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A label-free biosensor assay for botulinum neurotoxin B in food and human serum

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ABSTRACT

Botulinum neurotoxins (BoNTs) are among the most toxic substances known. Surveillance and diagnostics require methods for rapid detection of BoNTs in complex media such as foodstuffs and human serum. We have developed in vitro assays to specifically detect the protease activity of botulinum neurotoxin B (BoNT/B) on a time scale of minutes. Cleavage of the BoNT/B substrate VAMP2, a membrane SNARE protein associated with synaptic vesicles, was monitored using real-time surface plasmon resonance to measure vesicle capture by specific antibodies coupled to microchips. The assay is functional in low-ionic-strength buffers and stable over a wide range of pH values (5.5–9.0). Endoproteolytic cleavage of VAMP2 was detected in 10 min with 2 pM native BoNT/B holotoxin. Contamination of liquid food products such as carrot juice, apple juice, and milk with low picomolar amounts of BoNT/B was revealed within 3 h. BoNT/B activity was detected in sera from patients with type B botulism but not in healthy controls or patients with other neurological diseases. This robust, sensitive, and rapid protein chip assay is appropriate for monitoring BoNT/B in food products and diagnostic tests for type B botulism and could replace the current in vivo mouse bioassay.

Botulinum neurotoxins (BoNTs)[1] are potent poisons in humans, blocking acetylcholine release from motoneurons at the neuromuscular junction to produce a flaccid paralysis of skeletal muscles known as botulism, a life-threatening condition when the respiratory system is affected [1]. Thus, rapid and sensitive detection of BoNTs present at low concentrations in complex matrices is an important challenge. Although intoxication with BoNTs is generally accidental, their potential use as biological weapons is also a persistent threat. Botulism in humans is due predominantly to the ingestion of food contaminated with the BoNT serotypes A, B, and E, with BoNT/B being implicated in more than 50% of cases [2]. *Clostridium botulinum* produces BoNT/B as a di-chain protein. Endopeptidase activity resides on the light chain (BoNT/B–Lc), which is separated from the heavy chain by reduction of a disulfide bond in the cytosol of intoxicated neurons. BoNT/B–Lc specifically cleaves vesicle-associated membrane protein 2 (VAMP2, also called synaptobrevin), an integral membrane protein associated with 50 nm synaptic vesicles. Cleavage occurs at a single defined peptide bond, Q76–F77, that inactivates VAMP2, preventing it from driving Ca^{2+}-dependent vesicle fusion with the plasma membrane, thereby interrupting neuromuscular transmission [3].

There is no antidote for botulism. Specific treatment by immunotherapy must be performed rapidly if possible, within 24 h of the onset of symptoms [4,5]. Thus, in suspected cases, accurate diagnostic tests are crucial, not only to initiate therapy with anti-toxin antibodies but also to prevent others from being contaminated by food products from the same source. Because current laboratory tests often require days, diagnosis and the decision to use serotherapy are based uniquely on clinical observation of symptoms. Laboratory assays, including analysis of food and blood samples, are subsequently used to confirm clinical diagnosis and to establish

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[1] *Abbreviations used:* BoNT, botulinum neurotoxin; Lc, light chain; VAMP2, vesicle-associated membrane protein 2; LD_{50}, 50% lethal dose; SPR, surface plasmon resonance; CSP, cystein string protein; VGLUT1, vesicular glutamate transporter 1; mAb, monoclonal antibody; N-ter, N-terminal domain; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; Hc, heavy chain; cDNA, complementary DNA; octyl glucoside, *n*-octyl-β-D-glucopyranoside; DTT, dithiothreitol; SnH, 10,000 g brain supernatant fraction; RU, resonance units; LEMS, Lambert–Eaton myasthenic syndrome; GBS, Guillain–Barré syndrome; DMSO, dimethyl sulfoxide.

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the incriminated toxin serotype [2]. The "gold standard" of laboratory tests for botulism is an in vivo mouse lethality assay. This bioassay is sensitive and robust, detecting as little as 2–20 pg/ml neurotoxin (1 LD_{50}/ml). However, it is lengthy (24–48 h) and involves the sacrifice of numerous mice coinjected with neutralizing antibodies against different BoNTs to identify the serotype. In line with their high toxicity, BoNT serum concentrations are usually very low, and only 70% of clinically characterized cases of botulism are positive in the in vivo assay [6,7].

A variety of in vitro assays have been developed during recent years based on the cleavage of SNARE peptides measured using immunological, fluorimetric, and mass spectrometry methods [8–10]. These methods are specific and sensitive, use small sample volumes, and are well suited to large-scale applications such as screening for BoNT inhibitors. However, they are generally not very robust and, thus, are poorly adapted to direct detection of BoNTs in environmental or clinical samples owing to the presence of components that either interfere with toxin activity or result in nonspecific hydrolysis of substrates by endogenous proteases [4]. Thus, in vitro detection of BoNT activity in complex media must involve an initial toxin purification/concentration step. For BoNT/B, assays of this kind typically attain a sensitivity equivalent to that of the in vivo mouse assay in 6–8 h [11,12].

We previously showed that cleavage by BoNT/B of native VAMP2 in synaptic vesicles from rat brain or cell cultures can be accurately quantified by surface plasmon resonance (SPR) [13]. We now report a rapid and sensitive protein chip assay for BoNT/B in complex solution that is appropriate for food safety applications and the serological diagnosis of human botulism.

