Toxicon 57 (2011) 93–99

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Comparison of effects of botulinum toxin subtype A1 and A2 using twitch tension assay and rat grip strength test

Yasushi Torii [a,*], Naotoshi Kiyota [a], Nakaba Sugimoto [b], Yuichiro Mori [a], Yoshitaka Goto [a], Tetsuhiro Harakawa [a], Shinji Nakahira [a], Ryuji Kaji [c], Shunji Kozaki [d], Akihiro Ginnaga [a]

[a] The Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN), 1-6-1 Okubo, Kumamoto-shi, Kumamoto 860-8568, Japan
[b] Department of Molecular Therapeutics, Medical School of Osaka University, 2-2 Yamadaoka, Suita-shi, Osaka 565-0871, Japan
[c] School of Medicine, University of Tokushima, Faculty of Medicine, 18-15 Kuramoto-cho, Tokushima-shi, Tokushima 770-8503, Japan
[d] Department of Veterinary Sciences, School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku-oraikita, Izumisano-shi, Osaka 598-8531, Japan

ARTICLE INFO

Article history:
Received 30 June 2010
Received in revised form 3 September 2010
Accepted 19 October 2010
Available online 26 October 2010

Keywords:
Botulinum toxin
Twitch tension
Grip strength

ABSTRACT

Botulinum toxin type A is used as a therapeutic agent for some spastic neurological disorders. Type A organisms have been classified into four subtypes (A1 to A4) based on the amino acid sequence variability of the produced neurotoxin. At present, commercially available preparations of the toxin belong to subtype A1. To date, no study has compared the characteristics of the biological activity of toxins from different subtypes. We compared the efficacy of A1 toxin (LL toxin or neurotoxin: NTX) with that of A2 toxin (NTX) employing the twitch tension assay using the mouse phrenic nerve hemidiaphragm and grip strength test in rats. The inhibitory effects on neuromuscular transmission of A2NTX at pH 7.4 and pH 6.8 were 1.95 and 3.73 times more potent than those of A1LL, respectively. The 50% effective doses for the administered limb, the dose which caused a 50% reduction in grip strength, i.e. ED_{50}, of A1LL, A1NTX, and A2NTX were calculated as 0.087, 0.060, and 0.040 U/head, respectively. These doses for the contralateral limb, i.e. TD_{50}, of A1LL, A1NTX, and A2NTX were calculated as 6.35, 7.54, and 15.62 U/head, respectively. In addition, the time required for A2NTX-injected rats to recover the grip strength of the contralateral limb was 17 days, while that for rats injected with A1LL was 35 days. The results indicated that A2NTX is a more potent neuromuscular blocker than A1 toxins, and suggested that A2NTX will provide a preferentical therapeutic agent for neurological disorders.

1. Introduction

Botulinum toxins act at neuromuscular junctions and induce muscle relaxation by inhibiting acetylcholine release. The toxins cause muscle flaccidity, and have been developed and used as important therapeutic agents for neurological disorders such as blepharospasm, hemifacial spasm, and a variety of dystonias (Jankovic, 2004; Sadick, 2003). The toxins are protein complexes, called progenitor toxins, containing a 150-kDa neurotoxin (NTX) and non-toxic components. Type A progenitor toxins are classified by their molecular weight into three forms: LL toxin, 900 kDa; L toxin, 500 kDa; and M toxin, 300 kDa (Sakaguchi, 1983). The M toxin consists of an NTX and a non-toxic component exhibiting no hemagglutinin (HA) activity (described here as non-toxic non-HA: NTNH), and L and LL toxins are formed by conjugation of the M toxin with HA (Montecucco et al., 1996).

Recently, the amino acid sequences of toxins have been analyzed, and it has been shown that sequence variability occurs in NTX (Willems et al., 1993; Franciosa et al., 2004, 2006). Accordingly, type A organisms have been classified

* Corresponding author. Tel.: +81 96 344 1211; fax: +81 96 345 1345.
E-mail address: torii-ya@kaketsuken.or.jp (Y. Torii).

0041-0101/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2010.10.009

into four subtypes (A1 to A4) based on the amino acid sequence variability of NTX (Arndt et al., 2006). Botulinum toxin type A products used as treatment for neurologic disorders are LL toxin and NTX, produced by Clostridium botulinum subtype A1 (A1LL and A1NTX) (Dressler and Benecke, 2007). In A1 to A4 toxins, it was reported that A1 and A2 toxins showed no difference in mouse lethal toxicity (Sakaguchi et al., 1990; Tabita et al., 1991). However, there has been no report comparing toxin subtypes regarding their neuromuscular transmission inhibition and muscle relaxation as medicinal effects.

We purified NTX produced by *C. botulinum* subtype A2 (A2NTX). A2NTX shares 89% amino acid sequence homology with A1NTX (Willems et al., 1993). In this study, we compared the efficacy of A2NTX with that of commercial type A toxin products. The *in-vitro* assay involved a twitch tension assay using the mouse phrenic nerve hemidiaphragm, employed to compare neuromuscular transmission inhibition between A2NTX and A1LL. The *in-vivo* assay was a grip strength test involving rats, which was used to compare muscle flaccid paralysis among A2NTX, A1NTX, and A1LL.

2. Materials and methods

2.1. Purification of toxins

Botulinum neurotoxins type A (150 kDa, NTX) were prepared using a modification of a previously reported method (Sakaguchi et al., 1981). *C.botulinum* type A strains 62A and Chiba-H, which belong to subtypes A1 and A2, respectively, were cultured in PYG medium containing 2% peptone, 0.5% yeast extract, 0.5% glucose, and 0.025% sodium thioglycolate by allowing them to stand at 30 °C for 3 days. M toxin was purified from the culture fluid by acid precipitation, protamine treatment, ion-exchange chromatography, and gel filtration. Each subtype of M toxin was adsorbed onto a DEAE Sepharose column equilibrated with 10 mM phosphate buffer, and eluted with a 0–0.3 M NaCl gradient buffer for the separation of NTX from the non-toxic component. The NTXs were stored at −70 °C until use.

For the control, the commercial progenitor LL toxin (BOTOX®, Allergan Inc., Irvine, US, hereafter A1LL) was used.

2.2. Experimental animals

Female ICR/CD-1 mice (4 weeks of age, about 20 g, Charles River Laboratories Japan, Yokohama, Japan), male ddY mice (5 weeks of age, about 30 g, Charles River Laboratories Japan, Yokohama, Japan), and female S/D rats (8 weeks of age, about 200 g, Charles River Laboratories Japan, Yokohama, Japan) were used for the LD_{50}, twitch tension assay, and grip strength test, respectively. Animals were maintained under controlled light/dark conditions and had free access to food and water. This study was performed in accordance with the guidelines concerning experimental animals established by the Japanese Pharmacological Society, and was approved by the Animal Ethics Committee of our institute.

2.3. Toxin activity (mouse ip LD_{50}) measurements

The toxin activities of A2NTX, A1NTX, and A1LL were determined employing the mouse intraperitoneal LD_{50} method (Pearce et al., 1994), with one mouse ip LD_{50} = 1 unit (U). The mouse ip LD_{50} was determined by employing an assay involving 7 doses at a dilution interval of 1.25 and 20 animals per dose. The chosen evaluation period was the first 96 h after administration, and the LD_{50} was calculated using the probit method.

The specific activities of A2NTX and A1NTX were 93 and 63 U/ng of neurotoxin proteins, respectively. That of A1LL is reported to be about 20 U/ng of the toxin protein complex based on BOTOX® product information (Allergan Inc., 2010).

2.4. Twitch tension assay (in-vitro test)

The twitch tension assay was conducted as previously described, with modification (Sugimoto et al., 1992). Phrenic nerve hemidiaphragm preparations were isolated from ddY mice, and then transferred to oxygenated (95% O_2 + 5% CO_2) Krebs solution of the following composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.24; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; glucose, 10; pH 7.4. The costal margin of the muscle was fixed and the central tendon was connected to a tension transducer (Nihon Kohden, Tokyo, Japan) by a string. The preparation was suspended in a glass tissue chamber containing 10 mL of Krebs solution. The phrenic nerve was connected to a pair of platinum electrodes and stimulated with supramaximal rectangular pulses of 1 V amplitude and 10 ms duration at a frequency of 0.25 Hz. The tension transducer was connected to an amplifier (Nihon Kohden, Tokyo, Japan), and the muscle tension was recorded on paper using a pen recorder (Nihon Kohden, Tokyo, Japan). In addition, to emulate the possible muscle pH of patients with spasm, these twitch tension experiments were repeated under acidic conditions, with the pH brought to 6.8 using lactic acid (final concentration: 25 mM).

