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Direct biosensor detection of botulinum neurotoxin endopeptidase activity in sera from patients with type A botulism

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ABSTRACT

Botulinum neurotoxin A (BoNT/A) has intrinsic endoprotease activity specific for SNAP-25, a key protein for presynaptic neurotransmitter release. The inactivation of SNAP-25 by BoNT/A underlies botulism, a rare but potentially fatal disease. There is a crucial need for a rapid and sensitive *in vitro* serological test for BoNT/A to replace the current *in vivo* mouse bioassay. Cleavage of SNAP-25 by BoNT/A generates neo-epitopes which can be detected by binding of a monoclonal antibody (mAb10F12) and thus measured by surface plasmon resonance (SPR). We have explored two SPR assay formats, with either mAb10F12 or His_6-SNAP-25 coupled to the biosensor chip. When BoNT/A was incubated with SNAP-25 in solution and the reaction products were captured on a mAb-coated chip, a sensitivity of 5 fM (0.1LD_{50}/ml serum) was obtained. However, this configuration required prior immunoprecipitation of BoNT/A. A sensitivity of 0.5 fM in 10% serum (0.1 LD_{50}/ml serum) was attained when SNAP-25 was coupled directly to the chip, followed by sequential injection of BoNT/A samples and mAb10F12 into the flow system to achieve on-chip cleavage and detection, respectively. This latter format detected BoNT/A endoprotease activity in 50–100 μl serum samples from all patients (11/11) with type A botulism within 5 h. No false positives occurred in sera from healthy subjects or patients with other neurological diseases. The automated chip-based procedure has excellent specificity and sensitivity, with significant advantages over the mouse bioassay in terms of rapidity, required sample volume and animal ethics.

1. Introduction

Botulism is a potentially fatal but rare disease, often involving the ingestion of food contaminated with *Clostridium botulinum* neurotoxins (BoNTs). Toxins are absorbed in the intestine, and reach the neuromuscular junction via the circulation and the interstitial fluid. BoNTs act at the neuromuscular junction to inhibit neurotransmitter release, producing early symptoms such as blurred vision, ptosis, dry mouth and difficulty in swallowing (Lindstrom and Korkeala, 2006). Prompt immunotherapy with a trivalent anti-toxin (anti-BoNT/A, B and E toxin) is then required to forestall progression to descending paralysis, which can eventually compromise respiration.

The *in vivo* mouse bioassay is still the standard method for serological diagnosis of botulism, but fails to detect 20–30% of cases (McLauchlin et al., 2006; Wheeler et al., 2009; Sebald and Saimot, 1973; Mazuet et al., 2011). Its effectiveness depends on the concentration of BoNT in the circulation, which decreases rapidly following intoxination. The mouse bioassay is accurate at concentrations ≥ 1 LD_{50} but can take up to 4 days. At lower doses, mice may present characteristic symptoms that are reversed by neutralizing antibodies, but accurate diagnosis is a challenge. Furthermore the test requires large sample volumes, as typically several mice must be injected with 0.5–1 ml of serum/mouse, in the presence or absence of mono-specific neutralizing antibodies. There is thus a need for a rapid and sensitive *in vitro* test, based on the molecular properties of BoNTs, which can substitute for the mouse assay.

BoNTs are composed of two protein chains linked by a disulfide bridge and non-covalent interactions (Popoff and Poulain, 2010).

Abbreviations: BoNT, Botulinum neurotoxin; SPR, Surface plasmon resonance; RU, Resonance unit; BSA, Bovine serum albumin; LD_{50}, Median lethal dose; HEPES, 4-(2-Hydroxyethyl)-1-piperazine ethane sulfonic acid; SNAP-25, Synaptosomal-associated protein of 25 kDa; DTT, Dithiothreitol; HBS, HEPES-buffered saline; LOD, Limit of detection

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The heavy chain acts as a vector. It binds to receptors located at nerve terminals of the neuromuscular junction to assure internalization, and upon reduction of the disulfide bond, the light chain translocates into the cytoplasm. Light chains are metalloproteases with high substrate specificity, directed against single peptide bonds in SNARE proteins. SNAREs drive synaptic vesicle fusion and exocytosis of acetylcholine, thus their proteolytic inactivation blocks neuromuscular transmission presynaptically, resulting in muscle paralysis. Eight distinct BoNT serotypes (BoNT/A to H) are known (Popoff, 2014). Human botulism is mainly due to BoNT/A, B or E. Type A botulism has the highest incidence in the United States and constitutes the severest form (Arnon et al., 2001). In addition, BoNT/A is the most frequently used toxin in therapeutic applications to treat disorders resulting from hypercontraction or hypersecretion and in cosmetic applications (Dhaked et al., 2010).

