associated proteins (NAPs). The function of the NAPs is unclear but they consist of various hemagglutinin proteins and a non-toxic, non-hemagglutinin protein (East et al., 1996; Dineen et al., 2003; Smith et al., 2007). Because or its high toxic potency and ease or production BoNT is classified by the Centers for Disease Control as a class A bioterrorism agent (Arnon et al., 2001).

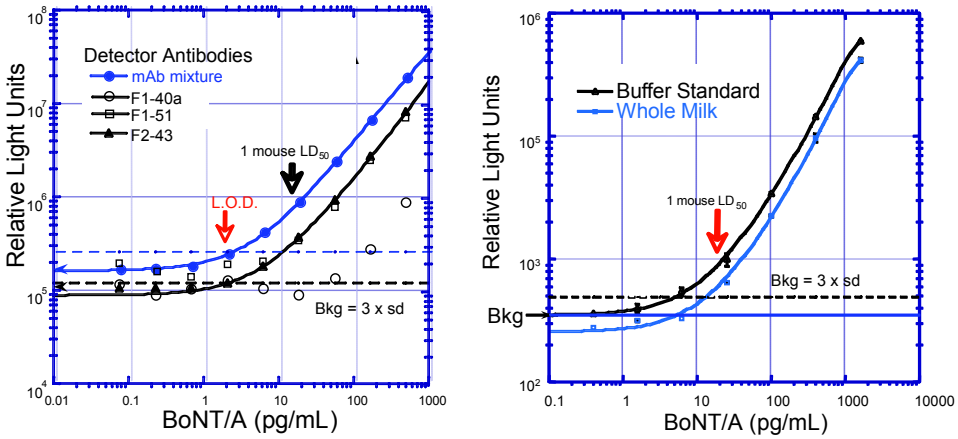
Detection of BoNT relies on use of the 'gold standard' mouse bioassay, a highly sensitive method with detection limits of 10–20 pg/mL (Ferreira et al., 2003; Wictome et al., 1999). However, the mouse bioassay is time consuming (up to four days), requires the use of large numbers of animals, has virtually no dynamic range, and relies on death as an endpoint. Furthermore, a subsequent neutralisation bioassay is required in order to confirm toxin presence and identify toxin serotype. Alternative *in vitro* methods that are rapid, sensitive, and toxin serotype-specific are highly desirable. To meet this need, numerous immunoassays have been developed, mostly sandwich (capture) enzyme-linked immunosorbent assays (ELISA) where toxin is captured by one antibody and then it is detected using a second antibody, the detection antibody. For the most part, these tests detect the presence of toxin but do not distinguish active from inactive toxin. Thus, performance of an immunoassay is influenced by the quality of the antibody pair, the nature of the stationary phase, and the label and/or substrate. A second category of *in vitro* tests for BoNT measures the activity of the toxin endopeptidase. However, many endopeptidase assays also incorporate specific antibodies to concentrate and present the toxin to an exogenous substrate (Rasooly et al., 2008; Bagramyan et al., 2008). Antibodies, therefore, represent the critical component of any immunoassay and many biosensors and their characteristics control assay performance. My laboratory has concentrated its efforts on development of a collection of high quality monoclonal antibodies (mAbs) to BoNT and NAPs in order to study toxin biology and build high performance immunoassays. Since the sandwich immunoassay is the most common format, antibody pairs (capture and detection) are the most useful for assay development.

2 Results

We have developed panels of mAbs for BoNT serotypes A, B, and E, as wells as antibodies to the NAPs of serotype A. The binding domain for most of the toxin specific mAbs have been localised to either the toxin LC, the translocation domain (HC_N) of the HC or to the receptor-binding domain of the HC (HC_C). The mAbs we isolated to BoNT/A bind only serotype A and have affinity dissociation constants in the low pM

range (Stanker et al., 2008; Scotcher et al., 2009a, 2009b). We were able to format these antibodies into a simple sandwich immunoassay with a limit of detection (LOD) of ~2 pg/mL [Figure 1(a)]. Using fragments of BoNT/B as immunogen, a collection of mAbs mostly specific for BoNT/B were isolated (Scotcher et al., 2010). Many of the mAbs developed to BoNT/B bind well in direct binding ELISA using immobilised toxin but failed to bind toxin in solution under physiological conditions. Thus they were not good candidates for capture antibodies. By modifying the screening strategy we were able to identify antibodies that do bind toxin in solution and allowed for construction of a sandwich ELISA with a lower LOD for BoNT/B of ~22 pg/mL. Likewise, we were able to isolate anti-serotype E mAbs that could be formatted into a sandwich ELISA. Unlike our previously developed mAbs, many of the mAbs binding the LC of BoNT/E have a wider specificity and were shown to bind serotypes C, B and F. The ability of these mAbs to neutralise toxin in vivo and to rescue animals following toxin exposure seems to correlate with the ability to bind toxin in solution. In addition to developing mAbs to the various toxin serotypes, we also have developed mAbs to the following BoNT/A NAPs: NTNH120, HA70, HA34 and HA17.