After the twitch tension had become stable, one of the toxins (diluted with 20 mM Tris–HCl containing 150 mM NaCl and 0.02% bovine serum albumin (pH 7.4 or 6.8)) was applied to the bathing solution, and the reduction in twitch tension was recorded. After the experiment, tensions were measured every 4 s from each data record, and these values were averaged over intervals of 1 min. The time required for the twitch tension to decline to $1/e$ of that observed immediately before toxin application was calculated.

2.5. Measurement of grip strength (in-vivo test)

We compared flaccid paralysis on the administered or contralateral side induced by A1LL, A1NTX, and A2NTX. The toxins were diluted two-fold serially to 0.15–2.4 U/mL for the former or at an interval of 40 U serially to 10–240 U/mL for the latter with physiological saline containing 0.5% human serum albumin. Rats were anesthetized by intraperitoneally injecting 40 mg/kg of sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan). After the disappearance of the eyelid reflex, the foreleg of the rat was shaved and 0.1 mL of each toxin concentration was injected into the

foreleg muscle (flexor digitorum muscle). For injection, an insulin syringe (Becton Dickinson, Tokyo, Japan) was used.

The grip strength was measured in the left and right forelegs of each rat using a Grip Strength Meter (Muromachi Kikai, Tokyo, Japan) and a modification of a previously reported method (Meyer et al., 1979). Each rat was fixed horizontally, and then pulled steadily by the root of the tail away from the T bar until its grip was broken. The peak of the grip strength was measured. Each rat was subjected to five such trials, and the average was used as the grip strength. The grip strength was measured at 0 (before administration), 1, 2, 3, 4, 7, and 14 days after injection. It is represented as the gram-force (gf).

To evaluate the duration of the muscle flaccidity-inducing effect of the toxin, the toxins were diluted to 0.12 U/mL with 0.5 w/v% human serum albumin-containing physiological saline and 0.1 mL of each toxin was injected into the muscle of the foreleg. The grip strength was measured until 66 days after injection.

To compare the duration of equivalent muscle flaccidity-inducing effects among the toxins, each toxin was diluted to the dose necessary to cause a 50% reduction in the grip strength on either the administered or contralateral side, and was injected into the muscle of the foreleg. The grip strength was measured until 35 days after injection. The variation range of the grip strength before injection was defined as the mean ± 2 S.D. The duration of the muscle flaccidity-inducing effect of the toxins was defined as the time required for recovery into this variation range of grip strength before injection.

2.6. Statistical analysis

The grip strength is expressed as the mean ± S.E.M., and the time-course is presented graphically. To evaluate the efficacy of the toxins, a regression line for each toxin was calculated for the peak effect versus the dose. Regression lines were used to calculate the doses causing 50% reductions in grip strength, and these values were termed the Effective Dose 50 (ED_{50}: administered side) and Toxic Dose 50 (TD_{50}: contralateral side), respectively.

The ED_{50} and TD_{50} of each toxin were analyzed using a nonlinear least-squares method employing SAS (SAS Institute Inc., ver. 9.1).

3. Results

3.1. Comparison of neuromuscular blocking activities of the toxins using the twitch tension assay

The inhibitory effects of A2NTX, A1NTX, and A1LL on neuromuscular transmission were determined based on the twitch tension assay. Dose-dependent shortening of the attenuation time of twitch tension was observed in this assay (Fig. 1). We plotted the logarithm of the time for twitch tension to decline to 1/*e* of that observed immediately before toxin application against the logarithm of toxin activity (LD_{50} doses), and the linearity of the regression line and homogeneity of variance were confirmed by regression analysis. Each toxin in the twitch tension assay could not be compared using identical toxin activities, because the employed doses of each toxin were different. However, comparison of the linear range of the dose–response relationship revealed that A2NTX was 1.21- and 1.95-times more potent than A1NTX and A1LL in its neuromuscular blocking activity, respectively. In addition, under acidic conditions (pH 6.8), the neuromuscular blocking activity of A2NTX was 3.73 times more potent than A1LL.

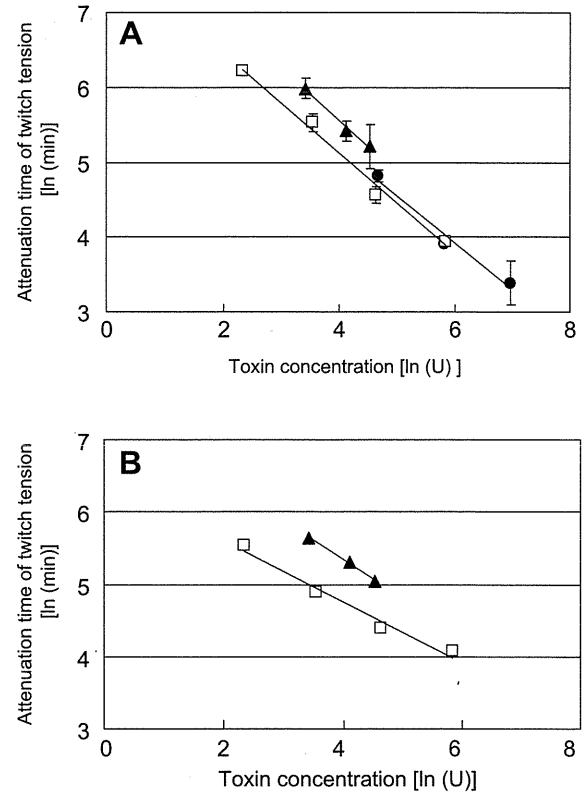


Fig. 1. Dose–response curves of A1LL, A1NTX, and A2NTX for the twitch tension assay using mouse phrenic nerve hemidiaphragm preparations. The response was expressed as the time required for twitch tension to decline to 1/*e* of that observed immediately before toxin application. (A) pH 7.4 (▲: A1LL, ●: A1NTX, □: A2NTX). (B) pH 6.8 (▲: A1LL, □: A2NTX). Each point is the mean ± S.D. (n = 3–5).

3.2. Comparison of muscle flaccidity induced by the toxins using the grip strength test

3.2.1. Time-course of grip strength on the administered side

To compare flaccid paralysis on the administered side among A2NTX, A1NTX, and A1LL, the grip strength was measured. It was found that the grip strength decreased with the LD_{50} concentration of toxins (Fig. 2). Grip strengths in the presence of A2NTX, A1NTX, and A1LL decreased until the 2nd, 3rd, and 3 days after toxin injection, respectively, and then recovered.

3.2.2. Time-course of grip strength of the contralateral foreleg after injection

The safety (diffusion) of each toxin was evaluated by periodically measuring the grip strength of the contralateral

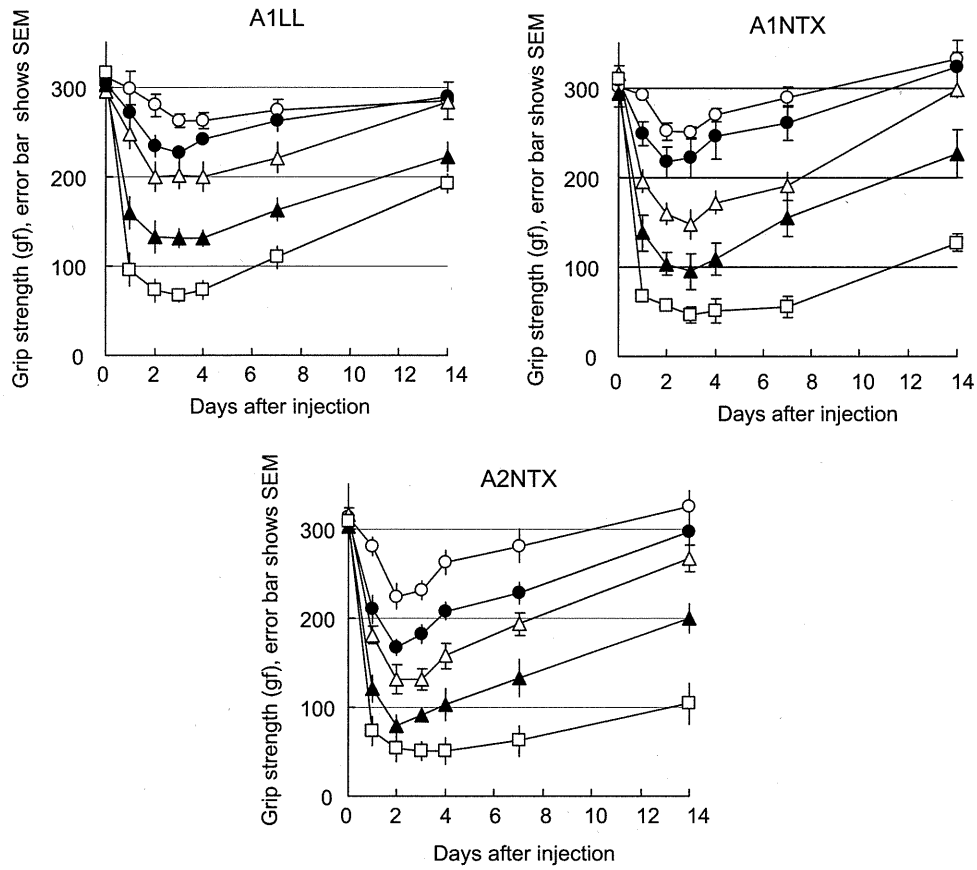


Fig. 2. Time-course of the grip strength on the administered side after toxin injection. Rats received A1LL, A1NTX, or A2NTX injection in the left foreleg (each at ○: 0.015 U, ●: 0.03 U, △: 0.06 U, ▲: 0.12 U, and □: 0.24 U). The grip strength was measured in the left foreleg of each rat at 0 (before administration), 1, 2, 3, 4, 7, and 14 days after injection. Each point is the mean ± S.E.M. ($n = 5$).

