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Studies on the dissociation of botulinum neurotoxin type A complexes

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ABSTRACT

The neurotoxins produced by the various strains of the anaerobic bacterium *Clostridium botulinum* naturally occur associated with complexing proteins which serve to protect the neurotoxins from the harsh environment of the mammalian gastrointestinal tract during bacterial invasion of the host. Three different complex species with the discrete sizes 19S (900 kDa, LL complex), 16S (500 kDa, L complex) and 12S (300 kDa, M complex) may be isolated from *C. botulinum* type A cultures. However, to affect their target cells these complexes must dissociate releasing the free 150 kDa neurotoxin.

This study assesses the stability of these *Clostridium botulinum* neurotoxin serotype A (BoNT/A) complexes and identifies factors which influence their dissociation. The knowledge gained with purified toxin complexes was subsequently employed to analyze the presence of such complexes in the freeze or spray-dried commercial BoNT/A products Botox and Dysport in comparison to the complexing protein free product Xeomin.

Purified 900 kDa and 500 kDa toxin complex preparations show a pH and time dependent release of the 150 kDa neurotoxin with a half-life of less than a minute at pH values >7.0. At pH values of 6.25 or less, the complexes are stable. Furthermore, dilution of concentrated 900 kDa complexes leads to dissociation into 500 kDa, neurotoxin containing complexes. Addition of sodium chloride as contained in isotonic saline leads to further disruption of these complexes resulting in the release of the free 150 kDa neurotoxin.

Examination of the commercial botulinum neurotoxin products Botox and Dysport using the same analytical procedures leads to the same conclusion: the dilution, drying and reconstitution processes of these products lead to a complete dissociation of 900 kDa complexes and 85% or more of neurotoxin are present in free form.

Conclusion: BoNT A toxin complexes have evolved to quickly respond to specific environmental changes by efficient release of the neurotoxin. During pharmaceutical production and reconstitution of BoNT A products, the same principles effect the quantitative dissociation of 900 kDa complexes and release of free neurotoxin prior to injection into target tissues.

1. Introduction

Botulinum neurotoxins are a group of proteinaceous toxins produced by various strains of the anaerobic bacteria *Clostridium botulinum* (serotypes A to G), *Clostridium butyricum*, *Clostridium baratii* and *Clostridium argentinens* (Schantz and Johnson, 1992). Botulinum neurotoxin serotype A (BoNT/A; (Niemann, 1992)) is one of the most potent poisons known to man causing flaccid muscle paralysis by blocking acetylcholine release at neuromuscular junctions

Abbreviations: kDa, kilodalton; AIEX, anion exchange (chromatography); BoNT/A, *Clostridium botulinum* neurotoxin serotype A; HA, hemagglutinin; HRP, horseradish peroxidase; HSA, human serum albumin; NaCl, sodium chloride; NT, neurotoxin; PBS, phosphate buffered saline; UC, ultracentrifugation.

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(Dressler et al., 2005; Schiavo et al., 2000). Paradoxically, the extraordinary potency, high specificity and long duration of effect of BoNT/A also make it an effective therapeutic muscle relaxant for clinical treatment of a variety of neuromuscular disorders and in cosmetic applications (Bhidayasiri and Truong, 2005; Carruthers and Carruthers, 2001; Montecucco and Molgo, 2005).

Botulinum neurotoxin is produced by *Clostridium botulinum* as a protein complex composed of the 150 kDa core neurotoxin (NT), various hemagglutinins (HAs) and a protein known as the non-toxin non-hemagglutinin (NTNH) (Inoue et al., 1996). Three different complex species with sizes of 19S (900 kDa), 16S (500 kDa) and 12S (300 kDa) may be isolated from *C. botulinum* type A cultures (Sugii and Sakaguchi, 1975).

The BoNT/A complex(es) dissociate at neutral to basic pH values (Wagman and Bateman, 1953) resulting in the free 150 kDa neurotoxin and high molecular weight hemagglutinin components (Boroff et al., 1966; DasGupta and Boroff, 1968; DasGupta et al., 1966). However, an in depth knowledge is lacking on the factors and conditions affecting dissociation, i.e. the pH and time dependence of the neurotoxin release in the infection process of hosts by *C. botulinum*. Furthermore, analytical studies of the biophysical status of neurotoxin in highly diluted and formulated BoNT/A preparations which are subjected to such conditions during their pharmaceutical production and administration to patients are missing despite widely perceived beneficial roles of 900 kDa complexes in the therapy with certain BoNT/A products.

In order to gain a more detailed understanding of the processes leading to the release of neurotoxin from their complexes as a prerequisite for accessing host cells or therapeutically targeted cells, we studied the influence of pH changes, protein concentration and presence of salts on the dissociation of BoNT/A containing complexes. To this end, we analyzed concentrated complexes isolated from clostridial fermentation broths as well as commercial products containing therapeutic concentrations of toxin using chromatographic procedures and sedimentation velocity analysis. The results of the study aid in understanding the processes leading to release of neurotoxin during invasion of the GI tract of hosts and in evaluating the role of complexing proteins in pharmaceutical preparations of BoNT/A as well as in their therapeutic application.

2. Materials and methods

2.1. Sample material

Concentrated BoNT/A complexes (Hall strain) were isolated as described (Malizio et al., 2000) with the exception of using size exclusion chromatography instead of crystallization as a polishing step.

For sedimentation velocity analysis, the complexes were subjected to an additional gel filtration step to yield isolated 900 kDa complexes.

The 150 kDa neurotoxin was isolated as described (Malizio et al., 2000).

The commercial products Botox and Dysport were acquired from pharmacies; Xeomin was obtained from Merz Pharmaceuticals; all product names are registered trademarks of Allergan Inc., Ipsen Ltd. and Merz Pharmaceuticals GmbH, respectively. All materials were used prior to the labeled expiration date.

Marker proteins for the calibration of sucrose gradients were purchased from GE Healthcare, Appli Chem and Sigma Aldrich.

2.2. Antibodies

Coating antibody:	Botulinum Antitoxin Type A (NIBSC, Potters Bar, United Kingdom)
Detection antibody NT:	Rabbit anti-BoNT/A serum (Merz Pharmaceuticals, Frankfurt Germany),
Detection antibody HA33:	Rabbit anti-HA33 (Acris Antibodies, Herford, Germany)
Secondary antibody:	HRP conjugated donkey anti-rabbit IgG (Perbio Science, Bonn, Germany)

2.3. Anion exchange chromatography analysis of intact and dissociated BoNT/A complexes

Samples were dissolved in AIEX Starting Buffer (10 mM Tris/HCl, 15 mM Bis-Tris/HCl, 0.1% Tween20 pH 6.0) and loaded onto a MonoQ 5/50 GL column (GE Healthcare). The column was washed with two column volumes of AIEX Starting Buffer; then the pH was adjusted by applying the appropriate basic buffer (10 mM Tris/HCl, 15 mM Bis-Tris/HCl, 0.1% Tween20; pH as required between 6.0 and 7.8) for the desired period of time. The resulting products were eluted from the column by applying a linear salt gradient in the respective basic buffer from 0 to 400 mM NaCl while collecting 250 µl fractions. Detection was performed by measuring UV absorbance at 280 nm and analyzing the peak fractions by SDS-PAGE in case of concentrated samples. Where small amounts of BoNT/A were used and/or protein excipients were present, the fractions were analyzed by ELISA.