A number of *in vitro* assays have been developed in the last decade, based mainly on immunodetection of the toxin heavy chain or on endopeptidase assays to reveal the enzymatic activity of the light chain (see for reviews, (Capek and Dickerson, 2010; Hakami et al., 2010)). The metalloprotease activity of BoNT/A light chain which specifically cleaves a single peptide bond (Q_{197}–R_{198}) in the synaptic SNARE SNAP-25 can provide a very sensitive means of detection and assays based on ELISA (Jones et al., 2008; Liu et al., 2012) and mass spectrometry (Barr et al., 2005) have been reported.

Biosensors have many applications in the detection of disease-related biomarkers, including antibodies and viruses. They have also considerable potential for the identification of food pathogens and toxins (Helmerhorst et al., 2012). Biosensors have been used to detect BoNT/A in optimized buffers using fluorescent or label-free readout (Bok et al., 2013; Frisk et al., 2009; Grate et al., 2009; Sun et al., 2009; Leveque et al., 2013; Marconi et al., 2008; Warner et al., 2009; Wei et al., 2011). Although the development of rapid automated detection provided by biosensors would be helpful for urgent diagnosis, detection of BoNT/A in serum, with a sensitivity appropriate for clinically relevant concentrations, has not yet been reported. Using antibodies directed against a neo-epitope generated by the enzymatic activity of BoNT/A we have explored the potential of SPR based-biosensors to detect toxin activity in serum. We report that injection of serum samples over a SNAP-25 substrate-chip permits for the first time the direct monitoring of circulating BoNT/A in patients with type A botulism.

2. Material and methods

2.1. Chemicals and reagents

His_6-SNAP-25, mAb 10F12 and anti-BoNT/A–Hc antibodies were prepared as described (Leveque et al. 2013; Mazuet et al. 2012). Amine coupling kits were from GEHealthcare and Biorad. $ZnCl_2$, Tween 20, DTT, iodoacetamide, Fc specific goat anti-mouse IgG and fatty acid-free bovine serum albumin were purchased from Sigma and Protein A Dynabeads from Invitrogen. Botulinum A neurotoxins: BoNT/A was from Metabiologics (Madison, WI, USA) at 3.5×10^7 LD_{50}/mg (1 mg/ml from Hall strain, MW 500,000 Da, 1 LD50/ml = 28 pg/ml = 54 fM), with frozen aliquots serially diluted immediately after thawing or produced and purified from Clostridium botulinum (A/B) strain NCTC2916 as previously described (Mazuet et al. 2012).

2.2. Sensor chip preparation

Preparation of the antibody chip: about 13,000 RU of 10F12 were coated at pH 5 on a CM5 chip using an amine coupling kit (GE Healthcare) in HBS buffer (10 mM HEPES-NaOH pH 7.4, 150 mM NaCl) at 25 °C. Mouse IgG was coupled under the same conditions and used as control for non-specific binding. Preparation of the SNAP-25 chip: His_6-SNAP-25 was amine coupled at pH 4.5 to a Biacore CM3 (GE Healthcare) or pH 4 to a ProteOn GLH (Biorad) sensor chips according to manufacturer's instructions and using HBS buffer as running buffer. The final density was 5000 RU on CM3 chips and 17,000 RU on GLH chips. Chips were conditioned by injection of cleavage buffer (50 mM HEPES-NaOH pH 7.4, 0.1% BSA, 10 mM DTT, 1% Tween 20, 100 μM $ZnCl_2$).

2.3. Determination of BoNT/A activity after immunoprecipitation

50 μl of Protein A Dynabeads were coated with 10 μg of affinity-purified anti-BoNT/A–Hc antibody. Beads were incubated for 1 h at 37 °C in 0.5 ml of serum in the presence or absence of serially diluted BoNT/A. Beads were washed briefly in 0.5 ml sodium phosphate-buffered saline (pH 7.2) containing 0.1% BSA and once in cleavage buffer without DTT. His-SNAP-25 (7 μM) was added to the beads in 100 μl cleavage buffer with 5 mM DTT and 0.5% Tween 20 and incubated for 5 h at 37 °C under mild agitation.