foreleg (right front leg) after the intramuscular injection of relatively large amounts of toxin into the left foreleg. It was found that the grip strength decreased according to the LD_{50} concentration of toxins (Fig. 3). The grip strength in the right foreleg, in which toxin was not injected, decreased until the 3rd or 4th day after toxin injection and recovered after the 7th day. The relationship between the grip strength and administered dose was calculated by regression analysis each measurement day, the log LD_{50} was plotted against the grip strength, and the linearity of the regression line and homogeneity of variance were confirmed by regression analysis. A1NTX of 20 and 24 U killed one of five rats, and A1LL of 24 U killed three of five rats. In contrast, all A2NTX-treated rats survived (Data not shown).

3.2.3. ED_{50} and TD_{50}

ED_{50} and TD_{50} were calculated from the peak decrease in the grip strength on the administered and contralateral side, respectively (Table 1). The ED_{50} for A2NTX was 0.040 U, which was lower than that for the other toxins. The ED_{50} values for A1NTX and A1LL were 0.060 and 0.087 U, respectively. The TD_{50} for A2NTX was 15.62 U, a higher dose than that for other toxins. The TD_{50} values of A1NTX and A1LL were 7.54 and 6.35 U, respectively.

3.2.4. Duration of muscle flaccidity induced by the toxins

We compared the duration of the muscle flaccidity-inducing effect of the toxins when the same dosage of each toxin was injected. The effects of A1LL, A1NTX, and A2NTX resolved at 35, 35, and 66 days after injection, respectively (Fig. 4A). A2NTX showed a longer duration of action than A1LL and A1NTX.

We also compared the duration of action of each toxin when adjusting the toxins to show equivalent muscle flaccidity-inducing effects. In spite of the different doses, recovery from all toxins occurred by 14 days after injection on the administered side (Fig. 4B). However, on the contralateral side, recovery occurred at 35, 35, and 17 days for A1LL, A1NTX, and A2NTX, respectively (Fig. 4C).

4. Discussion

We investigated whether the efficacy of botulinum toxin type A differed according to the type A structure or amino acid sequence of the toxin molecule. Our results indicate that A2NTX is more effective than A1LL and A1NTX, which have been used for commercial botulinum toxin type A products.

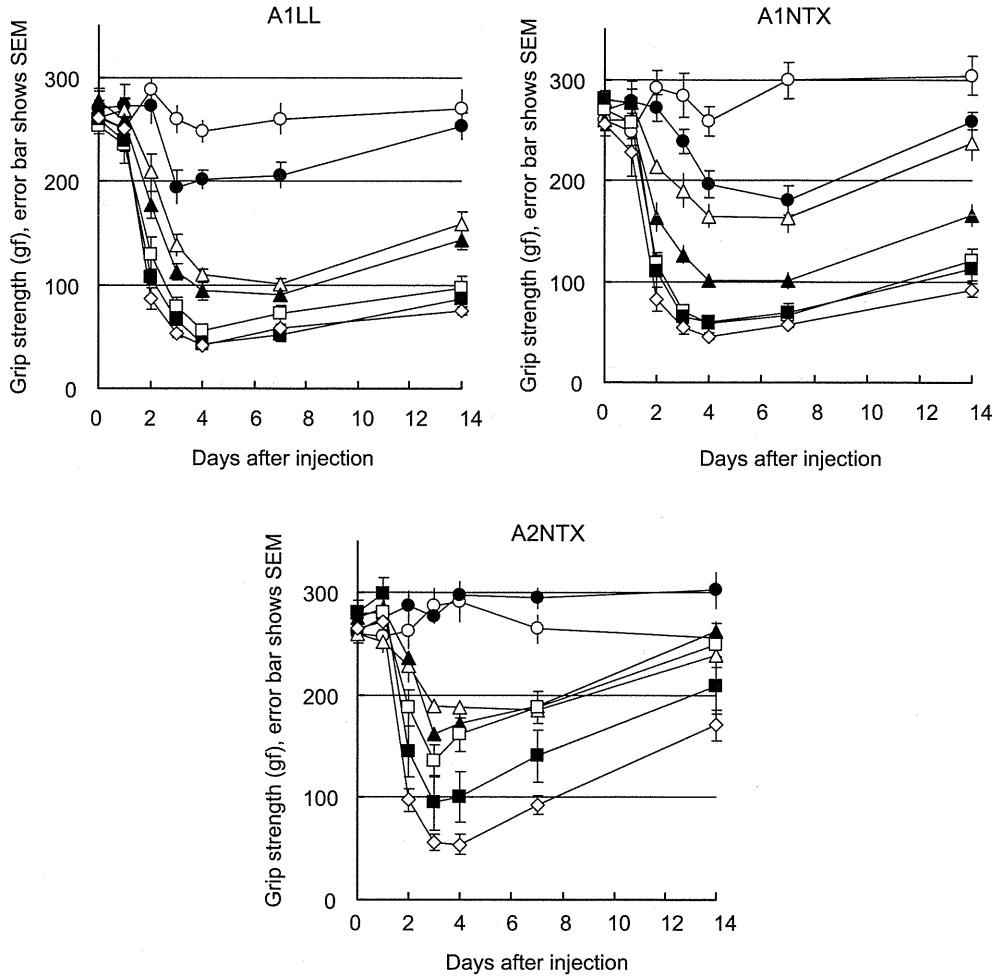


Fig. 3. Time-course of the grip strength of the contralateral foreleg after toxin injection. Rats received A1LL, A1NTX, or A2NTX injection in the left foreleg (each at ○: 1 U, ●: 4 U, △: 8 U, ▲: 12 U, □: 16 U, ■: 20 U, and ◇: 24 U). The grip strength was measured in the right foreleg of each rat at 0 (before administration), 1, 2, 3, 4, 7, and 14 days after injection. Each point is the mean ± S.E.M. ($n = 5$).

In *in-vitro* experiments using the twitch tension assay, A2NTX displayed about a 1.2- and 1.95-times higher inhibitory activity than A1NTX and A1LL, respectively. In a previous report, the inhibitory effect on neuromuscular transmission of the M toxin was compared with that of LL toxin, and M toxin was round to display about a 2-times higher inhibitory activity than LL toxin (Yoneda et al., 2005). The binding of a toxin to its receptor and subsequent endocytosis occur after the dissociation of NTX from the progenitor toxin complex. In this study, the difference in effectiveness might be thought to indicate that A1LL take long dissociation of HA and NTNH in the *in-vitro* experiment.

There is a possibility that areas of muscle damage in patients with spasm are acidic due to persistent muscle contraction. To reproduce this condition in *in-vitro* experiments, the effects of each toxin were determined under acidic conditions (pH 6.8 by the addition of lactic acid). Under these conditions, the neuromuscular blocking activity of A2NTX was 3.73 times more potent than that of A1LL, with a greater difference than at pH 7.4. In this study, the difference in effectiveness might be thought to indicate that the dissociation rate of A1LL at pH 6.8 is slower than at pH 7.4. This result suggests that that therapeutic effect of A2NTX is greater than that of A1LL in spasm patients.

To evaluate whether the *in-vitro* inhibitory effects of the toxins on neuromuscular transmission are correlated with their effects on muscle flaccidity, we conducted an investigation using the rat grip strength test. When we compared the ED_{50} values of the toxins (muscle flaccidity effect), we found that A2NTX was 2.1-times more effective than A1LL. This was virtually the same relationship between the toxins as seen in the twitch tension assay. These results indicate

Table 1
ED_{50} and TD_{50} for A1LL, A1NTX, and A2NTX.

Toxin	ED_{50} (U/head)[a]		TD_{50} (U/head)[b]	
	Value	95% confidence interval	Value	95% confidence interval
A1LL	0.087	0.07–0.10	6.35	5.75–7.24
A1NTX	0.060	0.05–0.07	7.54	6.61–8.51
A2NTX	0.040	0.03–0.05	15.62	13.18–23.44

[a] ED_{50}, dose at which a 50% reduction occurred in efficacy.
[b] TD_{50}, dose at which a 50% reduction occurred in safety.