2.4. Size analysis of protein complexes by sedimentation velocity analysis

Samples were diluted or reconstituted in the following buffers or solutions: PBS pH 7.4 (20 mM sodium phosphate/0.9% NaCl pH 7.4) or sterile isotonic saline (0.9% NaCl) or 50 mM sodium citrate pH 5.5. The dissolved samples were layered upon a sucrose gradient (10–40% sucrose in the appropriate buffer or solution) and centrifuged for 15 h at 5 °C and 300,000 × *g* in an SW60Ti swing-out rotor (Beckman Coulter). The resulting gradients were fractionated from the bottom overlaying paraffin oil and collecting 120 µl fractions. The fractions were analyzed by ELISA or BCA protein assay.

2.4.1. Calibration of the sucrose gradient

The sucrose gradient was calibrated using the following standard proteins: RNase, ovalbumin, conalbumin NT, aldolase, glucose oxidase, catalase, galactosidase, urease, alcohol oxidase, and thyroglobulin. Each of these proteins was dissolved in 0.9% NaCl to a final concentration of 5 mg/ml.

The standard proteins were centrifuged in separate gradients as described above. The fractions were analyzed by BCA assay and the resulting peak maximum was plotted against the respective molecular weight.

In order to confirm the separation quality during the study, at least one gradient containing appropriate marker proteins (e.g. NT101 and thyroglobulin) was included in every individual ultracentrifugation run.

2.5. Detection of NT and HA33 by ELISA

Sandwich ELISA systems were used for the detection of 150 kDa neurotoxin or HA33. The multiwell plates were coated with an equine polyclonal anti-BoNT/A serum (directed against all BoNT/A complex components, i.e. NT, hemagglutinins and NTNH). After a washing step with TBS-T (20 mM Tris/HCl/0.9% NaCl/0.1% Tween20 pH 7.6), the wells were blocked with 2% bovine serum albumin in 20 mM Tris/HCl, 0.9% NaCl pH 7.4. Subsequently, the wells were washed and incubated with the samples (diluted at least 1:5 in blocking solution to equilibrate pH and to reduce eventually interfering sucrose effects). Following an additional washing step, the wells were incubated with a polyclonal anti-NT serum (directed solely against the 150 kDa NT) or a polyclonal anti-HA33 antibody, both derived from rabbit. The wells were washed and a HRP-labeled goat anti-rabbit antibody was added. After incubation and washing, bound HRP was finally detected using the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB).

Each ELISA run was confirmed by applying an NT101 or BoNT/A 900 kDa complex standard on every individual plate.

3. Results

3.1. pH and time dependence of NT release

Previous studies on the influence of pH on the stability of BoNT/A complexes established a relative stability at pH 6 and lower and a dissociation above pH 7.5. Further information on the effects of pH between 6 and 7.5 is scarce, especially with regard to the kinetics of pH-induced complex dissociation. In order to study dissociation, concentrated solutions of BoNT/A complexes (10 μg) were applied to an anion exchange column at pH 6.0.The pH was then adjusted by incubating the column for a defined time period with a buffer of the desired pH value. The components of the dissociated neurotoxin complexes were then successively eluted from the column by a salt gradient at the same pH. The column fractions were then further analyzed by SDS-PAGE (Fig. 1A and B).

The BoNT/A complexes which elute as two partially resolved peaks (Fig. 1A, peaks 2a and 2b) at pH 6.0 dissociate further into an earlier eluting fraction (Fig. 1A, peak 1) and two later eluting peaks (Fig. 1A, peaks 3a and 3b) when the pH is increased toward physiological values (Fig. 1A, middle and lower panel). In order to correlate the eluted fractions with the composition of previously described neurotoxin containing complexes, we subjected equal volumes of each fraction to SDS-PAGE analysis under reducing conditions (Fig. 1B): In lane 2, the complexes prior to application to an anion exchange column serve as a control for total protein composition. All protein bands correspond to previously described BoNT/A complexes: NTNH, 106 kDa and 13 kDa proteolytic products of NTNH, neurotoxin protein bands (single, heavy and light chain), as well as hemagglutinins (HA52, HA33, HA19, HA17). Peak 2a (Fig. 1b, lane 3) contains a similar set and relative amounts of the control sample (Fig. 1b, lane 2) proteins except for minute amounts of 106 kDa and no 13 kDa protein: this pattern is indicative of 19S (LL or 900 kDa) and 16S (L or 500 kDa) complexes as established in (Sugii and Sakaguchi, 1975). The 19S (LL) complexes have been described as dimers composed of two 16S (L) complexes. Under the conditions applied in the anion exchange chromatography, both complexes have the same net charge and therefore are expected to co-elute at the same ionic strength. In contrast, peak 2b is enriched for the 106 kDa and also some detectable 13 kDa bands (Fig. 1B,

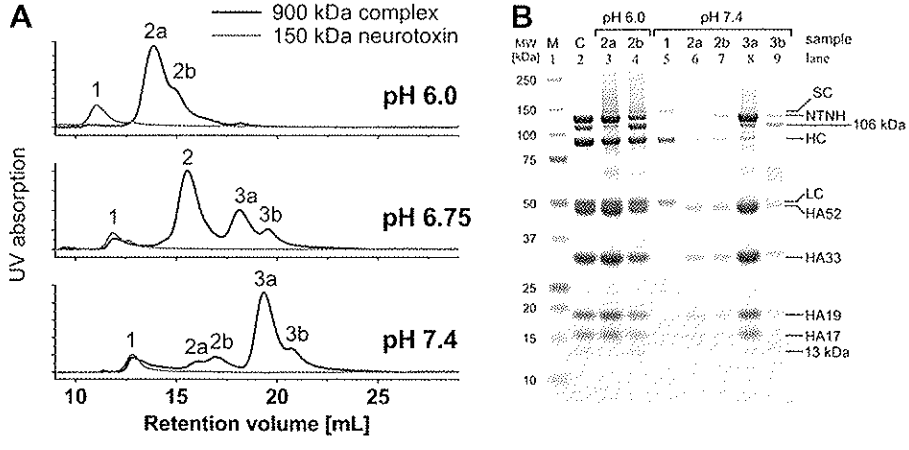

Fig. 1. (A): Anion exchange chromatographic separation of the 150 kDa neurotoxin and 900 kDa neurotoxin complex at pH 6.0, 6.75 and 7.4. Peaks are labeled as follows: 1 = neurotoxin (150 kDa); 2 = toxin containing complexes (2a = 900 kDa; 2b = 300 kDa); 3a and 3b = neurotoxin-free complex or complexing proteins. (B): Analysis of column fractions of 900 kDa complex (black chromatograms in panel A) by SDS-PAGE. Lane 1 (M) = molecular weight marker; lane 2 (C) = non-formulated 900 kDa BoNT/A complex; lane 3 (2a) and lane 4 (2b) = peaks 2a and 2b of pH 6.0 chromatogram in Fig. 1A; lane 5 to lane 9 = peaks 1, 2a, 2b, 3a and 3b of pH 7.4 chromatogram in Fig. 1A.