2.4. Sample preparation for on-chip cleavage

BoNT/A (Metabiologics) was thawed and serially diluted at room temperature in cleavage buffer. For measurement in serum, BoNT/A was serially diluted in 100% serum except for the last point which was diluted in cleavage buffer with a dilution factor of 10 or 4 to obtain a final serum concentration of 10% or 25%. To avoid an increase in ionic strength that interferes with assay sensitivity, the HEPES concentration in cleavage buffer was lowered to 10 mM for samples containing 25% serum. For cleavage time of 15 min and to ensure complete reduction of disulfide bonds, samples were pre-incubated for 15 min at 37 °C before analysis. This pre-reduction step was omitted for longer cleavage times (1–5 h). Serum samples in cleavage buffer were spun down once before loading in the biosensor sample rack refrigerated at 4 °C. 100 or 50 μl of serum from patients with type A botulism were diluted 4- or 8-fold, respectively, with cleavage buffer. Sera from healthy subjects or patients with other neuromuscular diseases were diluted 4-fold in the same conditions before analysis. Results were normalized to serum dilution.

2.5. SPR analysis

Measurement of SNAP-25 cleaved in solution: After incubation with BoNT/A captured on beads, the supernatant containing cleaved His-SNAP-25 was diluted 2-fold in 10 mM HEPES-NaOH pH 7.4, 0.3 M NaCl, 25 mM iodoacetamide, then injected for 5 min at 1 μl/min over a chip functionalized with mAb10F12. Sample buffer without His-SNAP-25 was injected to determine the background. Experiments were performed at 25 °C. The mismatch between the refractive index of the analyte solution, which contained a high detergent concentration, and the running buffer was corrected for by subtracting the SPR signal from blank samples.

For on-chip cleavage, SPR measurements were performed at 34 °C using the Biacore T200 (GEHealthcare) or the ProteOn (Biorad) apparatus with HBS or 10 mM Tris–HCl pH 7.4, 150 mM NaCl as running buffer, respectively. The flow rates were 1 μl/min with the Biacore and 25 μl/min with the ProteOn apparatus. For 5 h injections, samples were injected sequentially for 2 + 2 + 1 h in order to preserve biological activity at 4 °C, as enzymatic activity decays at 34 °C, over long injection periods. In all experiments,

cleavage buffer (with or without serum) in the absence of BoNT/A was injected onto a control lane coated with His-SNAP-25. Anti-mouse Fc antibody was used to increase SPR sensitivity. MAb10F12 (10 μg/ml) was preincubated with anti-mouse Fc antibody (10 μg/ml) and the complex was then injected for 2 min over all flow cells to measure cleaved SNAP-25. Alternatively mAb10F12 and anti-mouse antibody were injected sequentially. MAb binding was measured 10 s after the end of injection, and the chip was regenerated with an 8 s pulse of 10 mM glycine-HCl pH 2. The data obtained from the control flow cell were automatically subtracted from experimental measurements to yield the specific signal. Data were analyzed using a Biacontrol software (Biacore/GEHealthcare) and a ProteOn Manager v. 3.1.0.6 software (Biorad). The mean background was obtained by performing blank experiments using samples without BoNT/A, in the presence or absence of serum. The limit of detection (LOD) was defined as the mean of the background plus 3 × the standard deviation.

2.6. In vivo assay

Mice were injected intraperitoneally with 1 ml of diluted BoNT/A serum as described (Mazuet et al., 2012). Symptoms of botulism were monitored for 4 days. Experiments were performed in accordance with French and European community guidelines for handling laboratory animals.

2.7. Human serum samples

Sera from patients with clinically diagnosed botulism were collected by the National Reference Center for Anaerobic Bacteria and Botulism (Paris, France). These patients had BoNT/A intoxination confirmed using the mouse *in vivo* seroneutralization assay with serum titers above or equal to 1 LD_{50}/ml and identification of *C. botulinum* in the incriminated food. In 10 patients, BoNT subtype was determined by *C. botulinum* isolation from food or feces and neurotoxin gene sequencing. Samples had been stored frozen but thawed twice between *in vivo* titration and SPR analysis. Control sera from patients with chronic inflammatory demyelinating neuropathy (CIDP) were obtained from Dr Alain Creange and fulfilled the European Federation of Neurological Societies/Peripheral Nerve Society task force diagnostic criteria for CIDP (EFNS/PNS joint task force, 2010). Lambert–Eaton myasthenic syndrome sera (LEMS) were obtained from Nicole Moutot (INSERM UMR 1072, Marseille, France). Control human sera were obtained from the Etablissement Francais du Sang (Marseille, France).