Materials and methods

Chemicals and reagents

Antibodies against synaptic vesicles CSP (cystein string protein), VGLUT1 (vesicular glutamate transporter 1), vacuolar proton pump, synaptogyrin, and anti-SV2A were obtained as described previously [13,14]. Monoclonal antibodies (mAbs) against VAMP2–N-ter (N-terminal domain) (Cl 69.1), synaptotagmin 1 (Cl 41.1), and synaptophysin (Cl 7.2) were obtained from Synaptic Systems (Göttingen, Germany). A mAb, anti-VAMP77–83, was raised against a synthetic rat VAMP2 peptide, $_{77}$FETSAAK$_{83}$, with an additional C-terminal cystein residue allowing coupling to bovine serum albumin (BSA) for immunization. The mAb was selected by enzyme-linked immunosorbent assay (ELISA) with the immunogenic peptide. Nonimmune mouse immunoglobulin G (IgG) was obtained from Interchim (Montluçon, France). Recombinant His6-tagged BoNT/B–Hc (heavy chain) from *C. botulinum* BL6 was produced in *Escherichia coli* and purified on a cobalt column (Clontech). Polyclonal anti-BoNT/B was prepared as described previously [15] and immunopurified with recombinant BoNT/B–Hc immobilized on CNBr–Sepharose 4B (Amersham Biosciences). Rabbit anti-mouse Fcγ antibodies, sensor chips (CM3), and amine coupling kits were obtained from GE Healthcare. Purified *C. botulinum* neurotoxin serotype B, (0.5×10^8 LD_{50}/mg) was produced as described previously [16] (1 LD_{50}/ml corresponds to 20 pg/ml [133fM]). *C. botulinum* culture supernatant has a potency of 2×10^5 LD_{100}/ml. Complementary DNA (cDNA) encoding His-tagged BoNT/B–Lc was obtained as described previously [17]. *n*-Octyl-β-D-glucopyranoside (octyl glucoside), iodoacetamide, dithiothreitol (DTT), and fatty acid-free BSA were purchased from Sigma. Protein A agarose beads were obtained from Calbiochem. Control human sera were purchased from the Etablissement Français du Sang (Marseille, France).

Preparation of samples

SnH is the 10,000 g supernatant of a rat brain homogenate. SnH fractions were prepared as described previously [13]. Lyophilization was performed by Lyophal (Salon de Provence, France). SnH (100–300 μg/ml) was diluted in BoNT/B–Lc buffer (50 mM Hepes–NaOH, 0.16 mM DTT, 20 μM $ZnCl_2$, and 0.2 mg/ml BSA, pH 7.4) or in BoNT/B holotoxin buffer (5 mM Hepes–NaOH, 0.32 M sucrose, 25 mM DTT, 100 μM $ZnCl_2$, and 0.2 mg/ml BSA, pH 7.4) before incubation at 37 °C. BoNT/B reactions were stopped by 10-fold dilution in SPR analysis buffer (50 mM Tris–HCl and 400 mM NaCl, pH 7.4) plus 25 mM iodoacetamide when samples contained DTT. The effect of pH on native VAMP2 cleavage was determined with 50 mM Na acetate (pH 4.0, 4.3, or 5.5), 50 mM Mes (pH 6.5), 50 mM Hepes–NaOH (pH 7.4), 50 mM Tris–HCl (pH 8.0, 8.5, or 9.0), and 50 mM glycine–NaOH (pH 10.0). Buffers were prepared so that ionic strength did not exceed an equivalent of 100 mM NaCl. Reactions were stopped by adjusting to 400 mM NaCl before sample preparation for Western blotting. Liquid food media were obtained from three different commercial sources. Nonfat milk was diluted 3-fold, and dry skim milk was reconstituted at 3% in BoNT/B holotoxin buffer. Apple juice and carrot juice were centrifuged at 10,000*g*, and supernatants were diluted 2-fold in 20 mM Hepes–NaOH (pH 7.4). All samples were adjusted to 25 mM DTT and 100 μM $ZnCl_2$ and to pH 7.4 with NaOH if necessary. BoNT/B from the supernatant of a 4-day culture of *C. botulinum* type B (strain BL6) was diluted in human serum and injected intraperitoneally into 6 mice to determine the dilution yielding 1 LD_{50}/ml. This toxin was further diluted in different batches of human serum, and aliquots were frozen for parallel analysis in vitro and in vivo. All experiments were performed in accordance with French and European community guidelines for handling laboratory animals.

In vitro versus in vivo assays for serum BoNT/B

In vitro

Here 50 μl of protein A agarose beads was coated with 10–20 μg of affinity-purified anti-BoNT/B–Hc antibody and washed. Beads were incubated in 0.5 ml of serum for 1 h at 37 °C under mild agitation, washed briefly in 0.5 ml of sodium phosphate-buffered saline (pH 7.2) containing 0.1% BSA and once in 10 mM Hepes–NaOH and 320 mM sucrose (pH 7.4), and then incubated at 37 °C with 100 μl of SnH (600 μg protein/ml) in BoNT/B holotoxin buffer. Supernatants were diluted 50-fold in SPR analysis buffer.