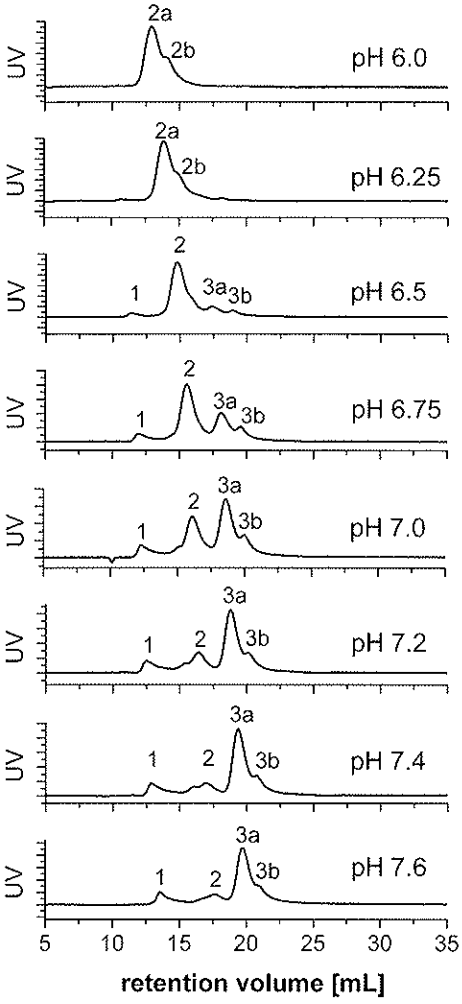

Fig. 2. Increasing dissociation of the 900 kDa neurotoxin complex at different pH values between 6.0 and 7.6. The 900 kDa neurotoxin complex was preincubated for 2 min at various pH values before the resulting molecular entities were separated by anion exchange chromatography. $n = 3$; representative experiment shown. The peaks were identified using SDS-PAGE (representative SDS-PAGE shown in Fig. 1B).

lane 4) which represent marker proteins for 12S (M) complexes as previously described in (Fujita et al., 1995).

The pharmacologically active 150 kDa neurotoxin is efficiently released from the complex at physiological pH values as can be seen by protein composition of the additional peak 1 (Fig. 1B, lane 5 showing neurotoxin protein bands: single, heavy and light chain). In contrast, late eluting fractions 3a and 3b (lanes 8 and 9) contain very low amounts of neurotoxin in the presence of a vast excess of the other complexing proteins. These fractions, therefore, resemble 19S (LL) and 16S (L) complexes and 12S (M) complexes (see also peaks 2a and 2b) after efficient release of neurotoxin. The minute quantities of neurotoxin co-eluting with these fractions (Fig. 1B, lane 8) either suggest rare re-adsorption and delayed release events of LL complexes or might represent minute subfractions of LL dimers with only a single molecule of neurotoxin released.

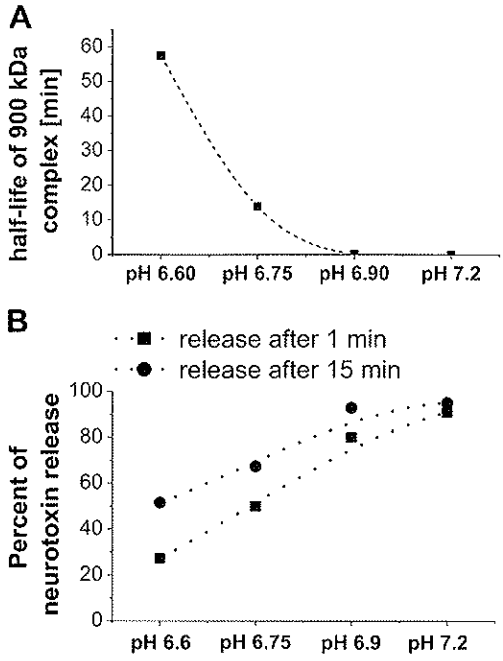

Fig. 3. Half-lives of the 900 kDa neurotoxin complex at different pH values between 6.6 and 7.2. The complexes disintegrate very rapidly in the presence of pH 6.9 and above with half-lives of less than 1 min. Disintegration of the complexes was determined by the experiments shown in Fig. 2.

We then closely examined the dissociation at different pH values between pH 6 and pH 7.6 using the same anion exchange procedure (Fig. 2): the resulting chromatograms show a gradual increase of dissociation with increasing pH. It can be noted that there are certain threshold pH values that can be recognized by the occurrence of maximal dissociation. Thus, dissociation of M complexes appears to be maximal at pH 6.75 whereas LL and L-complexes reach maximal dissociation at pH 7.4.

After having established an anion exchange chromatography protocol for reproducible determination of L and M complexes with defined pH threshold values for maximal dissociation, we studied the kinetics of complex dissociation. Complexes bound to the anion exchange column under acidic conditions were subjected to buffers with increasing pH for 1 min and 15 min, respectively, before elution. Surprisingly, the complexes disintegrate very rapidly at pH 6.9 and above with half-lives of less than 1 min (Fig. 3). At pH 6.75 and pH 6.6, the half-lives of the BoNT/A complexes are significantly longer (half-lives of 14 and 58 min, respectively) which correlate with submaximal dissociation observed under these pH conditions after 2 min (Fig. 2). These data strongly indicate that BoNT/A complexes are designed to rapidly release the neurotoxin once they leave acidic compartments and a neutral pH threshold is reached, e.g., in the intestinal environment.

3.2. Sedimentation velocity analysis of non-formulated BoNT/A complex

As shown in Figs. 1 and 2, anion exchange chromatography is a powerful method to study the rapid kinetics of

neurotoxin complex disintegration. At the same time, this method does not allow the determination of the relative molecular sizes of the eluted complexes, an important aspect for the verification of the conclusions drawn from the results. Furthermore, different changes in environment might lead to different dissociation patterns which could not be detected by separation methods based on net charge of the complexes: for example, 900 kDa and 500 kDa complexes are supposed to have the same overall net charge and thereby elute at the same ionic strength from the column. For determination of BoNT/A complex sizes, two methods have been successfully used in the past: gel filtration chromatography and sedimentation velocity analysis (Ohishi and Sakaguchi, 1975; Oishi and Sakaguchi, 1974; Sugii and Sakaguchi, 1975). We chose sedimentation velocity analysis because this ultracentrifugation technique does not rely on solid matrices which may influence the stability of the complex or create re-adsorption artifacts. Very importantly, it offers the opportunity to recover and analyze complexes in the extremely low therapeutically relevant concentrations (i.e. ng/ml) and in the presence of two to five thousand-fold excess of excipient proteins. Finally, there are no additional protectants needed to suppress adsorption which would also interfere with dissociation processes and/or recovery as well as analysis of these minute concentrations of BoNT/A complexes. To further reduce any complexities resulting from the presence of M- and L-complexes, we isolated 900 kDa (LL) by an additional preparative gel filtration step and used this as the starting material in the sedimentations velocity studies.