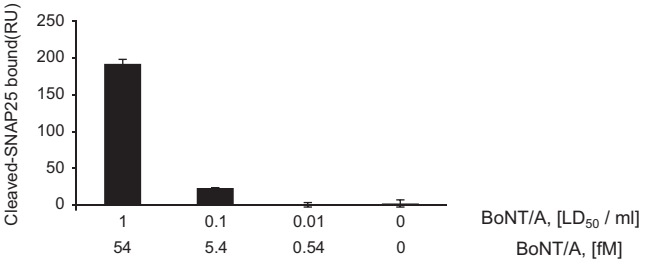

Fig. 1. Detection of BoNT/A immunoprecipitated from spiked serum samples. BoNT/A was spiked at the indicated concentrations in undiluted serum samples (0.5 ml), then immunoprecipitated using anti-BoNT/A HC antibodies bound to Protein A beads. The bead pellet was incubated with SNAP-25 (7 μM) in cleavage buffer for 5 h. The bead supernatant was alkylated, the ionic strength was adjusted and the amount of cleaved SNAP-25 analyzed, by injection over mAb10F12 immobilized on a chip. Representative of two independent experiments, results are means of triplicate injections ± SD.

3. Results and discussion

3.1. A monoclonal antibody chip to detect BoNT/A activity in serum

The monoclonal antibody mAb10F12 recognizes a C-terminal neoepitope generated when the endoprotease activity of BoNT/A light chain cleaves off a 9 amino acid fragment from the C-terminus of SNAP-25. However mAb10F12 does not react with full length SNAP-25 and thus provides a specific reagent to quantify BoNT/A activity using SPR (Leveque et al., 2013).

In order to adapt its use to clinical applications, experiments were carried out to assess BoNT/A detection in serum samples, initially using a format in which mAb10F12 was immobilized on a sensor chip. In this configuration an SPR signal is produced when the mAb captures, a SNAP-25 fragment generated by BoNT/A in the injected solution. To address the effect of serum in this BoNT/A detection assay, spiked BoNT/A samples were first incubated in the presence of an excess of SNAP-25 in cleavage buffer containing 10% human serum. The samples were then injected over the Biacore T200 sensor chip with two connected flow cells functionalized with either mAb10F12 or an irrelevant mAb to correct for non-specific binding. These experiments showed that injection of 10% serum into the flow cells induced a strong and fluctuating background signal, precluding accurate quantification of cleaved SNAP-25 (data not shown).

To circumvent interference from serum components binding to chip matrix, a polyclonal antibody against the heavy chain of BoNT/A was used to immunoprecipitate the toxin from undiluted BoNT/A-spiked serum samples, prior to the cleavage step. Antibody-beads with the bound BoNT/A were then washed and incubated with SNAP-25 in cleavage buffer containing DTT to fully activate the botulinum enzyme by reduction of the disulfide bonds linking the heavy and light chains. At the end of incubation, excess DTT was quenched and the bead supernatant containing cleaved SNAP-25 fragments was injected over a chip coated with mAb10F12. The results shown in Fig. 1 indicate that this method allows detection of BoNT/A activity $\geq$ 0.1 LD_{50}/ml ($\geq$ 5.4 fM), after 5 h substrate cleavage.

The sensitivity of this method was in the same range as that reported with mass spectrometry assay (0.36 LD_{50}/ml, (Parks et al., 2011)) and ELISA in which both anti-BoNT/A antibodies and substrate were coated on the plate (0.03 LD_{50}/ml, (Liu et al., 2012)). However, like all of the *in vitro* serological assays for BoNTs published to date, it required an initial immunoprecipitation step with anti-BoNT/A antibodies (Bagramyan et al., 2013; Dunning et al., 2012; Janardhanan et al., 2013; Jones and Marks, 2013; Mazuet et al., 2012; Parks et al., 2011; Stevens et al., 2013). *In vitro* detection of BoNT in the serum of patients with botulism is challenging due to matrix interference, small sample volumes, extremely low BoNT concentrations (< 1 pM) and requirement for a timely response. Immunoprecipitation concentrates the toxin and eliminates interfering molecules, allowing BoNT/A detection in sera from patients (Jones and Marks, 2013; Mazuet et al., 2012). However it is a time-consuming process, difficult to automate and requires additional reagents, including antibodies against all BoNT/A subtypes which do not inhibit enzymatic activity (Dunning et al., 2012; Kalb et al., 2009).