In vivo

Here 1 ml of serum was injected intraperitoneally into 2 mice. Symptoms of botulism were monitored for 4 days.

Measurement of VAMP2 cleavage

SPR experiments were performed on a Biacore 3000 apparatus (GE Healthcare) at 25 °C with a running buffer containing 50 mM Tris–HCl and 150 mM NaCl (pH 7.4) as described previously [13,14,17]. Briefly, mAbs directed against VAMP2 (Cl 69.1), synaptophysin (Cl 7.2), and nonimmune IgG (Jackson ImmunoResearch, Suffolk, UK) were captured (400–800 resonance units [RU]) by rabbit polyclonal anti-mouse Fcγ antibodies covalently immobilized on CM3 sensor chips. VAMP2 was quantified by injecting samples at a flow rate of 5 or 40 μl/min over functionalized sensor chips. VAMP2 proteolysis was measured by a decrease of synaptic vesicle binding to anti-VAMP2 antibodies. Signals obtained with nonimmune IgG were subtracted, and resonance units measured with anti-VAMP2 were normalized to the signal obtained with anti-synaptophysin antibodies. A schematic representation of the SPR method is illustrated in Fig. 1.

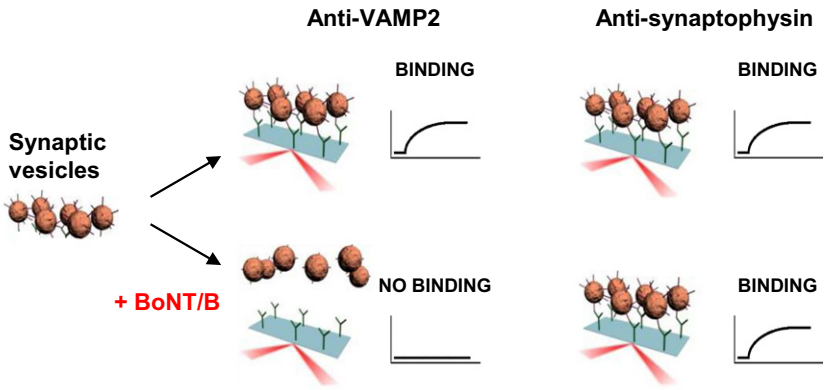

Fig.1. Schematic representation of the SPR biosensor method to assay BoNT/B activity. Synaptic vesicles were incubated in the presence or absence of BoNT/B and then injected on sensor chips functionalized with anti-VAMP2 antibodies or reference antibodies (anti-synaptophysin). BoNT/B treatment induces a decrease of synaptic vesicle binding only to anti-VAMP2 antibodies.

ELISA experiments were performed as described previously [17].

BoNT/B dose–effect curves were usually run once because errors on each data point are generally less than 10% of the indicated values.

BoNT/B detection in clinical samples

Sera from patients with clinically diagnosed botulism were obtained from the Institut Pasteur (Paris, France). These patients had BoNT/B intoxination confirmed using the mouse in vivo seroneutralization assay with serum titers between 0.5 and 8 LD50/ml. Samples had been stored for several weeks at 4 °C or frozen. Lambert–Eaton myasthenic syndrome (LEMS) and Guillain–Barré syndrome (GBS) sera were obtained from N. Martin-Moutot (INSERM UMR 641) and Hopital La Timone (J. Pouget, Pôle Neurosciences Clinique, Marseille, France), respectively.

Results

Lyophilized synaptic vesicles: a substrate to assay BoNT/B activity

We routinely use a frozen subcellular fraction (SnH) containing synaptic vesicles to assay the enzymatic activity of BoNT/B [13,17] and have shown that synaptic vesicles can be stably captured and their protein content can be analyzed by successive antibody injections [14]. To standardize and facilitate substrate storage and avoid variability in substrate quality, we lyophilized SnH. Following rehydration, we analyzed the integrity of antigens by SPR using antibodies against a panel of synaptic vesicle proteins. SnH batches that had been stored either frozen or lyophilized were diluted and injected into independent flow cells containing anti-synaptophysin antibodies or nonimmune IgG. The increase in SPR signal that results from synaptic vesicle binding to antibodies and nonspecific interactions with nonimmune control IgG was similar in both batches (not shown). The amplitude of the SPR signal generated for each epitope was very similar between frozen and lyophilized samples (Fig. 2A). These results demonstrate that lyophilization did not affect immunological detection of synaptic vesicle proteins in SnH.

To determine whether lyophilized SnH is a good substrate for BoNT/B, frozen or lyophilized batches were incubated in the presence of increasing concentrations of BoNT/B–Lc. VAMP2 cleavage was then measured by SPR or ELISA using antibodies against VAMP2–N-ter that is clipped off by the toxin. With SPR, VAMP2 is directly quantified by injecting SnH on a sensor chip functionalized with anti-VAMP2 and anti-synaptophysin antibodies. The ratio of vesicle capture by anti-VAMP2 versus anti-synaptophysin antibodies was calculated for each sample. Increasing BoNT/B–Lc concentrations inhibited synaptic vesicle capture by anti-VAMP2 antibodies but did not affect capture on anti-synaptophysin antibodies. With ELISA, vesicles were first captured on anti-synaptotagmin antibodies and then anti-VAMP2 immunoreactivity was measured. As illustrated in Fig. 2B, VAMP2 cleavage by BoNT/B–Lc was unaffected by the storage procedure, yielding EC_{50} values of 1.4 ± 0.5 pM for frozen SnH and 1.7 ± 0.6 pM for lyophilized SnH ($n = 3$). Lyophilized SnH batches were stable for at least 2 years (data not shown).