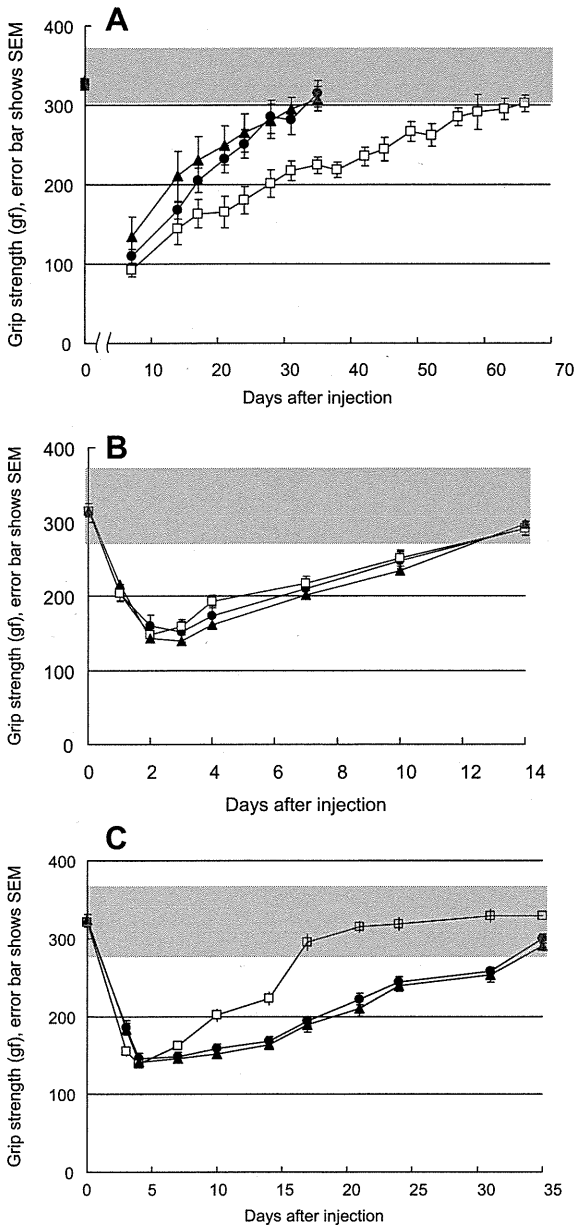


Fig. 4. The duration of the muscle flaccidity-inducing effects of the toxins (▲: A1LL, ●: A1NTX, □: A2NTX). The grey area shows the variation range (mean ± 2 S.D.) before injection. (A) Time-course of the grip strength on the administered side after toxin injection. Rats received 0.012 U of A1LL, A1NTX, or A2NTX. Each point is the mean ± S.E.M. ($n = 5$). (B) Time-course of the grip strength on the administered side after toxin injection. Rats received ED_{50} levels of A1LL, A1NTX, or A2NTX. Each point is the mean ± S.E.M. ($n = 5$). (C) Time-course of the grip strength on the contralateral side after injection. Rats received SD_{50} levels of A1LL, A1NTX, or A2NTX. Each point is the mean ± S.E.M. ($n = 5$).

that A2NTX is effective at half the usual dose of the conventional botulinum toxin type A product (A1LL).

When we compared the duration of the muscle flaccidity-inducing effect of the toxins, we found that a half-dose of A2NTX and one dose of A1LL were equivalent regarding the duration of the muscle-relaxing effect. When equivalent doses of the toxins were injected, A2NTX produced an effect with about a 2-times longer duration than that of A1LL. This result suggests that A2NTX might cause a lower incidence of adverse effects than the conventional botulinum toxin product (A1LL). Furthermore, as frequent administration might not be necessary, its injection might be unlikely to induce neutralization antibody production.

The diffusion of each toxin was evaluated by measuring the grip strength in the contralateral foreleg (toxin-untreated). A higher dose of A2NTX than that of A1 toxins (A1LL and A1NTX) was required to decrease the grip strength on the contralateral side. A2NTX diffused less effectively than A1 toxins. The difference in diffusion among these toxins may be due to the variation in the amino acid sequence of NTX. When it was given at a high dose, which caused reductions in grip strength on the contralateral side, recovery following the injection of A2NTX was more rapid than for A1 toxins. This result suggests that A2NTX is safer than A1 toxins. Thus, A2NTX has the potential to become a safer drug for human use than A1 toxins.

We measured the time-course of the grip strength on the administered side, and found that A2NTX acted more rapidly than A1LL or A1NTX. The conventional botulinum toxin type A products have been reported to require about 1 week until the effect is observed (Truong and Jost, 2006). These slow-acting agents contribute to patient distress. A2NTX provides a possible solution to the problem because it acts more quickly than the conventional toxin preparation. The muscle flaccidity-inducing effect of A2NTX peaked at 2 days, whereas that of A1LL and A1NTX peaked at 3–4 days. The difference in the onset of effect between A2NTX and A1 toxins may be due to the variation in the amino acid sequence of NTX.

In summary, in this study, we found the following: 1) The effects of neuromuscular transmission inhibition and muscle relaxation are more effective for A2NTX than A1LL and A1NTX. 2) A2NTX shows a lower-level diffusion than A1LL and A1NTX, requiring a higher dose to reduce the grip strength on the contralateral side.

Acknowledgements

The authors thank Mr. Ayataka Nagano and Mr. Yusuke Ohyama for providing A2NTX, Ms. Shiho Itai, Ms. Miho Shinmura, Ms. Satomi Munechika, and Ms. Kaori Harada for technical support, and Mr. Seiji Matsuo for statistical support. The present investigation was conducted with a part of financial support from the Society for the Japan Health Sciences Foundation.

Conflict of interest statement

None.

References

Arndt, J.W., Jacobson, M.J., Abola, E.E., Forsyth, C.M., Tepp, W.H., Marks, J. D., Johnson, E.A., Stevens, R.C., 2006. A structural perspective of the sequence variability within botulinum neurotoxin subtypes A1-A4. J. Mol. Biol. 29, 733–742.

BOTOX® Product Information, 2010. Allergan Inc.

Dressler, D., Benecke, R., 2007. Pharmacology of therapeutic botulinum toxin preparations. Disabil. Rehabil. 15, 1761–1768.

Franciosa, G., Floridi, F., Maugliani, A., Aureli, P., 2004. Differentiation of the gene clusters encoding botulinum neurotoxin type A complexes in Clostridium botulinum type A, Ab, and A(B) strains. Appl. Environ. Microbiol. 70, 7192–7199.
Franciosa, G., Maugliani, A., Floridi, F., Aureli, P., 2006. A novel type A2 neurotoxin gene cluster in Clostridium strain mascarpone. FEMS. Microbiol. Lett. 261, 88–94.
Jankovic, J., 2004. Botulinum toxin in clinical practice. J. Neurol. Neurosurg. Psychiatr. 75, 951–957.
Meyer, O.A., Tilson, H.A., Byrd, W.C., Riley, M.T., 1979. A method for the routine assessment of fore- and hindlimb grip strength of rats and mice. Neurobehav. Toxicol. 1, 233–236.
Montecucco, C., Schiavo, G., Rossetto, O., 1996. The mechanism of action of tetanus and botulinum neurotoxins. Arch. Toxicol. Suppl. 18, 342–354.
Pearce, L.B., Borodic, G.E., First, E.R., MacCallum, R.D., 1994. Measurement of botulinum toxin activity: evaluation of the lethality assay. Toxicol. Appl. Pharmacol. 128, 69–77.
Sadick, N.S., 2003. Botulinum toxin type B. Dermatol. Surg. 29, 348–351.
Sakaguchi, G., Ohishi, I., Kozaki, S., 1981. Biomedical aspects of botulism. In: Lewis, G.E. (Ed.), Purification and Oral Toxicities of Clostridium botulinum Progenitor Toxins. Academic Press, New York, pp. 21–34.
Sakaguchi, G., 1983. Clostridium botulinum toxins. Pharmacol. Ther. 19, 165–194.
Sakaguchi, G., Sakaguchi, S., Kamata, Y., Tabita, K., Asao, T., Kozaki, S., 1990. Distinct characters of Clostridium botulinum type A strains and their toxin associated with infant botulism in Japan. Int. J. Food Microbiol. 11, 231–242.
Sugimoto, N., Miyamoto, A., Horiguchi, Y., Okabe, T., Matsuda, M., 1992. Inhibition of neuromuscular transmission in isolated mouse phrenic nerve-diaphragm by the enterotoxin of Clostridium perfringens type A. Toxicon 30, 825–834.
Tabita, K., Sakaguchi, S., Kozaki, S., Sakaguchi, G., 1991. Distinction between Clostridium botulinum type A strains associated with food-borne botulism and those with infant botulism in Japan in intra-intestial toxin production in infant mice and some other properties. FEMS. Microbiol. Lett. 79, 251–256.
Truong, D.D., Jost, W.H., 2006. Botulinum toxin: clinical use. Parkinsonism. Relat. Disord. 12, 331–355.
Willems, A., East, A.K., Lawson, P.A., Collins, M.D., 1993. Sequence of the gene coding for the neurotoxin of Clostridium botulinum type A associated with infant botulinum: comparison with other clostridial neurotoxins. Res. Microbiol. 144, 547–556.
Yoneda, S., Shimazawa, M., Kato, M., Nonoyama, A., Torii, Y., Nishino, H., Sugimoto, N., Kozaki, S., Hara, H., 2005. Comparison of the therapeutic indexes of different molecular forms of botulinum toxin type A. Eur. J. Pharmacol. 31, 223–229.