3.3. Calibration of sucrose gradients using standard proteins

In order to be able to ascribe relative molecular sizes to the particles separated in the gradients, proteins with defined molecular weights were subjected to ultracentrifugation using sucrose gradients prepared according to the same protocol as used for analysis of BoNT/A complexes. As shown in Fig. 4A, proteins from relative molecular weights of 14 kDa up to 1338 kDa were readily separated and detected in the recovered fractions of these sucrose gradients. We then subjected highly concentrated, purified

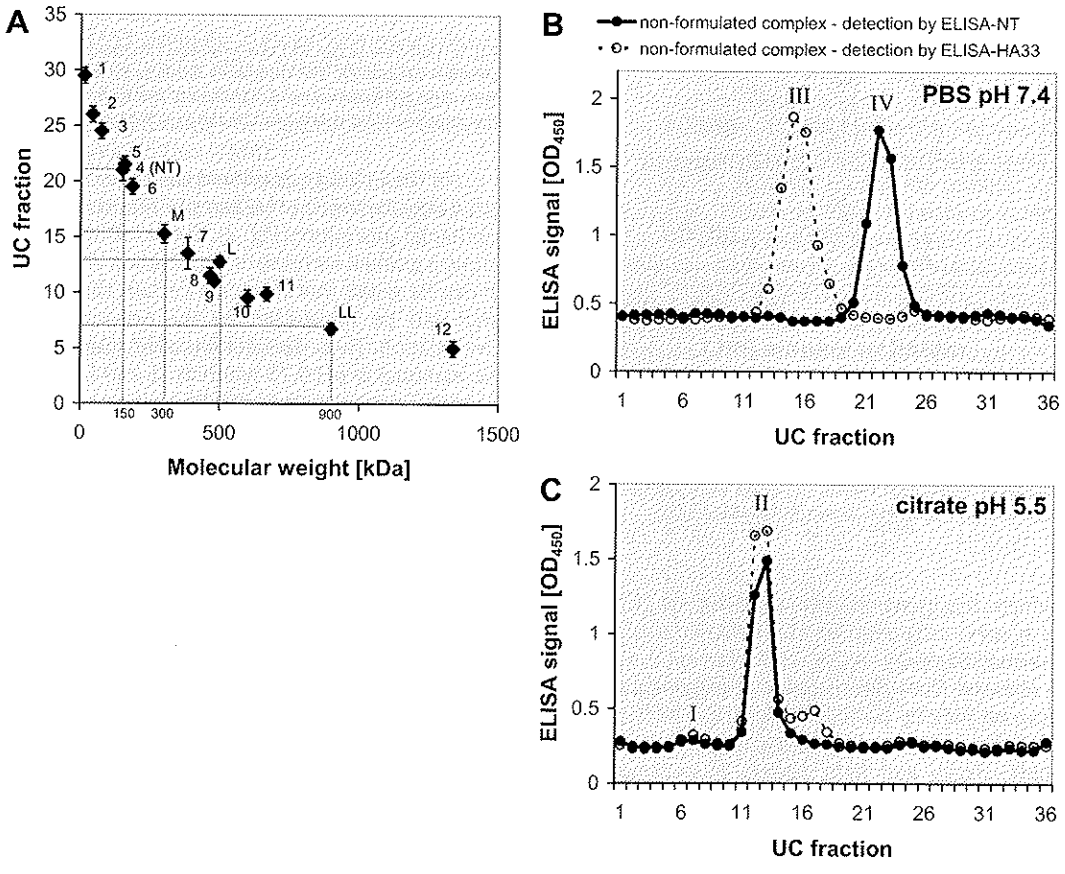

Fig. 4. (A): Calibration of the ultracentrifugation sucrose gradient. The following proteins were used: 1: RNase (13.7 kDa); 2: Ovalbumin (43 kDa); 3: Conalbumin (75 kDa); 4: NT (150 kDa); 5: Aldolase (158 kDa); 6: Glucose oxidase (186 kDa); 7: Catalase (385 kDa); 8: Galactosidase (465 kDa); 9: Urease (480 kDa); 10: Alcohol oxidase (600 kDa); 11: Thyroglobulin dimer (669 kDa); 12: Thyroglobulin tetramer (1338 kDa). Mean fractions of the BoNT/A complexes: M: BoNT/A M-Complex (300 kDa, 12S); L: BoNT/A L-Complex (500 kDa, 16S); LL: BoNT/A LL-Complex (900 kDa, 19S). (B and C): Analysis of non-formulated BoNT/A complex size at 40 ng/ml. by sedimentation velocity analysis. Peaks are labeled as follows: I: neurotoxin containing 19S (~900 kDa) complex, II: neurotoxin containing 16S (~500 kDa) complex, III: neurotoxin-free complex, IV: free 150 kDa neurotoxin. (B): Sedimentation profile after dilution with PBS pH 7.4; fraction analysis by ELISA-NT and ELISA-HA33, respectively. (C): Sedimentation profile after dilution with citrate pH 5.5; fraction analysis by ELISA-NT and ELISA-HA33, respectively. The HA33 positive shoulder in fraction 17 has not been further identified.

neurotoxin as well as BoNT/A complexes to the ultracentrifugation procedures and detected the proteins in the expected gradient fractions using UV absorption measurements (Fig. 4A). Thus, purified neurotoxin distributed to fractions at the upper end of the gradient containing the lower sucrose concentrations, mean fraction 21 (for comparison: glucose oxidase MW 158 kDa, mean fraction 21.5). The 900 kDa (LL) complexes localized to the lower parts of the gradients containing the higher sucrose concentrations, mean fraction 7 (for comparison thyroglobulin dimer, MW 669 kDa, mean fraction 10; thyroglubulin tetramer, MW 1338, mean fraction 5).

After verification of the correct separation of proteins and protein complexes to their expected positions in the sucrose gradients using high (UV detectable) concentrations, we applied therapeutic concentrations of 900 kDa complexes (4.0 ng in 100 µl buffer) and employed ELISA for monitoring fractions for neurotoxin content as well as for the complexing protein HA33. Sedimentation velocity was carried out on two separate sucrose gradients at pH values 5.5 and 7.4, respectively: For dissociation analysis, aliquots of fractions were equilibrated and subjected to ELISA for neurotoxin as well as HA33 proteins.