Effects of buffer composition on BoNT/B activity

Routine assays for the enzymatic activity of BoNT/B were carried out in Hepes–NaOH buffer at pH 7.4. We found that, when added separately, neither 5% dimethyl sulfoxide (DMSO), 0.32 M sucrose, 0.02% Triton X-100, nor 25 mM DTT affected toxin activity in Hepes–NaOH buffer (not shown). We next explored the influence of pH and ionic strength. SnH was incubated with BoNT/B–Lc in buffers covering the pH range of 4.0–10.0. Cleavage of native VAMP2 was then analyzed by Western blotting with an antibody directed against VAMP2–N-ter and an anti-syntaxin 1 antibody as a loading control. The results, illustrated in Fig. 3A, indicate that 10 nM BoNT/B–Lc had no effect at pH 4.0 or 10.0. However, distinct cleavage of VAMP2 occurred between pH 5.5 and 9.0. SnH was then treated with a range of BoNT/B–Lc concentrations between pH 5.5 and 10.0 and analyzed by SPR. Globally similar EC_{50} values in a range of 50–150 pM were measured from pH 5.5 to 9.0 (Fig. 3B), whereas no activity was detected at pH 10.0. The effect of ionic strength on the enzymatic activity of BoNT/B–Lc was examined in a buffer containing 0, 100, or 400 mM NaCl. The data (Fig. 3C) show a strong decrease in toxin activity at 100 and 400 mM NaCl with EC_{50} values of 107 pM and 3.7 nM respectively, yielding 54- and 1900-fold increments compared with 0 mM NaCl ($EC_{50} = 2$ pM). These results indicate that BoNT/B is fully active in a large range of pH values. Although we found a slight decrease in activity with Pipes and phosphate buffer at pH 7.4 (data not shown), BoNT/B activity appears to be fairly insensitive to the nature of the buffer but strongly affected by ionic strength.

Rapid detection of BoNT/B activity

A sensor chip was coated with antibodies against the synaptic vesicle proteins VAMP2 and synaptophysin as well as nonimmune

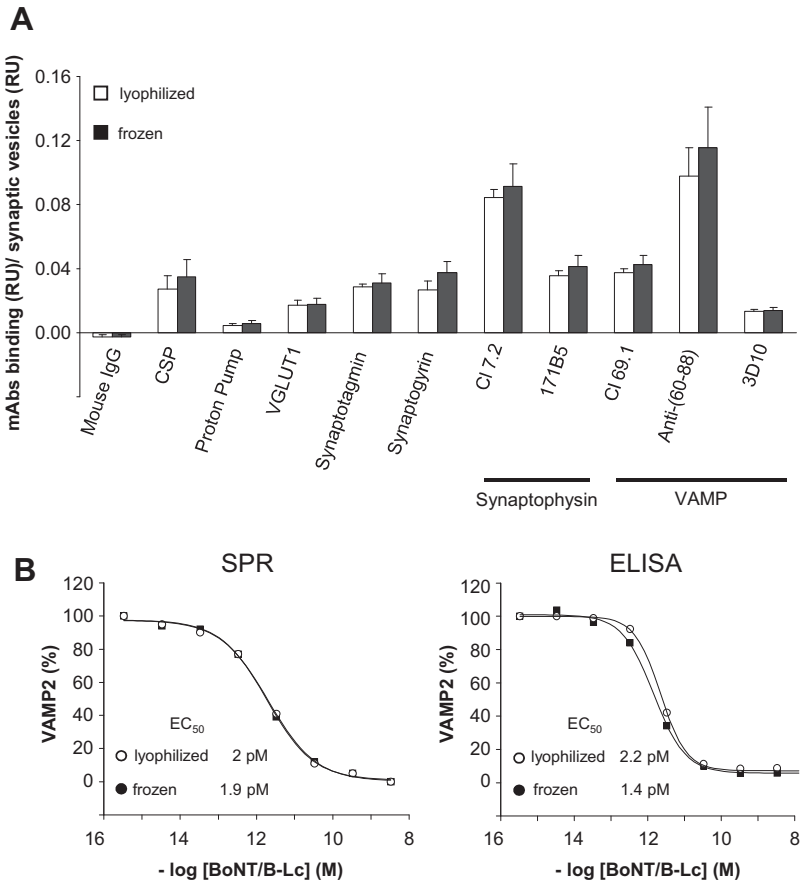

Fig.2. Characterization of membrane preparations for BoNT/B–Lc assay. (A) Synaptic vesicles from frozen or lyophilized SnH were immunocaptured on different flow cells of a sensor chip. Antibodies against the indicated synaptic vesicle proteins were then injected consecutively, and the amount of antibody bound (normalized with respect to the quantity of captured vesicles) was measured by SPR. (B) Frozen or lyophilized SnH was incubated with increasing concentrations of BoNT/B–Lc for 3 h and analyzed by either SPR or ELISA. Results are representative of five independent experiments.