Figure 1 Competition ELISA detection of BoNT, (a) detection of toxin in buffer (b) BoNT detection in whole milk, blue line verses buffer, black line



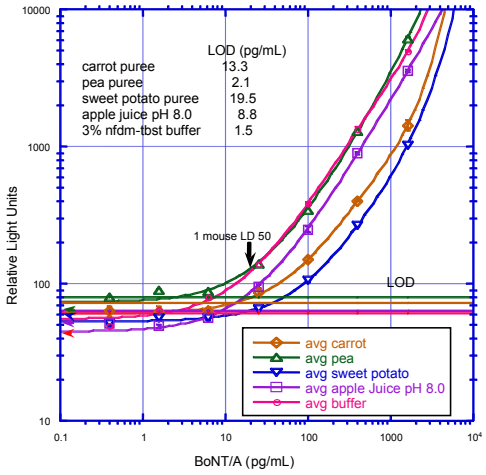
(a) (b)

Notes: Red arrow indicates LOD. Dashed lines represent the average background plus three standard deviations. Solid blue line, mixture of the three antibodies shown individually in black. Data represents average of three replicates, bar = one standard deviation.

Performance of the sandwich ELISAs for measuring BoNT serotypes A and B has been evaluated in a variety of foods. Shown in Figure 1(a) are results obtained using a sandwich ELISA incorporating a luminescent substrate to detect BoNT/A. Both the capture and detector antibodies are mAbs. In these experiments individual detector antibodies as well as a mixture of three detector mAbs were used. The LOD, defined as the average of the background (zero toxin) plus three times the standard deviation, is ~14 fM (2.1 pg/mL) using detector antibodies F1-51 or F2-43 individually or in a mixture. Less sensitive detection was obtained using mAb F1-40. The three detector mAbs used as a mixture were previously shown not to compete with each other for

binding. Clearly, this ELISA is roughly ten-fold more sensitive than the mouse bioassay. Shown in Figure 1(b) are results obtained when BoNT spiked milk was analysed. These same antibodies have been used to format lateral flow assays (Ching et al., 2012) and an assay based on an electrochemiluminesent detector using the meso scale discovery (MSD) instrument (Figure 2). Using the MSD platform an LOD ranging from 2–5 pg/mL was observed with extremely low background levels and standard deviations. Analysis of a number of different food samples spiked with BoNT/A demonstrated detection levels at 25 pg/mL (Figure 2). At these low toxin levels, recoveries varied from 20 to 100% but at higher toxin levels, > 2 ng/mL, recoveries closer to 100% were routinely observed. Toxin recovery was dependent on sample preparation and the type of food analysed. In those cases where the spiked samples were split and analysed by the mouse bioassay the LOD, depending on the type of food being analysed, was ~25 pg/mL. Sandwich immunoassays for toxin serotypes B an E also have been developed.

Figure 2 Electrochemiluminesent detection of BoNT/A in various foods



Notes: MAb F1-2 was used as capture and a mixture of mAb F1-40a, F1-51 and F2-43 as detector antibodies. Solid lines represent the LOD calculated for each food. Data represents average of three replicates, bar = one standard deviation.

A collection of mAbs specific for the toxin NAPs were developed using recombinant GST-NAP fusion proteins. Initial efforts have focused on serotype A and include antibodies to the NTNH120, HA70, HA34 and the HA17 proteins. Epitopes for the anti-HA70 mAbs have been localised to the HA50 and HA20 post-translational cleavage products of HA70 seen in serotype A. The anti-HA70 mAbs have been used to format a sandwich ELISA.

3 Conclusions

We have developed a series of mAbs and formatted these into sensitive sandwich immunoassays capable of detecting BoNT and toxin associated proteins in the low pg/mL range. Since BoNT is secreted as a toxin complex, we expect NAP to be present in naturally or intentionally contaminated foods. Thus, we anticipate using the anti-NAP

mAbs to develop multi-target assays that will enhance confidence in positive and negative findings. Furthermore, combining anti-NAP and anti-toxin antibodies into a defined mixture may enhance assay sensitivity for BoNT.

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