At neutral pH conditions, the neurotoxin localized to the fractions representing free neurotoxin (peak III, Fig. 4B, solid lines). The HA33 protein appeared in fractions of higher buoyant density indicating that is still associated with complexes of approximately 400 kDa (peak IV, Fig. 4B, interrupted lines). When diluted and separated at acidic pH values (i.e. using citrate buffer pH 5.5), more than 90% of the detected neurotoxin were not found in the fractions corresponding to 900 kDa complexes but it appeared to be associated with complexes corresponding to a molecular weight of approx. 500 kDa (peak II, Fig. 4C, solid lines). The same distribution was found for HA33 (peak II, Fig. 4C, interrupted lines) which indicates that both proteins are associated in these complexes. The relative molecular weight of 500 kDa was highly indicative of the previously described L-complexes and the co-localization of the two proteins further hints at a scenario according to which the 900 kDa complexes break apart at low concentrations. An alternative but rather unlikely explanation would be that the higher sucrose concentrations in the ELISA samples might have interfered with detection of the two proteins in the high density fractions of the gradients despite dilution of the ELISA samples before binding to the antibodies on the microtiter plates. To rule out such interference and to find further evidence for a reproducible dissociation of 900 kDa complexes at low concentrations, we subjected decreasing concentrations of 900 kDa complexes to ultracentrifugation under acidic conditions and analyzed the distribution of neurotoxin as well as HA33. At a 900 kDa complex concentration of 100 µg/ml, all of the neurotoxin is detected in the fractions containing the 900 kDa in the previous calibration study using UV absorption (compare peak I in Fig. 5A lowest panel with location of mean fraction for 900 kDa in Fig. 4A). This result demonstrates that the absence of neurotoxin signals at the expected 900 kDa fractions could not have been a cause of interference because the summarized absolute absorption values recorded for each gradient were comparable and proportional to the concentration of 900 kDa concentration employed regardless of association with 900 kDa or 500 kDa complexes.

Importantly, by decreasing the concentration of 900 kDa complexes, increasing concentrations of neurotoxin were

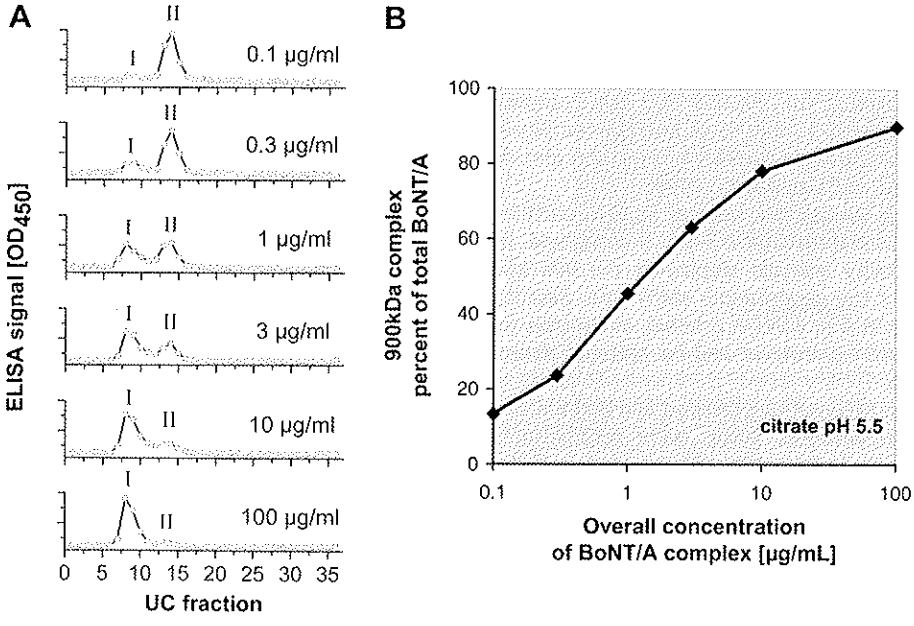

Fig. 5. Concentration-dependent dissociation of non-formulated 900 kDa BoNT/A complex. The non-formulated BoNT/A complexes were diluted to various concentrations from 100 µg/ml to 0.1 µg/ml and subjected to sedimentation velocity analysis. Peaks are labeled as follows: I: neurotoxin containing 19S (~900 kDa) complex, II: neurotoxin containing 16S (~500 kDa) complex. (A): Representative sedimentation profiles after dilution with citrate pH 5.5; fraction analysis by ELISA-NT. (B): Dependency of BoNT/A complex size from overall concentration of BoNT/A complex at pH 5.5: relative area of 900 kDa peak ($n = 3$).

detected in the fractions containing 500 kDa complexes (peak II, Fig. 5A from lowest to upper panel) which confirms the single observation made earlier (Fig. 4C) and which confirms the hypothesis that 900 kDa complexes efficiently disintegrate into 500 kDa at lower concentrations. In all gradients, the HA33 signals co-localized to the same fractions as the neurotoxin (data not shown) as it was the case in the initial experiments (Fig. 4C) which further substantiated the conclusion that the 900 kDa complexes break apart into 500 kDa complexes when diluted under acidic conditions (Fig. 5A and B) as opposed to further release of neurotoxin during dilution under neutral conditions (Fig. 4B).

Taken together, our dissociation studies aid in understanding underlying principles controlling the release of neurotoxin from their complexes. The composition and architecture of 900 kDa complexes, therefore, seems to have evolved, on the one hand, to protect the neurotoxin in the acidic, proteolytic compartments, whereas, on the other hand, upon only brief exposure to neutral pH conditions the complexes rapidly and efficiently can release the neurotoxin leaving shells composed of non-toxic proteins behind. In addition, the interaction of the two L-complexes constituting the 900 kDa complexes seems to be quite weak enabling formation of 500 kDa complexes by mere dilution even in acidic compartments.

3.4. Sedimentation velocity analysis of commercial products

After having established a system for reliable analysis of particle sizes in dilute solutions of BoNT/A, we asked the question whether the therapeutic BoNT/A products containing complexing proteins provide intact BoNT/A complexes in the reconstituted products for injection into target tissues. The results of these studies are important to evaluate the benefits and risks of further use of complexing proteins for the therapy with botulinum neurotoxin. Currently, several different pharmaceutical products are available which contain BoNT/A. For example, Botox is advertised as containing the purified 900 kDa complex, Dysport as consisting of a mixture of BoNT/A complexes and Xeomin as pure 150 kDa neurotoxin free of complexing proteins (Carruthers and Carruthers, 2008).

In the following studies, the sizes of BoNT/A particles within the commercial products were investigated by sedimentation velocity analysis employing the 900 kDa complexes used for the experiments above and, Xeomin, a complexing protein-free BoNT/A product, as controls for intact and dissociated complexes and for released neurotoxin, respectively.