3.2. Direct detection of BoNT/A enzymatic activity using a substrate-chip assay

In order to eliminate the immunoprecipitation step and establish a more direct detection procedure, we investigated a recently developed on-chip format (Leveque et al., 2013). In this configuration, SNAP-25 was coated on a flow cell surface of a ProteOn XPR36 biosensor apparatus and a BoNT/A sample was injected at

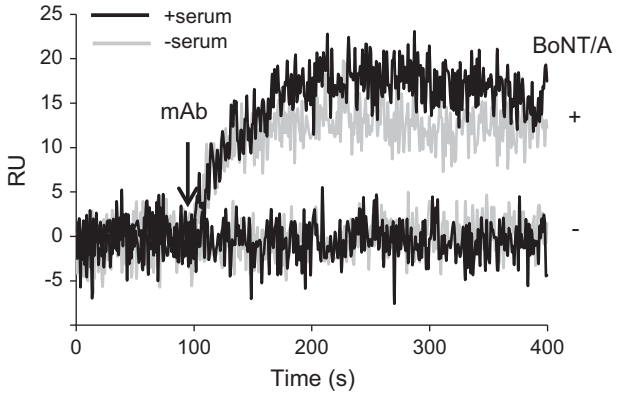

Fig. 2. Detection of BoNT/A activity in samples containing serum. Cleavage buffer was injected over a SNAP-25 coated chip for 15 min in the presence (black trace) or absence (gray trace) of 10% serum and in the presence or absence of BoNT/A (1 LD_{50}/ml, i.e. 54 fM). Representative of two independent experiments performed on the ProteOn XPR36 apparatus. Note that blank serum did not produce non-specific binding of mAb10F12.

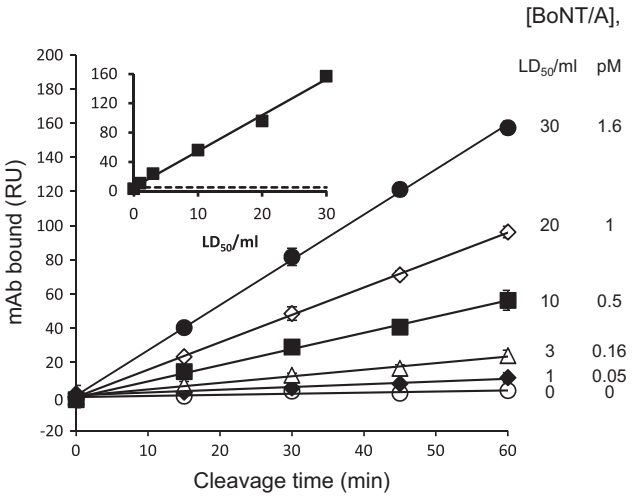

Fig. 3. Dose-dependence and kinetics of the SNAP25-chip assay. Six samples containing the indicated concentrations of BoNT/A were injected in parallel for 15 min. Cleaved SNAP25 was measured after mAb10F12 binding. This procedure was then repeated three times to give a total cleavage time of 60 min. Inset: dose–SPR response (RU) obtained at 1 h. Dashed line denotes the mean background level plus three SD. Representative of two independent experiments, results are means of mAb10F12 binding signal ($n=3 \pm$ SD).

34 °C into the flow system. The proteolytic product generated on the chip by BoNT/A was then detected by injection of mAb10F12. Spiked samples containing BoNT/A (10 LD_{50}/ml, i.e. 540 fM) were diluted 10-fold in cleavage buffer and injected for 15 min over the SNAP-25-coated chip. The injection of samples containing serum resulted in a large SPR signal, corresponding to the adsorption of serum proteins on the flow cell during the period in which substrate cleavage occurred (data not shown). However, after BoNT/A treatment, the subsequent injection of mAb10F12 gave a similar increase in resonance signal, irrespective of whether the chip had been exposed to serum or not (Fig. 2, upper traces). Signals close to zero were obtained in both conditions in the absence of BoNT/A (Fig. 2 lower traces). Similar results were obtained using the Biacore T200 biosensor with positive SPR signal of 18 ± 5 RU in the presence or absence (20 ± 4 RU) of serum ($n=5$, from 5 independent experiments and different serum samples). We also observed that cleavage could be measured in samples containing 25% serum (data not shown). These results indicate that the on-chip format allows direct measurement of BoNT/A activity in the presence of serum. Because substrate cleavage and detection on the chip surface are carried out sequentially, this method avoids interference with the SPR signal generated when serum components bind non-specifically to the chip. The detection of BoNT/A activity in presence of serum was surprising, since 10% serum strongly inhibited the activity of BoNT/B (Ferracci et al., 2011). Thus BoNT/B may be more sensitive to interference from serum than BoNT/A (Ravichandran et al., 2006). Another reason might be due to intrinsic properties of the on-chip configuration (Deere et al., 2008; Lee et al., 2008). Indeed, BoNT/A is a basic protein and thus likely to accumulate in the gel phase of the sensorchip, which is composed of negatively-charged dextran or alginates, whereas mostly acidic serum proteins may be excluded.