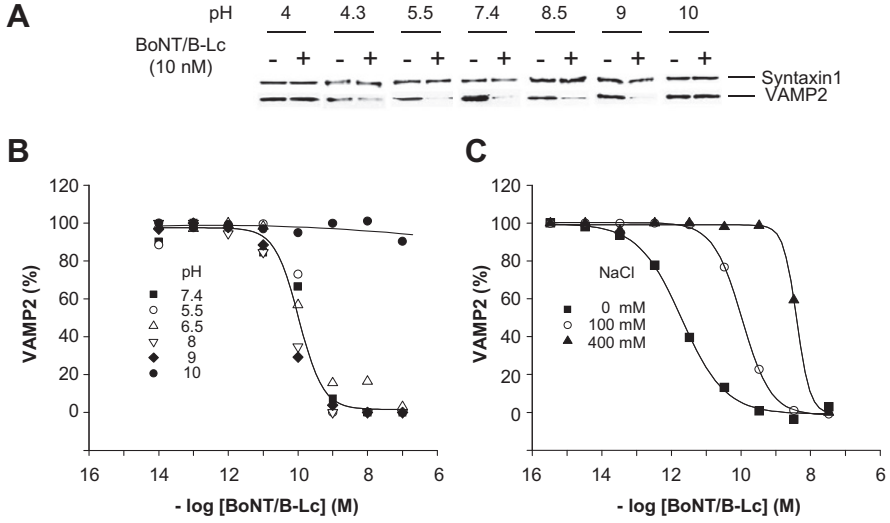

Fig.3. Effects of pH and ionic strength on BoNT/B–Lc activity. (A,B) SnH was diluted in different buffers at the indicated pH values, incubated with BoNT/B–Lc for 30 min, and then analyzed by Western blotting (A) or SPR (B). (C) SnH was incubated with BoNT/B–Lc in cleavage buffer containing the indicated concentrations of NaCl for 3 h. SPR results are representative of at least two independent experiments.

control antibodies. When a rat brain fraction containing synaptic vesicles (SnH) was injected into the flow buffer, the SPR signal generated by vesicle binding to immobilized antibodies changed as a function of time and injection flow rate. With a 40 μl/min flow rate, robust binding to antibodies was measured in 1 min. Preincubation of SnH with BoNT/B–Lc led to a reduction in the SPR signal with anti-VAMP2 antibodies (Fig. 4A). As little as 10 pM BoNT/B–Lc was detected in 2 min (1 min of incubation and 1 min of SPR

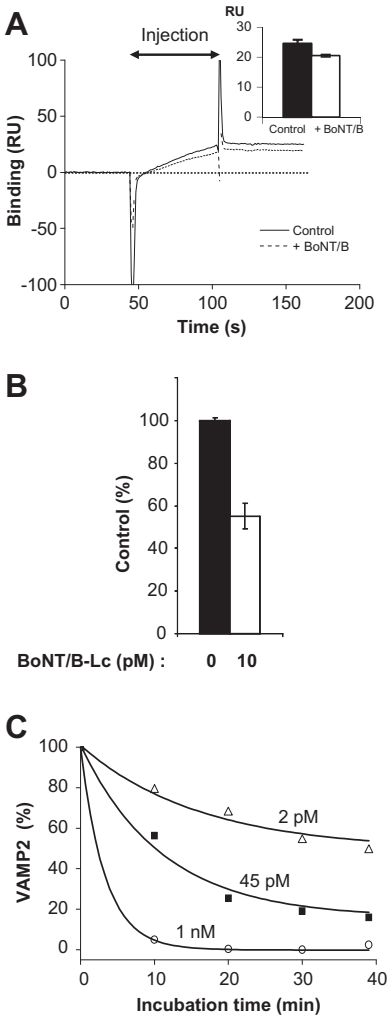

Fig.4. Rapid detection of BoNT/B activity. (A) SnH was incubated at 37 °C for 3 h in the presence (dashed trace) or absence (solid trace) of 0.2 pM BoNT/B–Lc. The superimposed sensorgrams illustrate synaptic vesicle capture on anti-VAMP2 antibodies and the decrease in response caused by BoNT/B action, and the inset indicates a decrease of 16 ± 3% (triplicate analysis). (B) SnH was incubated with 10 pM BoNT/B–Lc for 1 min and then analyzed by SPR. Results are normalized to the signals measured on anti-synaptophysin antibodies (means ± standard deviations, $n = 3$). (C) SnH was incubated with the indicated concentrations of BoNT/B holotoxin. Aliquots were removed at the indicated times and analyzed by SPR. Data are representative of two independent experiments.

analysis), yielding a 45% decrease in vesicle binding to VAMP2 (Fig. 4B). Note that the SPR signal with anti-VAMP2 antibodies was normalized with respect to that obtained with antibodies against synaptophysin, which is not a BoNT/B substrate. This internal standard compensates any variability in the absolute amount of sample injected.

To measure the endoprotease activity of native BoNT/B, 25 mM DTT was introduced in samples to release the catalytic activity of the light chain. VAMP2 cleavage, which proceeds as soon as disulfide bonds are reduced, was time and concentration dependent (Fig. 4C). At 2 pM (15 LD_{50}/ml), BoNT/B induced a 20 ± 4% decrease in VAMP2 in 10 min ($n = 3$ from three independent experiments), whereas total cleavage was achieved with 1 nM BoNT/B in 10 min.