J Pharmacol Sci 117, 275 – 285 (2011)

Journal of Pharmacological Sciences

Full Paper

Type A1 but Not Type A2 Botulinum Toxin Decreases the Grip Strength of the Contralateral Foreleg Through Axonal Transport From the Toxin-Treated Foreleg of Rats

Yasushi Torii[1,*], Norio Akaike[2], Tetsuhiro Harakawa[1], Keiko Kato[3], Nakaba Sugimoto[4], Yoshitaka Goto[1], Shinji Nakahira[1], Tomoko Kohda[5], Shunji Kozaki[5], Ryuji Kaji[6], and Akihiro Ginnaga[1]

[1]*The Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN), 1-6-1 Okubo, Kumamoto-shi, Kumamoto 860-8568, Japan*
[2]*Research Division for Life Science, Kumamoto Health Science University, 325 Izumi-machi, Kumamoto-shi, Kumamoto 861-5598, Japan*
[3]*Department of Animal Medical Sciences, Kyoto Sangyo University, Motoyama, Kamigamo, Kita-ku, Kyoto-shi, Kyoto 603-8555, Japan*
[4]*Department of Molecular Therapeutics, Medical School of Osaka University, 2-2 Yamadaoka, Suita-shi, Osaka 565-0871, Japan*
[5]*Department of Veterinary Sciences, School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku-oraikita, Izumisano-shi, Osaka 598-8531, Japan*
[6]*School of Medicine, University of Tokushima Faculty of Medicine, 3-18-15 Kuramoto-cho, Tokushima-shi, Tokushima 770-8503, Japan*

Received July 9, 2011; Accepted October 25, 2011

Abstract. The adverse effects of botulinum LL toxin and neurotoxin produced by subtype A1 (A1LL and A1NTX) are becoming issues, as the toxins could diffuse from the toxin-treated (ipsilateral) to contralateral muscles. We have attempted to produce neurotoxin from subtype A2 (A2NTX) with an amino acid sequence different from that of neurotoxin subtype A1. We measured the grip strength on the contralateral foreleg as an indicator of toxin spread from the ipsilateral to contralateral muscles. Doses of 0.30 log U or above of A1LL and A1NTX reduced the contralateral grip strength, whereas a dose of 0.78 log U of A2NTX was required to do so. We investigated the route of toxin spread using denervated, colchicine-treated, and antitoxin-treated rats. A1LL was transported via axons at doses higher than 0.30 log U and via both axons and body fluid at about 0.80 log U or a higher dose. Interestingly, A2NTX was transported via body fluid at about 0.80 log U or a higher dose, but not via axons to the contralateral side. It was concluded that A1LL and A1NTX decreased the grip strength of the toxin-untreated foreleg via both axonal transport and body fluids, while A2NTX was only transported via the body fluid.

Keywords: botulinum toxin, grip strength (rat), neurotomy, colchicine, axonal transport

Introduction

Botulinum toxins have been researched and developed for use as important therapeutic agents for neurological disorders such as blepharospasm, hemifacial spasm, various dystonias, and overactive bladder (1 – 3). The toxins are protein complexes containing a 150-kDa neurotoxin (NTX) and nontoxic components. Type A protein complexes, called progenitor toxins, have molecular weights of 900 (LL toxin), 500 (L toxin), or 300 (M toxin) kDa (4). LL and L toxins have nontoxic components exhibiting hemagglutinin (HA) activity, whereas the nontoxic components of M toxin have no HA activity. The NTX component consists of heavy (100 kDa) and light (50 kDa) chain components held together by a disulfide bond (5). The heavy chain contains the translocation (N-termi-

*Corresponding author. torii-ya@kaketsuken.or.jp
Published online in J-STAGE on November 26, 2011 (in advance)
doi: 10.1254/jphs.11121FP

nal region of the heavy chain) and cell-binding domain (C-terminal region of the heavy chain). The light chain contains the endopeptidase domain, which cleaves proteins associated with intracellular vesicular transport, such as SNAP-25 (synaptosomal-associated protein of 25 kDa) for type A toxin, and consequently inhibits acetylcholine release from neurons, leading to paralysis. Type A organisms have been classified into five subtypes (A1 – A5) based on the amino acid sequence variability of the produced NTX (6, 7). Botulinum toxin type A products, which are used as a treatment for neurologic disorder, are produced from LL toxin or NTX derived from subtype A1 organisms (8). The toxins show high-level efficacy at very low doses, but their adverse effects are becoming an issue. In the treatment for torticollis, cervical dystonia, and cosmetic cases, patients showed dysphagia or respiratory compromise (9 – 11). In clinical studies of treatment for spasm, patients who received high-dose toxin showed weakness around the site of administration as well as symptoms of botulism (12 – 14). The U.S. Food and Drug Administration announced that the effects of botulinum toxin could spread from the injection site to other areas of the body, causing symptoms similar to those of botulism (15). The major problem with the toxin products was the relaxation of non-target muscles due to spread. It was reported that this spread might be the result of transport of the toxin via nerves (16).

To search for a toxin product with fewer adverse effects, we produced NTX from subtype A2 (A2NTX), with an amino acid sequence different from that of NTX subtype A1. In this study, to compare the spread dose of LL toxin from subtype A1 (A1LL), NTX from subtype A1 (A1NTX), and A2NTX, we conducted grip strength tests involving the side contralateral to the toxin-treated foreleg in rats as an indication of spread. To investigate the route of toxin spread, we carried out grip strength tests of the contralateral side of rat models treated with neurotomy or colchicine before the administration of toxins and in the rats treated with antitoxins after toxin administration. In addition, we used immunoblotting to determine if toxins were present in the contralateral foreleg after toxin administration.

Materials and Methods

Purification of toxins

Botulinum type A neurotoxins (150 kDa, NTX) were prepared as described in the previously reported method, with modifications (17). *Clostridium botulinum* type A strains 62A and Chiba-H, which belong to subtype A1 and A2, respectively, were cultured in PYG medium containing 2% peptone, 0.5% yeast extract, 0.5% glucose, and 0.025% sodium thioglycolate by allowing them to stand at 30°C for 3 days. M toxin was purified from the culture fluid by acid precipitation, protamine treatment, ion-exchange chromatography, and gel filtration. Each M toxin subtype was adsorbed onto a DEAE Sepharose column equilibrated with 10 mM phosphate buffer and eluted with a 0 – 0.3 M NaCl gradient buffer for NTX and non-toxic component separation. The different types of NTX were stored at −70°C until use.

For the test control, commercial progenitor LL toxin (BOTOX; Allergan Inc., Irvine, CA, USA) was used.

Experimental animals

ICR/CD-1 mice (4 weeks of age, female, about 20 g; Charles River Laboratories Japan, Yokohama) and S/D rats (8 weeks of age, female, about 200 g; Charles River Laboratories Japan) were used for the toxic activity assay and grip strength test, respectively. Animals were maintained under controlled light/dark conditions and had free access to food and water. This study was performed in accordance with the guidelines concerning experimental animals established by The Japanese Pharmacological Society and was approved by the Animal Ethics Committee of our institute.

Toxic activity measurements

The toxic activities of A1LL, A1NTX, and A2NTX were determined employing the mouse intraperitoneal (i.p.) LD_{50} method (18). The mouse i.p. LD_{50} was determined using a 7-dose assay with a dilution interval of 1.25 and 20 mice per dose. The evaluation period was the first 96 h after administration, and the LD_{50} was calculated by the probit method. One mouse i.p. LD_{50} was defined as 1 unit (U).

Investigation of toxin spread

We compared flaccid paralysis of the contralateral side muscles to that of those injected with A1LL, A1NTX, and A2NTX as an indicator of toxin spread (adverse effect). The toxins were serially diluted to 1.00 – 1.90 log U/mL with physiological saline containing 0.5% human serum albumin (diluent). Rats were anesthetized by the i.p. injection of 40 mg/kg of sodium pentobarbital (Kyoritsu Seiyaku, Tokyo). After the disappearance of the eyelid reflex, the foreleg of the rat was shaved and 0.1 mL of each toxin concentration was injected into the foreleg muscles using an insulin syringe (Becton Dickinson, Tokyo).