The freeze-dried preparations, Dysport and Xeomin, and the spray-dried preparation, Botox, were reconstituted with the designated buffers PBS pH 7.4, non-buffered isotonic saline pH approx. 5.8, and 50 mM sodium citrate pH 5.5. The final neurotoxin concentrations were 800 U/mL for Botox, Dysport and Xeomin according to the labeled nominal biological activity. In parallel, concentrated purified 900 kDa complexes, as used in the experiments above, were diluted using the same diluents to a final concentration of 40 ng/mL. In each case, 100 μl of these samples were then layered on a separate sucrose gradient. Centrifugation, fractionation and ELISA analyses were performed as described above. Each BoNT/A product, Botox, Dysport, Xeomin and the diluted 900 kDa complex control, was analyzed at least three times under all three conditions. Fig. 6 shows the ELISA results of one representative batch of each drug product. The calculated mean quantities of complexes and released neurotoxin in the different products under the described conditions are summarized in Table 1. Thus, at pH 7.4, the neurotoxin is quantitatively detected in the fractions corresponding to 150 kDa (Fig. 6A) in all preparations. Comparable to the previous results for pH-induced dissociation of 900 kDa proteins (Fig. 4B and C), the HA33 in Botox, Dysport and the control 900 kDa complexes again, predominantly sedimented at much higher molecular weights of approx. 400 kDa (Fig. 6B)

If reconstituted and separated at even slightly acidic pH values (0.9% NaCl or citrate pH 5.5), at least 90% of the control 900 kDa complexes appeared in the fractions corresponding to a molecular weight of approx. 500 kDa. Only traces of neurotoxin and HA33 could be detected in the fractions corresponding to 900 kDa (Fig. 6C–F). Moreover, neither Botox nor Dysport show significant amounts of neurotoxin associated with 900 kDa molecular weight entities (Fig. 6C and E). In contrast, in a distribution pattern similar to that observed under pH 7.4 conditions, the neurotoxins of Botox and Dysport mainly sedimented at 150 kDa congruent with the distribution pattern of the purified neurotoxin in Xeomin (Fig. 6C and E). Again, the HA33 component could be observed predominantly at approx. 400 kDa (Fig. 6D and F). Only 10–20% of the neurotoxin in reconstituted Botox preparations could be detected in fractions corresponding to 500 kDa particles (Fig. 6C and E).

Thus, the dissociation of BoNT/A neurotoxin complex at physiological pH values could also be proved in commercially available products. No significant difference could be observed concerning the neurotoxin release at pH 7.4 between the different investigated BoNT/A complex products, i.e. Botox, Dysport, and non-formulated complex.

Surprisingly, the BoNT/A complex particle size analysis revealed that none of the tested products contained 900 kDa BoNT/A complexes even under conditions known to stabilize the complex. In fact, even if reconstituted with slightly acidic solvents (0.9% saline or citrate pH 5.5), more than 85% of the neurotoxin of Botox and all detectable neurotoxin of Dysport was present in free form (Fig. 6C, E, Fig. 7, Table 1). Therefore it can be concluded that the analyzed pharmaceutical preparations do not contain the neurotoxins in 900 kDa complexes. Only a minute fraction of neurotoxin in Botox is present in 500 kDa complexes which will most probably immediately release the remaining neurotoxin when injected into the interstitial space of target tissues where neutral pH conditions prevail.

4. Discussion

For the efficient infection of mammalian hosts by Clostridia, the active neurotoxin protein is encapsulated by a protective shell composed of several proteins. This toxin complex appears to act like a shuttle delivering active 150 kDa neurotoxin to the intestinal mucosa and,