Detection of BoNT/B activity in complex media

SPR was used to detect BoNT/B in liquid food products that constitute a frequent source of intoxication in humans. BoNT/B activity was evaluated in apple juice, carrot juice, 3% powdered milk, fresh nonfat milk, and control buffer spiked with toxin. BoNT/B activity was detected in all liquid food samples (Fig. 5), yielding EC_{50} values of 2 ± 0.9 pM in carrot juice ($n = 3$), 4 ± 0.7 pM in apple juice ($n = 3$), 8 ± 4 pM in powdered milk ($n = 3$), and 20 ± 8 pM in nonfat milk ($n = 3$), with detection thresholds (set at 20% VAMP2 cleavage) of 0.3 ± 0.06, 0.3 ± 0.1, 2 ± 1, and 5 ± 3 pM, respectively (2–37 LD_{50}/ml). Thus, SPR analysis was compatible with all liquid food samples tested, although sensitivity was decreased at least 50-fold compared with control conditions (EC_{50} = 0.04 ± 0.007 pM).

Detection of BoNT/B activity in serum after immunoprecipitation

We analyzed BoNT/B activity in human sera. Preliminary experiments indicated that sera needed to be diluted 10-fold (not shown) because the high concentrations of DTT required for light chain activation induced rapid coagulation. Thus, SnH was incubated for 3 h at 37 °C in 10% human serum containing a range of BoNT/B concentrations, and results were compared with standard conditions. The EC_{50} of BoNT/B increased 30-fold from 44 ± 10 fM in the control to 1.2 ± 0.2 pM (0.3–10 LD_{50}/ml) in 10% serum (data not shown). Thus, the inhibitory effect of the serum, in addition to the need for a 10-fold dilution, resulted in an assay that is approximately 100-fold less sensitive than the in vivo mouse assay.

To increase sensitivity to a level compatible with detection in clinical samples, we introduced an additional step in which BoNT/B was first immunoprecipitated from undiluted serum using anti-BoNT/B–Hc antibodies. Unpurified BoNT/B was diluted in sera from a sample containing a concentration of 1 LD_{50}/ml, which was the lowest concentration inducing a toxic effect in mice in this experiment. Sample aliquots were injected (1 ml/mouse, 2 mice) or incubated (0.5 ml) with anti-BoNT/B–Hc antibodies on protein A agarose beads. The immunoprecipitated pellet was then incubated with SnH, and the time course of VAMP2 proteolysis was monitored using SPR (Fig. 6). Cleavage of VAMP2 was already maximal after 1 h incubation at 1 LD_{50}/ml and was clearly detectable at 0.1 LD_{50}/ml (28% cleavage). VAMP2 cleavage increased with time, and BoNT/B activity was detected (25% cleavage) at 0.01 LD_{50}/ml after 4–5 h of incubation.

To investigate whether this method can detect BoNT/B activity in bona fide clinical specimens, a retrospective study of sera from 16 patients was undertaken. BoNT/B was immunoprecipitated from 0.5 ml of serum and incubated with synaptic vesicles, and VAMP2 was measured by SPR. The cleavage of VAMP2 occurred at distinct rates with different samples; three representative sera from BoNT/B patients (open symbols) are illustrated in Fig. 7A. In contrast, VAMP2 was perfectly stable in healthy control sera (black squares).

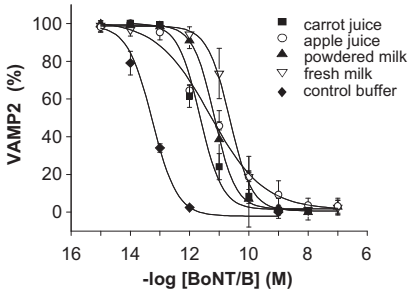

Fig.5. Detection of BoNT/B activity in liquid food products. SnH (100 μg/ml) was diluted in apple juice, carrot juice, reconstituted 3% powdered milk, fresh milk, or control buffer containing the indicated concentrations of BoNT/B and then incubated for 3 h at 37 °C. Samples were analyzed on a CM3 sensor chip carrying anti-VAMP2 and anti-synaptophysin antibodies. Results are means (± standard deviations) of three independent experiments.

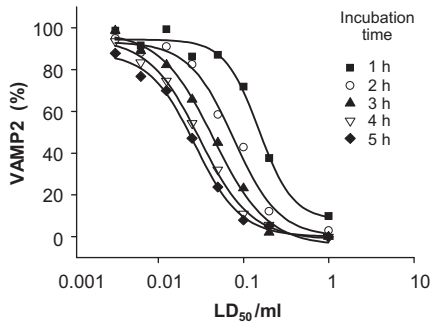

Fig.6. In vitro assay versus in vivo assay of BoNT/B in serum. *Clostridium botulinum* type B culture supernatant was serially diluted in human serum, and samples were analyzed in vivo in 2 mice per dilution and in vitro. Sera spiked with the indicated amounts of BoNT/B were incubated for 1 h at 37 °C with anti-BoNT/B–Hc beads. Beads were washed, and then SnH was added. SPR analysis of supernatant aliquots was performed after the indicated times. Data are representative of two independent experiments.