3.3. Time and dose-dependency of the SNAP-25 substrate chip assay

In order to explore the dose-dependency of the assay, human serum was spiked with BoNT/A at concentrations ranging from 1 to 30 LD_{50}/ml (54 to 1620 fM) and 10-fold dilutions were injected on the ProteOn XPR36 biosensor, which allows parallel analysis. Each concentration was injected four times for 15 min each (60 min in total) followed by detection with mAb10F12. After each 15 min round of BoNT/A injection, the chip was totally regenerated by a pulse of acidic pH permitting cumulative detection. Time courses were linear (Fig. 3) and a dose–response curve was plotted using the SPR signal (RU) measured at 1 h for 5 different BoNT/A concentrations (Fig. 3 inset). 10 LD_{50}/ml (540 fM) serum were detected in 15 min (final on-chip concentration=1 LD_{50}/ml, i.e. 54 fM) and 1 LD_{50}/ml (54 fM) serum (final on-chip concentration=0.1 LD_{50}/ml, i.e. 5.4 fM) in 1 h (Fig. 3). The detection of 1 LD_{50}/ml (54 fM) in serum was confirmed using the Biacore T200 apparatus yielding 9.4 ± 1.4 RU whereas serum alone gave -1.7 ± 1.2 RU ($n=4$ from independent experiments and different serum samples). The chip-based assay performed in the presence of serum is thus time- and concentration-dependent and compatible with detection of 1 LD_{50}/ml (54 fM) BoNT/A within 1 h. Moreover these results indicate that a single flow cell coated with SNAP-25 can be reused several times allowing multiple assays on the same chip.

3.4. Sensitivity limit of the on-chip assay

The sensitivity limit of the on-chip method for detection of BoNT/A activity in serum was determined using a Biacore biosensor and an increased cleavage time. This apparatus offers a high signal/noise ratio and allows reduced sample consumption compatible with prolonged injections at a low flow rate (1 μl/min). To reduce potential loss of enzymatic activity due to warming in the injection syringe, samples were maintained on a rack at 4 °C and aliquots were injected sequentially. Control sera were spiked with 1 LD_{50}/ml (54 fM) BoNT/A, diluted 10-fold in cleavage buffer and aliquots were injected. A mean SPR signal of 59 ± 12 RU ($n=5$ independent experiments, using different human sera) was obtained after 5 h. This value is clearly above the limit of detection (LOD) of 2 RU, determined from experiments with blank sera ($n=5$ from independent experiments and different serum samples). No effect of serum hemolysis on enzymatic detection was noted (data not shown). These results show that the enzymatic activity of BoNT/A was stable for several hours when diluted in different sera.

With a cleavage step of 5 h, BoNT/A concentrations as low as 0.01 LD_{50}/ml (i.e. 0.1 LD_{50}/ml, 5.4 fM in the serum sample) were reliably measured (8.7 ± 2 RU, $n=4$ from independent experiments and different serum samples). This corresponds to 0.28 pg/ml of BoNT/A (540 attomolar). Sensitivity was comparable to that measured in the absence of serum (Leveque et al., 2013). The detection

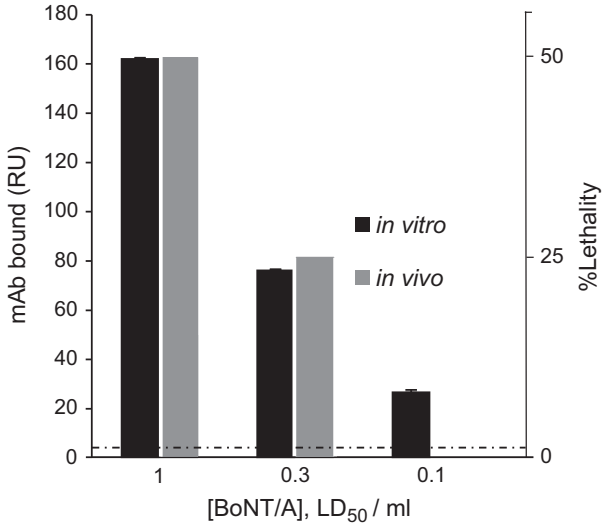

Fig. 4. Comparison of the SPR assay and the mouse bioassay at low serum BoNT/A concentrations. Serum samples spiked with BoNT/A were diluted 4-fold in cleavage buffer and analyzed using the SPR on-chip assay. The dashed line represents the LOD (2 RU from 5 independent experiments with different sera). SPR results are representative of 2 independent experiments; results are means of mAb10F12 binding signal ($n=3\pm$SD).

threshold could probably be reduced even further if the cleavage time was prolonged.