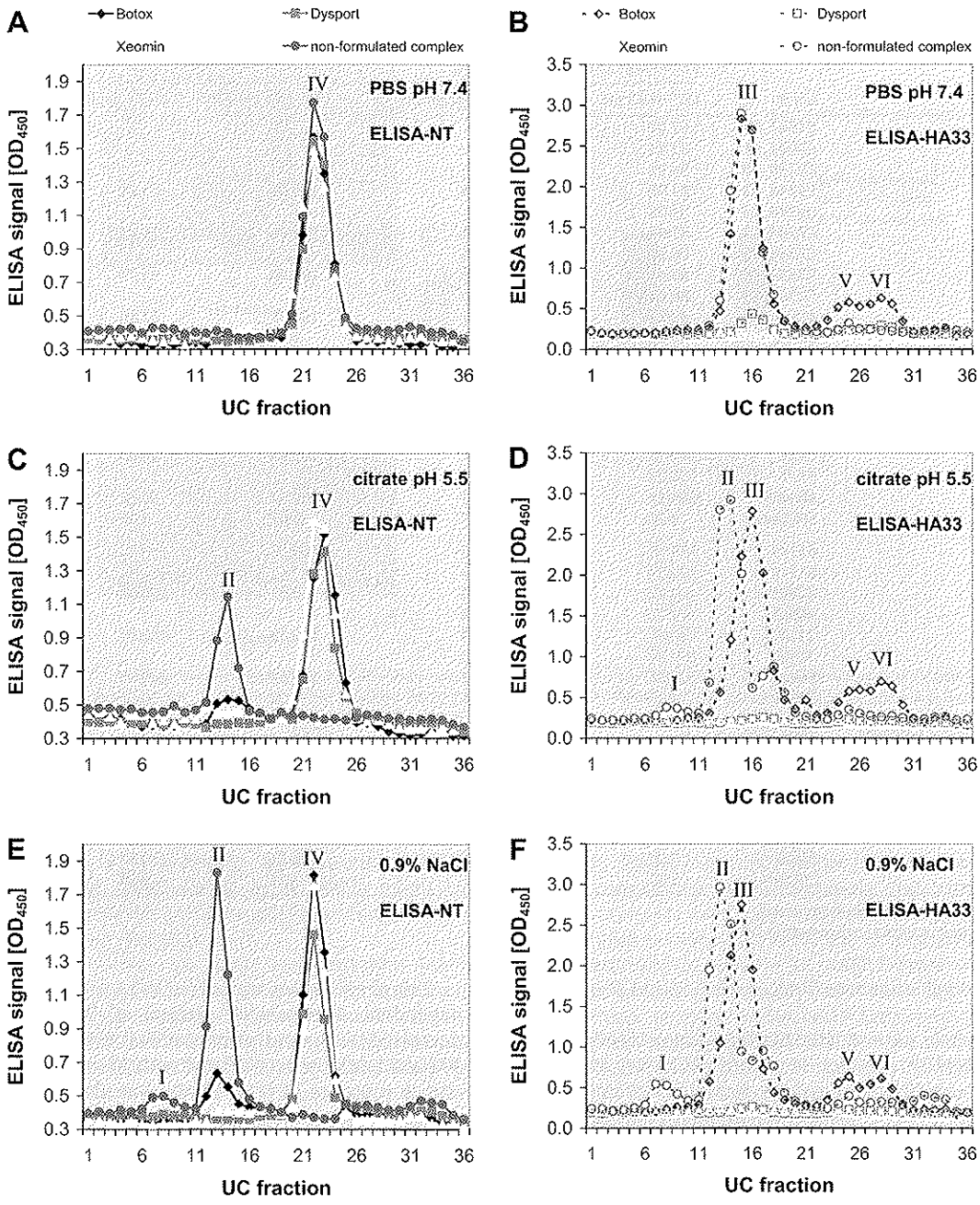

Fig. 6. Analysis of BoNT/A products by sedimentation velocity analysis. Peaks are labeled as follows: I: neurotoxin containing 19S (~900 kDa) complex, II: neurotoxin containing 16S (~500 kDa) complex, III: neurotoxin-free complex, IV: free, 150 kDa neurotoxin, V and VI: unknown HA33-containing peaks <100 kDa. 80 U Botox, 80 U Dysport 80 U Xeomin or 4 ng non-formulated complex (equimolar to Botox) were loaded onto the sucrose gradient ($n = 3$ or higher; representative sedimentation profiles shown). (A) and (B): Sedimentation profile after reconstitution at pH 7.4 with PBS; fraction analysis by ELISA-NT and ELISA-HA33. (C) and (D): Sedimentation profile after reconstitution at pH 5.5 with citrate; fraction analysis by ELISA-NT and ELISA-HA33. (E) and (F): Sedimentation profile after reconstitution with sterile saline; fraction analysis by ELISA-NT and ELISA-HA33.

subsequently, blood from where it spreads to target cells to elicit the typical paralytic symptoms and finally kills the invaded host.

The occupancy of the shuttle complex with neurotoxin is known to be pH controlled. Thus, at low pH values, the neurotoxin associates with the complex and is thereby protected against the harmful environmental conditions occurring in the stomach (Chen et al., 1998; Fu et al., 1998). In contrast, at neutral pH values, the neurotoxin is released from the shuttle. Although this mechanism was described decades ago (DasGupta and Boroff, 1968; DasGupta et al., 1966; Sugii and Sakaguchi, 1975; Wagman and Bateman, 1953), little is known about the kinetics of neurotoxin release and the stability of the complexes toward other

Table 1
Summary of BoNT/A market product analysis.

		Percentage of non-complexed NT				
Product	Lot #	UC[a]–NaCl pH ~ 5.8	UC[a]–citrate pH 5.5	UC[a]–PBS pH 7.4	AIEX[b] pH 6.0	AIEX[b] pH 7.4
Botox®	C1641_C1	97.4%				
	C2001_C1	93.8%				
	C2230_C3	94.9%				
	C2344_C3	87.9%	86.6%	100.0%	67.2%	100.0%
	C2344_C3	89.0%				
	C2364_C3	85.6%	85.5%	100.0%	70.5%	100.0%
	C2364_C3	86.9%	86.6%			
	C2384_C3	85.4%	79.2%	100.0%	65.7%	100.0%
	C2384_C3	86.8%	78.4%			
	Mean	*89.7%*	*83.2%*	*100.0%*	*67.8%*	*100.0%*
	Std.Dev.	*4.5%*	*4.1%*	*0.0%*	*2.5%*	*0.0%*
Dysport®	656L	100.0%	100.0%	100.0%	50.8%	100.0%
	656L	100.0%	100.0%			
	677N	100.0%	100.0%	100.0%	58.2%	100.0%
	677N	100.0%	100.0%			
	687E	100.0%	100.0%	100.0%	58.5%	100.0%
	687E	100.0%	100.0%			
	Mean	*100.0%*	*100.0%*	*100.0%*	*55.8%*	*100.0%*
	Std.Dev.	*0.0%*	*0.0%*	*0.0%*	*4.4%*	*0.0%*
XEOMIN®	61111	100.0%	100.0%	100.0%	100.0%	100.0%
	61111	100.0%	100.0%			
	40801	100.0%	100.0%	100.0%	100.0%	100.0%
	40801	100.0%	100.0%			
	21103	100.0%	100.0%	100.0%	100.0%	100.0%
	21103	100.0%	100.0%			
	Mean	*100.0%*	*100.0%*	*100.0%*	*100.0%*	*100.0%*
	Std.Dev.	*0.0%*	*0.0%*	*0.0%*	*0.0%*	*0.0%*
Non-formulated complex	20071014	0%	0%	100%	0%	87%
	20071014	0%	0%			
	20071014	0%	0%	95%	0%	87%
	20071014	0%	0%			
	20071014	0%	0%	93%	0%	78%
	20071014	0%	0%			
	Mean	*0.0%*	*0.0%*	*96.2%*	*0.0%*	*84.0%*
	Std.Dev.	*0.0%*	*0.0%*	*3.5%*	*0.0%*	*5.3%*

[a] UC = ultracentrifugation experiment.
[b] AIEX = anion exchange chromatography.

environmental influences, such as dilution and presence of salts.

In this study, we evaluated the dissociation kinetics of the 900 kDa BoNT/A complex at various pH values and environmental conditions to identify factors which destabilize the BoNT/A complexes in addition to the pH environment.

The data presented on concentrated 900 kDa complexes confirm the previously demonstrated and marked pH dependence, however, in our study it becomes evident that the release occurs rapidly once a certain pH threshold is encountered. At neutral pH values, more than 80% of the neurotoxin complexes dissociate within less than a minute (Fig. 2). This time frame is extremely short when compared to the onset of botulism symptoms following neurotoxin ingestion. Interestingly, the remaining complexing proteins are still aggregated in particles of 400 kDa (Fig. 1A) suggesting that BoNT/A complexes rather act as a stable protection and release device and not only resemble scaffolds which collapse once the neurotoxins are released. We then asked whether other environmental factors can trigger dissociation events and learned that 900 kDa complexes can disassemble under defined conditions without releasing the neurotoxin, i.e. after dilution in acidic solutions (Fig. 5A).

This phenomenon turned out to be strictly concentration dependent. The relative amount of 900 kDa complex decreased continuously from >85% at 100 μg/ml to less than 10% at 0.1 μg/ml even if diluted with 50 mM citrate at pH 5.5 and in presence of HSA. Finally, we identified even mild changes in salt concentration which lead to release of the neurotoxin from 500 kDa complexes. This concentration and salt-dependence of the shuttle could be another mechanism by which an efficient release of the neurotoxin is facilitated after passage of the stomach in order to enable toxin uptake by the infected host, i.e. after passage of complexes through the intestinal mucosa into the interstitial fluid.

As several conditions affecting dissociation of BoNT/A complexes and release of neurotoxin were identified by testing a few external stimuli possibly relevant during the host invasion process, there might be other factors and conditions which trigger these events as well. In particular, bile salts and interactions of complexes with the glycocalyx through their hemagglutinins pose potential factors for the regulation of complex dissociation and release of neurotoxin.