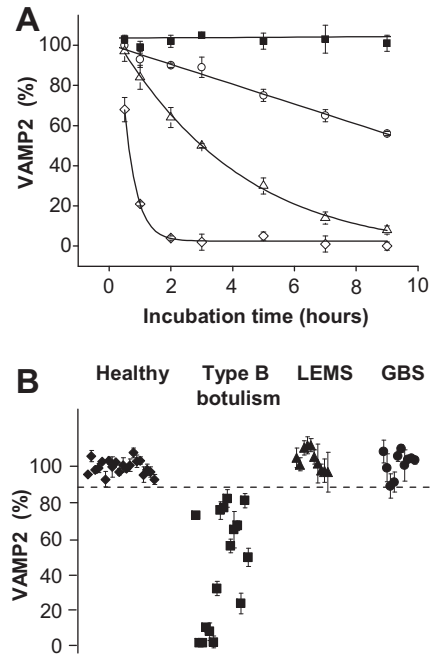

Fig.7. SPR detection of BoNT/B in the sera of patients with type B botulism. Sera were immunoprecipitated for 1 h, followed by incubation with SnH for the indicated times (A) or for 5 h (B), and then were analyzed by SPR. (A) Kinetics of VAMP2 cleavage by BoNT/B from sera of 3 patients (open symbols) or mean of 10 healthy control sera (■). (B) Results from 20 healthy sera and sera from 16 patients with type B botulism, 10 patients with LEMS, and 10 patients with GBS. The dashed line represents the threshold for positivity, representing the mean of the 20 healthy sera (▲) minus 3× standard deviation. Error bars represent 1 standard deviation calculated from three injections.

After 5 h incubation, VAMP2 proteolysis reached at least 15% with samples from all patients with type B botulism. In contrast, VAMP2 remained intact ($M = 100 \pm 3.7\%$) with 20 healthy control sera (Fig. 7B) or sera from patients with LEMS or GBS, two other conditions that affect neuromuscular transmission and can produce paralysis (Fig. 7B). In these conditions, we consider serum samples as positive for BoNT/B activity when they yield values inferior to the mean of the control group minus three standard deviations, represented by the dashed line in Fig. 7B. Hence, 16 of 16 patients (100%) with type B botulism were seropositive in this assay.

We next checked whether the loss of VAMP2 immunoreactivity resulted from cleavage at the Q76–F77 peptide bond, which is the specific endoproteolytic target of BoNT/B [18]. For this purpose, we used a mAb, anti-VAMP77–83, that recognizes the new amino terminus of the VAMP2 membrane-anchored C-terminal peptide (77–116) generated by BoNT/B action and, thus, recognizes only the BoNT/B-cleaved form of VAMP2. This antibody labels VAMP2 in cultured hippocampal neurons only after BoNT/B treatment (see Supplementary Fig. S1 in supplementary material) and reacts exclusively with the residual VAMP2 fragment associated with synaptic vesicles or with a recombinant VAMP2 fragment anchored in proteoliposomes following treatment with BoNT/B (data not shown). SnH was processed as above with sera from 2 patients and a healthy control. Vesicles were immobilized on a chip by an anti-synaptophysin antibody. Monoclonal anti-VAMP–N-ter, anti-VAMP77–83, or anti-synaptotagmin antibodies were then injected, and antibody binding to the captured vesicles was measured by SPR. Consistent with data in Fig. 7, in sera from both patients, BoNT/B activity resulted in a decrease in binding of anti-VAMP–N-ter antibody compared with control serum (Fig. 8). In contrast, mAb anti-VAMP77–83, which recognizes only the product of proteolysis by BoNT/B, displayed increased binding with sera from botulism patients. Finally, the SPR signal with a control mAb directed against another synaptic vesicle protein (synaptotagmin) was unchanged.

Discussion

SPR analysis of native VAMP2 associated with synaptic vesicles constitutes a new method useful for rapid detection for both food

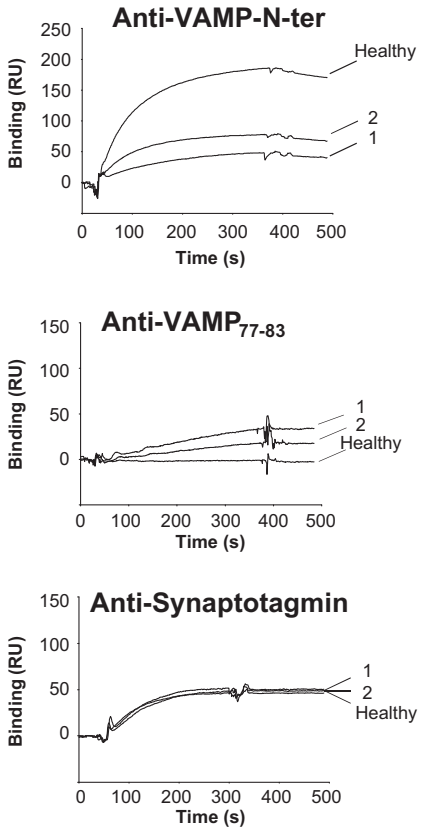

Fig.8. Detection of the BoNT/B-cleaved VAMP2 fragment associated with synaptic vesicles. Anti-BoNT/B beads were incubated with sera from a healthy control or 2 patients with type B botulism (1 and 2). After incubation with SnH, synaptic vesicles (~2000 RU) were stably captured on sensor chips via anti-synaptophysin antibodies. Synaptic vesicle epitopes were then detected by injection of the mAbs anti-SV2 (10 μg/ml), anti-synaptotagmin (10 μg/ml), anti-VAMP–N-ter (10 μg/ml), and anti-VAMP77–83 (25 μg/ml). Results were corrected with signals obtained with anti-SV2 antibodies and are representative of three independent experiments.