To confirm and extend our evaluation of assay sensitivity, a direct comparison of the on-chip assay and the mouse assay was performed. BoNT/A was produced and purified as described (Mazuet et al., 2012). Serial dilutions ranging from 30 to 0.1 LD_{50}/ml were prepared in undiluted healthy serum pooled from several subjects and aliquots were frozen. As expected, the *in vivo* assay indicated that 50% of the mice died at 1 LD_{50}/ml and the lowest BoNT/A concentration producing a lethal effect was 0.3 LD_{50}/ml (Table S1). Spiked samples between 1 and 0.1 LD_{50}/ml serum (100 μl) were diluted 4-fold in cleavage buffer and injected for 5 h over independent flow cells coated with SNAP-25.

As depicted in Fig. 4, the SPR assay gave a strong signal at 1 LD_{50}/ml serum that decreased with BoNT/A concentration. A concentration of 0.1 LD_{50}/ ml serum (final dilution during the cleavage step=0.025 LD_{50}/ml) yielded 28 RU, a value largely above the LOD (dashed line). Taking into account the intrinsic variation in potency estimated for BoNT/A from both sources, our results indicate that similar detection thresholds can be achieved with commercial or in-house BoNT/A. The *in vitro* test thus allows detection of serum concentrations of BoNT/A that are not detected by the *in vivo* assay (0.1 LD_{50}/ml *versus* 0.3 LD_{50}/ml) and in less time (5 h *versus* 96 h). The on-chip method was then tested for its capacity to identify BoNT/A activity in authentic sera from patients with type A botulism.

3.5. Measurement of BoNT/A activity in sera from patients with type A botulism

A retrospective study was carried out on sera from 11 human patients intoxicated with BoNT/A. All of these sera were stored frozen and BoNT/A positivity determined in the mouse bioassay with specific neutralizing anti-BoNT/A serum. Sera were diluted in cleavage buffer, injected for 5 h onto a SNAP-25 chip in a Biacore apparatus and cleaved-SNAP-25 was measured via mAb10F12 binding (Fig. 5). All patients sera (11/11) containing BoNT/A1 (7 samples) or BoNT/A2 (3 samples), or an undetermined (1 sample) toxin A subtypes gave positive signals ranging from 13 to 2600 RU (Fig. 5). 21 healthy control samples gave a mean signal of -1.8 ± 2 RU resulting in a LOD of 5 RU (dashed line). 22 control sera from patients with Lambert–Eaton myasthenic syndrome (LEMS) or chronic inflammatory demyelinating neuropathy (CIDP), two diseases that affect neuromuscular transmission and can produce paralysis and symptoms similar to botulism (Fig. 5), were indistinguishable from the healthy control group.

This detection method relies on the use of a mAb that binds specifically to a neo-epitope generated when the endoprotease activity of BoNT/A cleaves the Q_{197}–R_{198} peptide bond of SNAP25. As BoNT/A is the only BoNT that cleaves this peptide bond, our test is BoNT/A- toxinotype specific. However, to date, seven different BoNT/A subtypes have been described according to differences in amino acid sequence. They all cleave SNAP-25 at the same peptide bond but with different efficacies (Whitemarsh et al. 2013). The assay we have established revealed BoNT/A activity in all 11 patients, irrespective of whether they were intoxicated with the most prevalent subtypes A1 or A2. However it remains to be determined whether the method we describe will effectively detect the A3 to A7 subtypes.

All patient sera tested in the mouse bioassay contained ≥ 1 LD_{50}/ml. However the enzymatic activity of some samples measured by SPR was less than predicted by mouse toxicity. This may be due to a pre-analytical problem, such as loss of activity during storage since the initial mouse bioassay. Unfortunately the available sample volumes were too small to repeat the mouse assays in parallel and compare simultaneous *in vivo* and *in vitro* assays. As all the serum samples had not previously been precisely titered by the mouse assay, an accurate correlation between *in vivo* and *in vitro* data could not be established. However the 2 highest SPR signals (2600 and 550 RU) in Fig. 5a corresponded to high titers in the mouse assay (80 and 8 LD_{50}/ml, respectively). A strict correlation between enzymatic activity and toxicity in the mouse bioassay is unlikely, as the former only requires a biologically active BoNT/A light chain, whereas the latter depends on associated heavy and light chains, which must both be functional to achieve *in vivo* targeting, translocation and SNAP-25 cleavage. Moreover true titer determination *in vitro* requires simultaneous measurement of a calibration curve that is not possible using the Biacore T200 apparatus.