The grip strength of the left and right forelegs of each rat was measured using a Grip Strength Meter (Muromachi Kikai, Tokyo) and the modification of a previously reported method (19). Two Grip Strength Meters were placed side by side, and a T-bar was attached to each. A

rat gripped the T-bar with each foreleg and was fixed horizontally, and then the rat was pulled steadily by the root of its tail away from the T-bar until its grip was broken. The peak grip strength was measured. The grip strength of each rat was measured five times, and the average was used. The grip strength was measured at 0 (before administration), 2, 3, 4, and 7 days after injection. The rat grip strength was expressed in units of gram-force (gf).

Investigation of route of toxin spread using a neurotomy model

To investigate whether toxins are transported via nerves to the contralateral muscles, denervation of the left brachial plexus of toxin-treated muscle was carried out as follows (Fig. 1): rats underwent general anesthesia by i.p. injection of pentobarbital sodium at 40 mg/kg, and regional anesthesia around the surgical site by the intramuscular injection of 5 mg/kg xylazine (Nippon Zenyaku Kogyo, Koriyama). A surgical incision was made in the left thoracic region, exposing the left brachial plexus. The division of this plexus was ligated at two sites and cut between those sites. After the surgical operation, the muscle and skin were sutured, and 75 mg of kanamycin (Meiji Seika, Tokyo) was injected subcutaneously (s.c.) to prevent infection. The non-neurotomy group received a sham operation.

A1LL and A1NTX were serially diluted with the diluent to 1.60 – 1.90 log U/mL and A2NTX, to 1.80 log U/mL. A volume of 0.1 mL with each toxin at various concentrations was injected into the left foreleg muscles in the neurotomy and non-neurotomy groups. The vehicle group was injected only with the diluent.

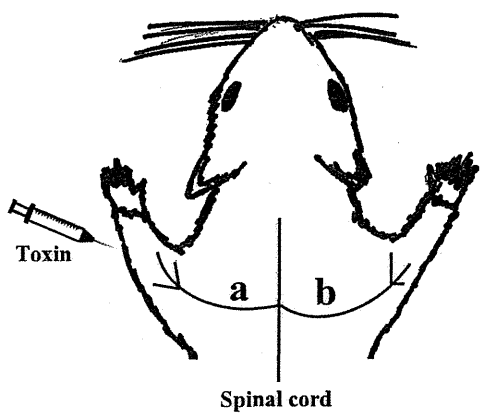


Fig. 1. Treatment of the neurotomy and colchicine models. a: left brachial plexus was cut (neurotomy model) or was treated with colchicine (left colchicine model), b: right brachial plexus was treated with colchicine (right colchicine model). The toxins were injected into the left foreleg in these models.

The grip strength was measured at 0 (before administration), 2, 3, and 4 days after administration.

Investigation of route of toxin spread using a colchicine model

To investigate whether the toxins are transported axonally to the contralateral foreleg, the left and right brachial plexus of rats was chemically treated with colchicine (Wako, Osaka) (left and right colchicine model, respectively) (Fig. 1). Colchicine selectively inhibits axonal transport by acting on neuronal microtubules (20). The left or right brachial plexus was surgically exposed in the same way as in the neurotomy model and then covered with agars containing colchicine (10 μg). The agars were themselves covered with Vaseline (Wako) to avoid the leakage of colchicine into non-target tissues. The non-colchicine–treated rat group received a sham operation.

A1LL and A1NTX were diluted to the dose of 0.60 log U and injected into the left foreleg muscles in these colchicine models and non-colchicine–treated groups. A2NTX was diluted to the dose of 0.78 log U and injected in the left colchicine– and non-colchicine–treated groups. The grip strength was measured at 0 (before administration), 2, 3, and 4 days after administration.

Investigation of route of toxin spread employing treatment with antitoxin

To investigate whether toxins are transported via the body fluid, we intravenously administered antitoxin following toxin injection. Antitoxin against type A1 toxin (type A1 antitoxin) was equine-derived Japanese standard botulinum antitoxin type A (National Institute of Infectious Diseases, Tokyo). Antitoxin against type A2 antitoxin (type A2 antitoxin) was the equine-derived F(ab')2 fragment, obtained as follows: Horses were immunized with toxoid of type A2 toxin, and sera were collected. The F(ab')2 fragment was purified by peptic digestion from immunoglobulin which was extracted from the obtained sera.

The dose of toxins used was higher than the previous one because the toxins may possibly be transported through pathways other than axonal transport. A1LL and A2NTX were diluted to the dose (A1LL: 0.84 log U, A2NTX: 1.20 log U) that caused a 50% reduction in the grip strength of the contralateral foreleg, as reported in a previous experiment (21), and injected into the left foreleg muscles. In the antitoxin-treated groups, A1LL- or A2NTX-treated rats were intravenously given 1 unit of type A1 or A2 antitoxin (A1-antitoxin– and A2-antitoxin–treated groups, respectively) at 1 h after toxin administration. One unit of these antitoxins can neutralize 4.00 log U of type A1 or A2 toxin, respectively (22). In addition, in the prepared neurotomy model, A1LL and anti-

toxin type A1 were administered in the same way. These antitoxin doses were set so that the antitoxin dose did not affect the grip strength of the toxin-injected foreleg. The grip strength was measured at 0 (before administration), 2, 3, and 4 days after administration.

Immunoblotting

To show the presence of the toxins in the contralateral foreleg, we investigated whether SNAP-25 was cleaved by the toxins in the contralateral nerve ends. The presence of cleaved SNAP-25 was demonstrated by immunoblotting, according to the previously described method (16). The neurotomy and left colchicine–treated models were used. Control rats received a sham operation. A1LL and A2NTX were each diluted to the appropriate dose (A1LL: 0.60 log U, A2NTX: 0.78 log U) and injected into the left foreleg muscles. The right foreleg muscles and nerve were isolated at 4 days after injection. The muscles and nerve were homogenized, and proteins were extracted with lysis buffer of the following composition: 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 10% glycerol, 20 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na_2MoO_4, 0.5 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 0.01 mM PMSF. Protein extracts were separated by electrophoresis and electroblotted to a PVDF membrane (GE Healthcare Japan, Hino), which was then incubated with primary antibody (anti-botulinum toxin type A-cleaved SNAP-25) for 1 h at room temperature. The anti-botulinum toxin type A-cleaved SNAP-25 was produced as described previously method (23). Blots were then reacted with HRP-conjugated anti rabbit IgG (GE Healthcare Japan) and developed by ECL (GE Healthcare Japan).

Statistical analysis

The grip strength is presented as the mean ± S.E.M., and the time course is shown graphically. The change in the grip strength of each toxin-treated group, neurotomy model, colchicine model, antitoxin-treated group, and vehicle group over the 4 days after administration was analyzed by one-way ANOVA, Tukey's test, and two-way ANOVA. SAS (ver. 9.1; SAS Institute Japan, Tokyo) was employed for statistical analysis. The significance level was set to two-sided 5%. The *P*-value was rounded to 4 decimal places.

Results

Comparison of the adverse effect of toxins on contralateral muscles

The adverse effect of each toxin was evaluated by measuring the grip strength of both forelegs after intramuscular injection into the left foreleg. The grip strength decreased with increasing toxin concentration in the contralateral foreleg (Fig. 2). The grip strength of the toxin-treated (ipsilateral) foreleg was about 0 gf at all toxin concentrations used (data not shown).