safety and diagnosis. Synaptic vesicles contained in a nonpurified brain fraction constitute a cheap and abundant substrate that can be lyophilized and incubated in various complex media without degradation. Furthermore, SnH pretreated with a range of BoNT/B concentrations and then lyophilized could provide standards for internal calibration of assays. Taking advantage of the near real-time analysis provided by SPR, the activity of 2 pM (15 LD_{50}/ml) BoNT/B was measured in only 10 min. Thus, our in vitro test provides the basis for rapid surveillance.

Previous reports using peptide substrates indicated that the enzymatic activity of BoNT/B was strongly inhibited by moderately acidic or basic pH [18–20]. However, it was not determined whether pH primarily affected enzyme activity or substrate conformation. Our results clearly show that, compared with synthetic substrate, the enzymatic activity on the "physiological" target is not affected by slightly acidic or basic pH, probably due to greater stability of the native substrate conferred by membrane anchoring [21]. However, an effect of membrane environment on the stability of BoNT/B–Lc cannot be excluded [22]. These findings are consistent with the idea that structural Zn^{2+} ions remain associated with BoNT/B in this pH range and that secondary and tertiary structures of the toxin are not modified at pH values between 6.0 and 8.0 [18]. On the other hand, in agreement with data using peptide substrates, high salt strongly inhibited the proteolysis of native VAMP2 by BoNT/B, probably by interfering with substrate recognition [18–20].

BoNT/B was directly assayed at low picomolar concentrations in liquid food products such as carrot or apple juice and milk. Similar sensitivity was obtained in serum provided that it was diluted. Our results demonstrate direct detection of BoNT/B activity at low concentrations in complex media. Detection thresholds in complex media are, however, higher than those in an optimized buffer where sensitivity reached femtomolar levels. Immunoprecipitation of toxin, to remove interfering substances from sera, dramatically improved the detection threshold for BoNT/B activity. The time required to ensure detection is dependent on serum toxin levels. A detection limit of 0.01 LD_{50}/ml was achieved in 4–5 h. This constitutes a gain in sensitivity of approximately 50- to 100-fold over previously reported in vitro assays that incorporated an immunocapture step before cleavage [11,12]. Our results provide the first in vitro assay sensitive enough to routinely detect BoNT/B in serum samples from patients with botulism. This assay could be particularly useful for early diagnosis of infant botulism in which only limited serum volumes are generally available.

Contrary to control sera, all of the serum samples from patients affected by type B botulism were positive in our assay, even samples containing low BoNT/B concentrations that produced symptoms in mice but were not lethal. This laboratory test could be helpful for physicians because some cases of botulism are not recognized when symptoms are mild and/or patients are misdiagnosed (most often as GBS) [23]. Unfortunately, serum volumes available for our retrospective study were insufficient to reevaluate LD_{50} values in parallel with the in vivo assay. Further investigation is needed to examine whether there is a correlation between the two methods. However, it is not really expected that the in vitro BoNT/B assay, which detects enzymatic activity of the light chain, will correlate with the in vivo assay, which requires a functional heavy chain to ensure toxin binding and transport into nerve terminals prior to VAMP2 cleavage. In line with this, only a few molecules of BoNT/B–Lc are needed within the cytoplasm to paralyze a nerve ending [19]. The potent toxicity of BoNTs relies on the heavy chain, which binds with high affinity to motoneuron terminals, thereby concentrating the toxin at its site of action even when serum levels are very low. Hence, a significant advantage of our assay is that it can detect BoNT/B activity in poorly conserved samples in which the heavy chain is not fully functional or has separated from the light chain. Finally, the high sensitivity of the in vitro assay could be helpful for measurement of low serum concentrations of BoNT/B in patients that currently escape laboratory detection and for clinical studies to monitor circulating toxin levels during patient follow-up [24]. It should be noted that this assay can also detect the enzymatic activity of tetanus toxin that cleaves VAMP2 at the same scissile bond as BoNT/B. The addition of specific antibodies directed against BoNT/B or tetanus toxin during the cleavage step would be useful to discriminate between these two toxins [25].

This assay offers dual advantages of rapidity conferred by real-time measurement and sensitivity through the analysis of native substrate by SPR. It constitutes a realistic alternative to the in vivo assay currently performed to diagnose botulism. We recently reported an SPR assay for BoNT/A [17] and are currently developing methods for BoNT/E. It is expected that simultaneous analysis of botulinum serotypes can be achieved with a single sensor chip functionalized with specific antibodies directed against VAMP and different epitopes on SNAP25 that specifically distinguish between cleavage by BoNT/A and that by BoNT/E. Because botulism requires immediate treatment, this assay should help to orient diagnosis and determine whether serotherapy should be initiated. Furthermore, the ability to rapidly identify which BoNT serotype is involved would allow immunotherapy based on serotype-specific human mAbs rather than polyspecific (anti-BoNT/A, -/B, and -/E), equine polyclonal antibodies that can induce adverse secondary effects [26,27].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.11.045.

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