Obviously sera that yield a high SPR value in 5 h (Fig. 5a) could be analyzed using a shorter cleavage time. An experiment using the serum sample with the highest SPR titer was performed *in vitro* using a rapid protocol. As shown in Fig. 5b, 15 min was sufficient to reveal the enzymatic activity of circulating BoNT/A. When laboratory diagnosis is required, a preliminary test could thus be achieved within minutes to determine whether the patient has a high serum titre (e.g. 30–60 min for [BoNT/A] >1 LD_{50}/ml) and then the cleavage step prolonged with intercalated detection steps to adapt to lower titers.

4. Conclusion

We have explored two assay formats to detect BoNT/A in serum at a concentration as low as 0.1 LD_{50}/ml. While the format incorporating a BoNT/A immunoprecipitation step could allow detection from diverse complex media, the on-chip cleavage format offers the advantages of simplicity and automation compatible with the use of serum samples. Samples and mAb10F12 are diluted and placed on a sample rack and the SPR apparatus pilots the serum injection and detection procedures. In 1 h the biosensor can detect 0.1 LD_{50}/ml in 10% serum which corresponds to 1 LD_{50}/ml in the initial serum sample. For comparison, the mouse bioassay requires around 12 h to detect 1 LD_{50}/ml. ELISA and mass spectrometry are also effective in this concentration range, but are not faster (20–30 h) than the mouse assay. In 5 h the SPR

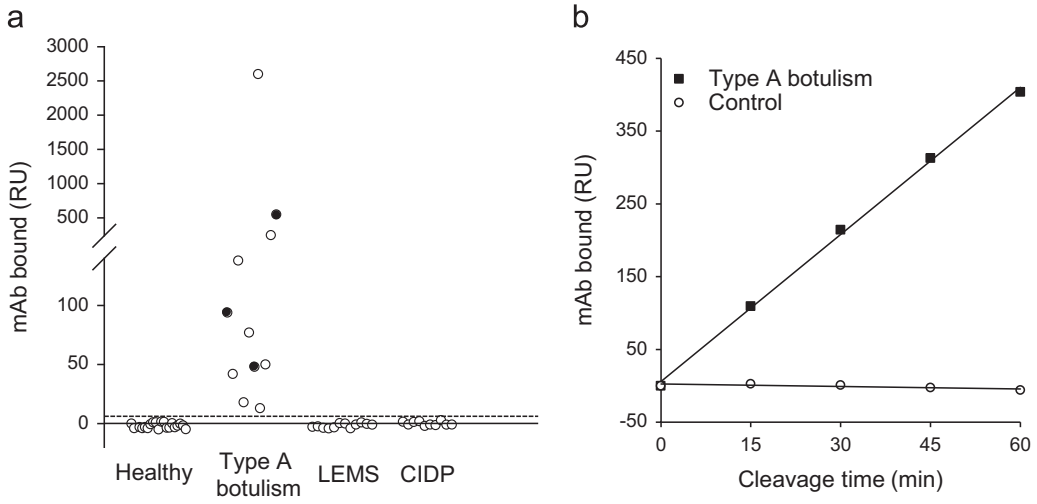

Fig. 5. Detection of BoNT/A activity in sera from patients with type A botulism. (a) Sera were diluted in cleavage buffer and injected for 5 h over a SNAP-25-coated chip followed by detection of cleavage with mAb10F12. Sera were from healthy subjects ($n=21$), patients with type A botulism ($n=11$, open circles=BoNT subtype A1; black closed circles=BoNT subtype A2), Lambert–Eaton myasthenic syndrome ($n=12$, LEMS) and chronic inflammatory demyelinating polyneuropathy ($n=10$, CIDP). The dashed line indicates the threshold for positivity taken as the mean+3 × the SD of the healthy control group. (b) Serum from a patient with type A botulism or a control serum were diluted 8-fold in cleavage buffer, incubated for 15 min at 37 °C and injected onto a SNAP-25 chip 4 × 15 min with mAb10F12 detection after each round of injection.

procedure attains sensitivity similar to the one obtained with a dual coated ELISA (Jones and Marks, 2013). The main advantages of biosensor-based assays *versus* ELISA are rapidity and flexibility, allowing the cleavage time to be adapted to the BoNT/A concentration in serum.

In conclusion the automated biosensor assay we report has major advantages over the mouse bioassay in terms of rapidity, sensitivity, reduced sample volume and ethical considerations regarding animal testing. It is expected to provide a significant improvement for laboratory diagnostics and may be further developed for pharmacokinetic applications related to the therapeutic and cosmetic use of BoNT/A.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.02.015.

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