However, the various stimuli which lead to release of neurotoxin, at the same time, increase its accessibility to digestive enzymes and thereby degradation in the less

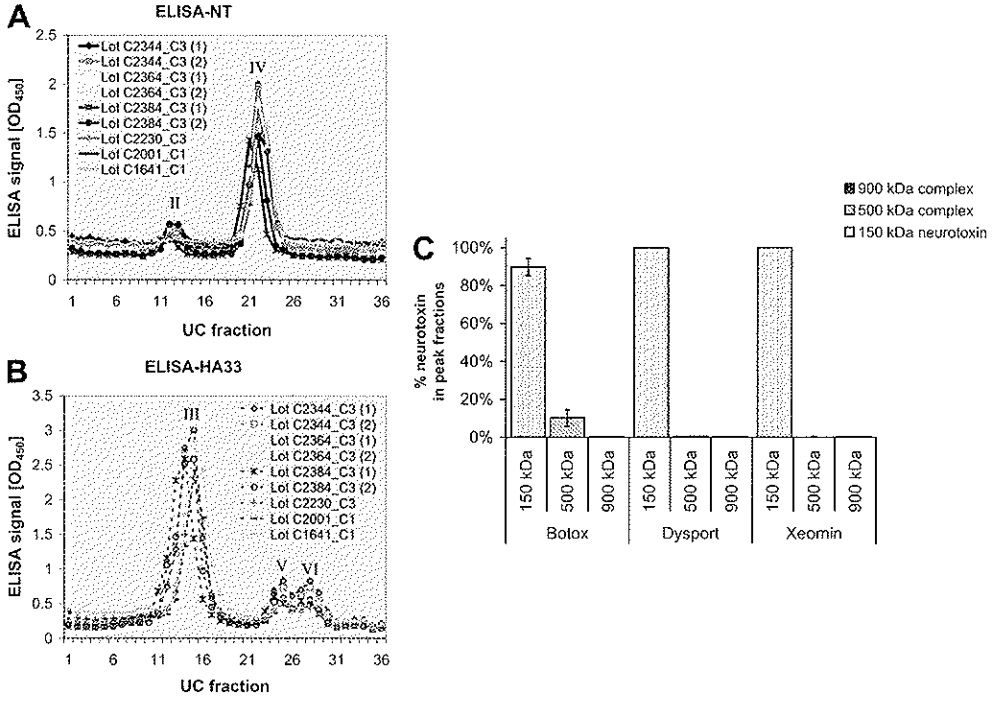

Fig. 7. Analysis of BoNT/A complex size of commercially available Botox lots ($n = 9$) by sedimentation velocity analysis. Peaks are labeled as follows: II: neurotoxin containing 16S (~500 kDa) complex, III: neurotoxin-free complex, IV: free, 150 kDa neurotoxin. Sedimentation profile of non-formulated BoNT/A complex after dilution with either 0.9% NaCl/0.5% HSA or with 0.94% sucrose/0.2% HSA; fraction analysis by ELISA-NT.

acidic but proteolytic compartments of the intestinal tract. The approx. 100.000 fold higher doses observed for the oral toxicity of BoNT/A complexes – despite their stability at low pH – compared to parenterally administered BoNT/A complexes (Ohishi, 1984; Ohishi et al., 1977) might be explained by efficient release and concomitant degradation of neurotoxin in the intestinal tract.

Furthermore, neither the exact transportation route across the intestinal walls nor the nature of the transported neurotoxin, i.e. free or complexed, are understood in detail. Additional studies will be needed to identify which factors are the most relevant for efficient invasion of the hosts by neurotoxin and which might be promising targets for the prevention of *C. botulinum* poisoning.

Finally, our data on the dissociation of BoNT/A complexes in therapeutic products provide insights into some quality aspects of these highly potent therapeutic products shortly prior to their application in patients. This is the first study which determines the status of neurotoxin complexes in reconstituted BoNT/A products. An earlier report focused on the concentrated drug substance of Botox which, in analogy to the concentrated 900 kDa complexes used in our studies, seems to consist of bona fide 900 kDa complexes (Lietzow et al., 2008 and Fig. 4A).

To explain the results obtained for the reconstituted drug products, it is important to emphasize that based upon the dilution studies the existence of a 900 kDa complex at pharmacologically relevant concentrations would appear to be unlikely (Fig. 5A and B). Additionally, factors occurring during production and the use of BoNT/A products can trigger the release of neurotoxin as well (Fig. 6E and F). These two factors: dilution and/or dissociation by addition of salt (Figs. 5 and 6) explain the total absence of 900 kDa complexes in reconstituted BoNT/A products Botox and Dysport. Surprisingly, none of the tested commercial products contain the pure 900 kDa BoNT/A complex even under conditions known to stabilize the complex (Inoue et al., 1996) and in contrast to recently published data (Chapman et al., 2007; Lietzow et al., 2008).

Since further production steps such as formulation, filling, freeze or spray drying and finally reconstitution for injection all represent additional changes in the environment of highly diluted BoNT/A complexes, the high degree of neurotoxin release in the products is now readily understandable. After reconstitution according to manufacturers' instructions with sterile saline (0.9% NaCl, pH ~5.8) and analysis by sedimentation velocity ultracentrifugation, even more free neurotoxin was determined for Botox (89.7% ± 4.5%) and Dysport (100%) (Table 1). The remaining neurotoxin of Botox (10.3% ± 4.5%) was found to be associated with complexes in the range of 500 kDa (Table 1).

The role and importance of the clostridial complexing proteins in clinical efficacy and safety is disputed. It has been claimed that the complexing proteins serve to enhance activity (Sharma and Singh, 2004), stabilize and protect the neurotoxin (Brin, 2009) or inhibit neurotoxin diffusion into adjacent tissues (Sharma et al., 2003). In light of these new observations on neurotoxin release from 900 kDa neurotoxin complexes and the data on reconstituted commercial BoNT/A products, past perceptions on the proposed beneficial effects of complexing proteins in a therapeutic setting can no longer be supported. On the

contrary, our data on complex dissociation in commercial BoNT/A products are in agreement with previously published studies demonstrating comparable diffusion characteristics of the neurotoxin in Botox, Dysport, both containing complexing proteins, and Xeomin, which is free from complexing proteins, after injection into muscles (Carli et al., 2009). It can be concluded that neurotoxin diffusion is not impeded by any association with complex species as the 900 kDa complexes are already dissociated when the highly diluted and dried BoNT/A formulations are reconstituted. Moreover, potentially harmful effects of the complexing proteins in the tissue should be studied.

In consequence, the necessity of including complexing proteins in medicinal formulations to improve therapeutic outcome as claimed for neurotoxin products containing complexing proteins must be questioned. Especially, since the therapeutic equipotency of new generation BoNT/A products free of complexing proteins such as Xeomin and first generation BoNT/A products still containing complexing proteins such as Botox has been demonstrated in clinical trials (Benecke et al., 2005; Carli et al., 2009; Jost et al., 2005; Roggenkamper et al., 2006; Wohlfarth et al., 2007, 2008).

Conflict of interest

None declared.

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