The decrease in the grip strength of the contralateral

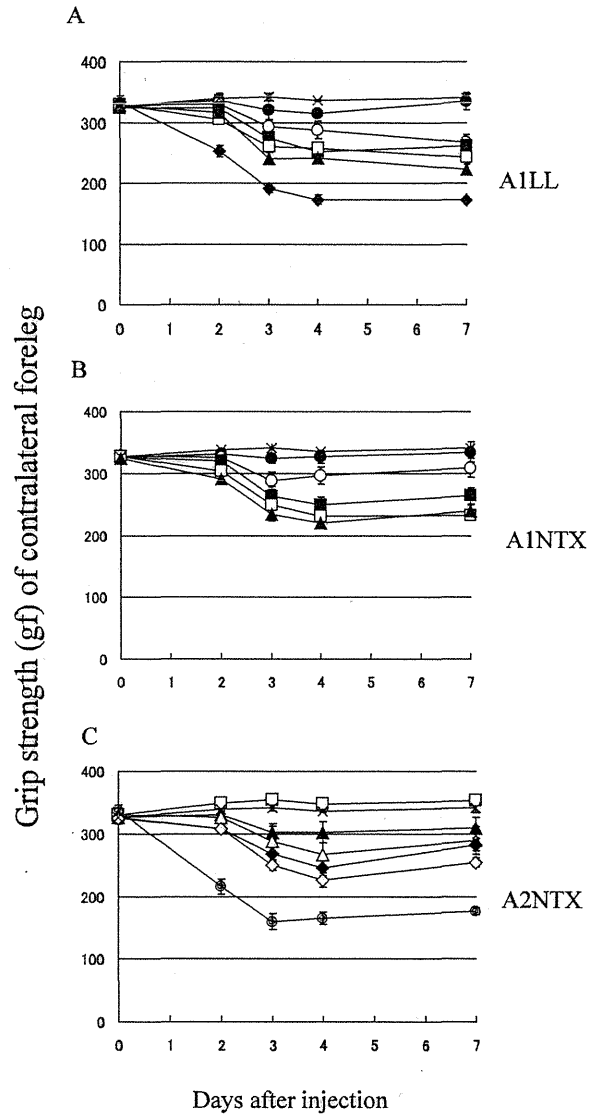


Fig. 2. Time-course changes in the grip strength of the contralateral forelegs after toxin injection in rats. Rats were injected with A1LL (A), A1NTX (B), or A2NTX (C) at various concentrations (0 – 1.20 log U) into left foreleg muscles. The grip strength was measured in the contralateral foreleg of each rat before (0) and at 2, 3, 4, and 7 days after injection. Each point is the mean ± S.E.M (n = 5). 0 (closed circle), 0.30 (open circle), 0.48 (closed square), 0.60 (open square), 0.70 (closed triangle), 0.78 (open triangle), 0.84 (closed diamond), 0.90 (open diamond), 1.20 (double circle) log U, and Vehicle (cross mark).

foreleg reached a maximum on day 4 after administration. Thus, the change during the 4-day period was calculated by subtracting the value before administration from that on day 4. The change over the 4 days after injection between toxin-treated and vehicle groups was analyzed by one-way ANOVA. A1LL and A1NTX showed a significant difference from the vehicle at 0.30 log U or more, respectively. In contrast, A2NTX showed a significant difference at a dose higher than 0.78 log U (Fig. 3).

Effects of A1LL and A1NTX on contralateral muscles in the neurotomy model

To investigate the route of toxin spread to the contralateral foreleg, a rat neurotomy model was used. The change over the 4 days after injection among neurotomy, non-neurotomy, and vehicle groups was analyzed using two-way ANOVA and Tukey's test. Two-way analysis of variance (toxins dose by neurotomy treated) was performed among neurotomy, non-neurotomy, and vehicle groups. All groups of A1LL and A1NTX showed a significant difference from the vehicle group (main effect of toxin dose: $P < 0.0001$, main effect of neurotomy treated: $P < 0.0001$, toxins dose by neurotomy treated: $P = 0.0008$

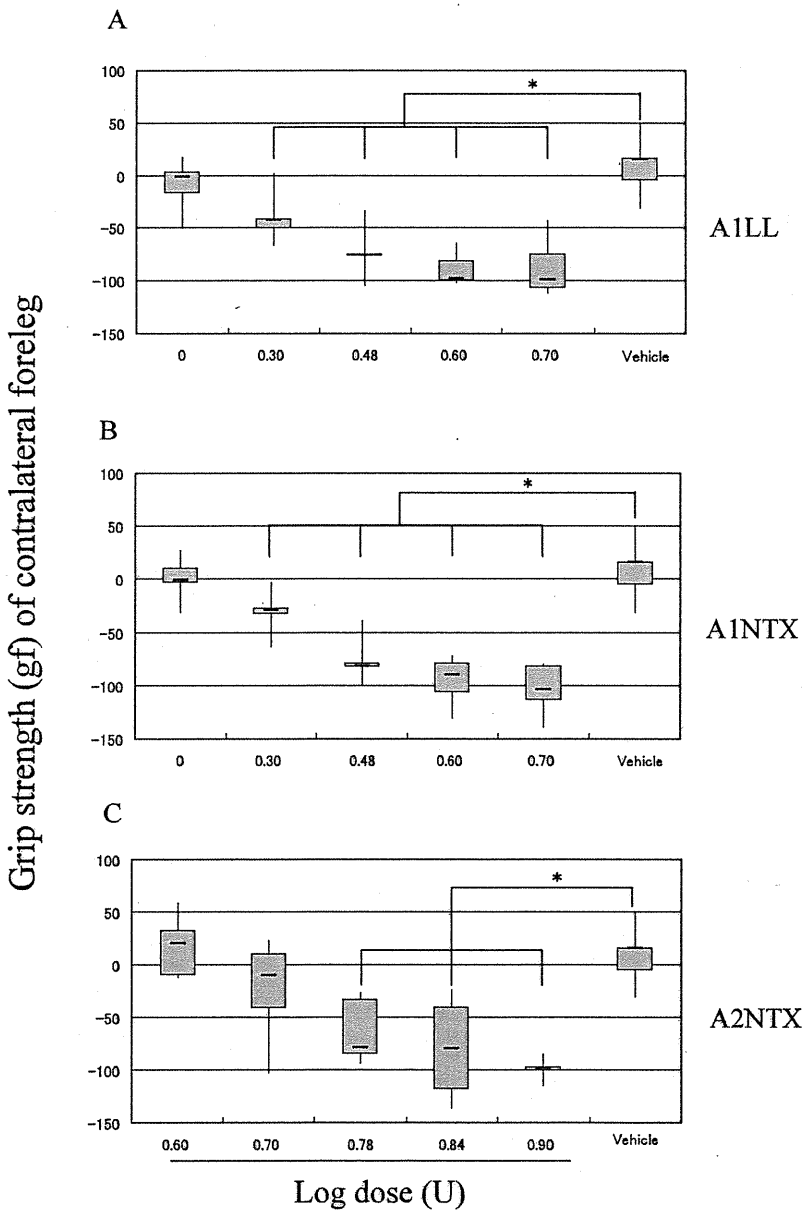


Fig. 3. Change in the grip strength of the contralateral foreleg during the 4 days after toxin injection [A1LL (A), A1NTX (B), and A2NTX (C)] (n = 5; $*P < 0.05$, one-way ANOVA). A1LL, A1NTX, and A2NTX showed a significant difference from the vehicle at more than 0.30, 0.30, and 0.78 log U, respectively.

in A1LL; main effect of toxin dose: $P < 0.0001$, main effect of neurotomy treated: $P < 0.0001$, toxins dose by neurotomy treated: $P = 0.0028$ in A1NTX). Tukey's test was performed among the groups. In the neurotomy group, the grip strength of the contralateral foreleg after the injection of 0.78 log U or less of A1LL or A1NTX did not decrease, and there was no significant difference between the neurotomy and vehicle groups ($P = 0.1859$, $P = 0.8453$ in A1LL and A1NTX at 0.78 log U, respectively). In contrast, the grip strength decreased in the non-neurotomy group, and there was a significant difference between the neurotomy and non-neurotomy groups ($P < 0.0001$ in A1LL and A1NTX at 0.78 log U). Moreover, a significant difference was observed between the neurotomy and vehicle groups at 0.90 log U of each toxin ($P = 0.0346$, $P = 0.0140$ in A1LL and A1NTX, respectively) (Fig. 4). In the neurotomy and non-neurotomy groups, the grip strength of the ipsilateral foreleg was about 0 gf (data not shown).

Effects of A1LL and A1NTX on the contralateral muscles in the colchicine model

The results using the neurotomy model suggested that both A1LL and A1NTX were transported to the contralateral muscles via a nerve pathway. In order to clarify this toxin pathway, left and right colchicine model rats were produced by treating the left and right brachial plexus with colchicine, respectively. The left and right colchicine models were blocked regarding axonal transport from the ipsilateral muscle to the spinal cord and from the spinal cord to the contralateral muscle, respectively. The change over the 4 days after injection among colchicine-treated, non-colchicine–treated, and vehicle groups was analyzed using one-way ANOVA and Tukey's test.

The left colchicine– and non-colchicine–treated groups were injected with 0.60 log U of A1LL or A1NTX. The grip strength of the contralateral foreleg did not decrease in the left colchicine–treated rats, similar to the results seen in the neurotomy model. One way ANOVA was performed, and there was a significant difference among the left colchicine–treated, non-colchicine–treated, and vehicle groups ($P < 0.0001$ in A1LL and A1NTX). Tukey's test was performed, and there was no significant difference between left colchicine–treated and vehicle groups ($P = 0.8862$ and $P = 0.6132$ in A1LL and A1NTX, respectively). In contrast, the grip strength of the contralateral foreleg decreased in the non-colchicine–treated group, resulting in a significant difference between the left colchicine– and non-colchicine–treated groups ($P < 0.0001$ in A1LL and A1NTX) (Fig. 5A).

The right colchicine– and non-colchicine–treated groups were injected with 0.60 log U of A1LL or A1NTX. The grip strength of the contralateral foreleg did not decrease in the right colchicine–treated rats, similar to the results seen in the left one. One way ANOVA was performed, and there was a significant difference among the right colchicine–treated, non-colchicine–treated, and vehicle groups ($P < 0.0001$ and $P = 0.0002$ in A1LL and A1NTX, respectively). Tukey's test was performed, and there was no significant difference between the right colchicine–treated and vehicle groups

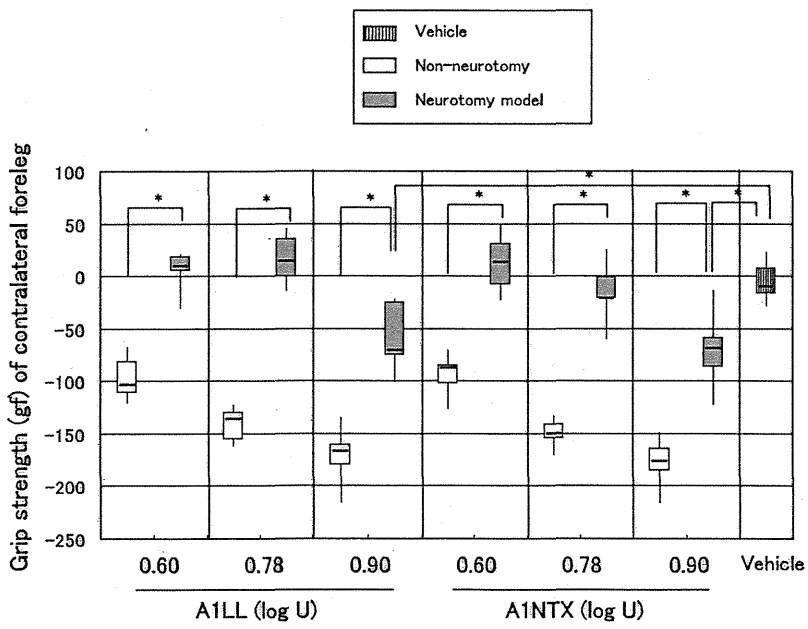


Fig. 4. Change in the grip strength of the contralateral foreleg in the neurotomy and non-neurotomy models during the 4 days after A1LL or A1NTX injection (n = 5; *$P < 0.05$, Tukey's test). Denervation was performed at the left brachial plexus, and then denervated rats received an injection of A1LL or A1NTX at 0.60, 0.78, and 0.90 log U into the left foreleg. There was no significant difference between neurotomy and vehicle groups at 0.78 log U or less of each toxin, but a significant difference appeared at 0.90 log U. At all doses used, the grip strength of the non-neurotomy group significantly differed from that of the neurotomy group.

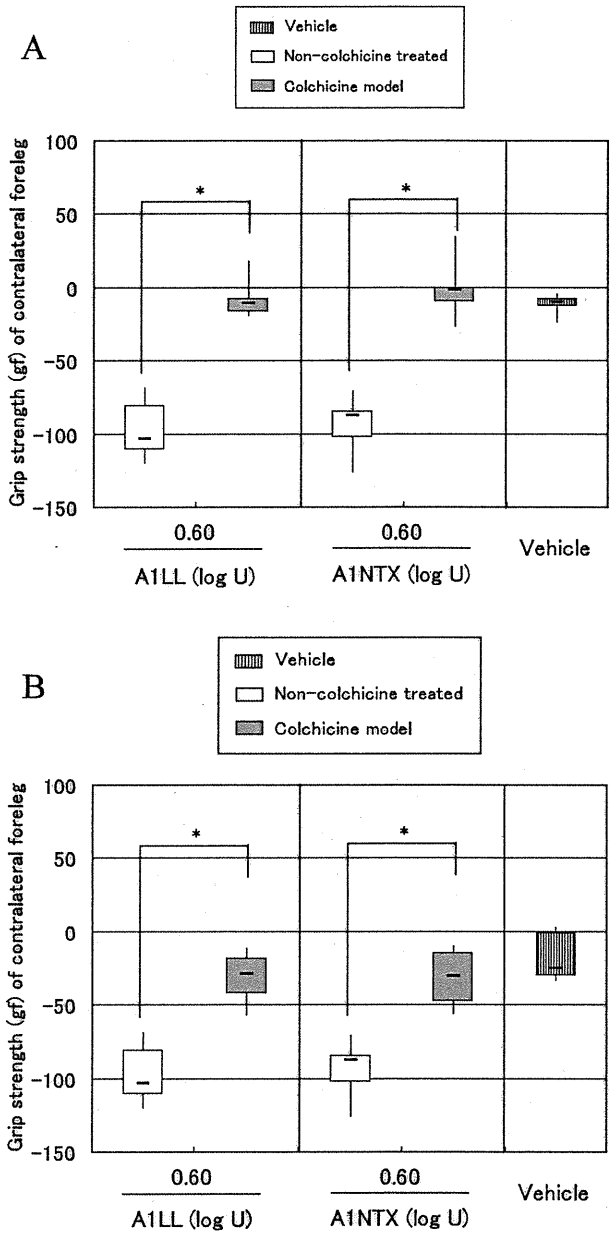


Fig. 5. Change in the grip strength of the contralateral foreleg in colchicine model rats during the 4 days after toxin injection (n = 4 – 5; *$P < 0.05$, Tukey's test). A: The left brachial plexus of rats was treated with colchicine, and then the rats received 0.60 log U of A1LL or A1NTX in the left foreleg. The grip strength of the contralateral foreleg did not decrease in the left colchicine model rats. There was no significant difference between the left colchicine model and vehicle rat groups, but there was a significant difference between the left colchicine model and non-colchicine–treated rat groups. B: The right brachial plexus of rats was treated with colchicine, and then the rats received 0.60 log U of A1LL or A1NTX in the left foreleg. The grip strength of the contralateral foreleg did not decrease in the right colchicine model rats. There was no significant difference between the right colchicine model and vehicle rat groups, but there was a significant difference between the right colchicine model and non-colchicine–treated rat groups.

($P = 0.5896$ and $P = 0.5560$ in A1LL and A1NTX, respectively). In contrast, the grip strength of the contralateral foreleg decreased in the non-colchicine–treated group, resulting in a significant difference between the right colchicine– and non-colchicine–treated groups ($P < 0.0001$ and $P = 0.0018$ in A1LL and A1NTX, respectively) (Fig. 5B).

In the left colchicine–, right colchicine–, and non-colchicine–treated groups, the grip strength of the ipsilateral foreleg was about 0 gf (data not shown). To verify whether the colchicine-treated nerve was intact, the left brachial plexus of toxin-nonadministered colchicine model rats was stimulated 7 days after surgery. Muscular contraction of the left foreleg was normal on electrical stimulation.

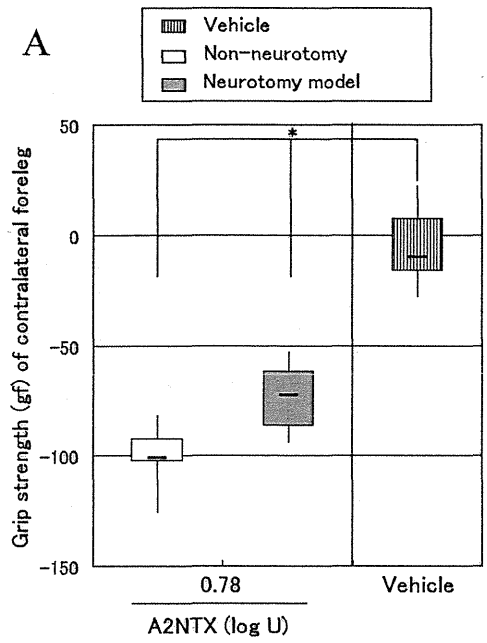
Effect of A2NTX on the contralateral muscles in the neurotomy and colchicine model

We determined the grip strength in the neurotomy and non-neurotomy groups treated with 0.78 log U of A2NTX. One way ANOVA was performed, and there was a significant difference among the neurotomy, non-neurotomy, and vehicle groups ($P = 0.0017$). Tukey's test was performed, and neurotomy and non-neurotomy groups were significantly different from the vehicle group ($P = 0.0093$ and $P = 0.0020$ in the neurotomy and non-neurotomy groups, respectively). Unexpectedly, there was no significant difference in the effect of A2NTX between the neurotomy and non-neurotomy groups ($P = 0.6609$) (Fig. 6A). The grip strength in the colchicine- and non-colchicine–treated groups treated with 0.78 log U of A2NTX significantly differed from that of the vehicle group ($P < 0.0001$, in colchicine- and non-colchicine–treated groups). There was no significant difference in the effect of A2NTX between the colchicine- and non-colchicine–treated groups ($P = 0.8297$) (Fig. 6B). In all groups, the grip strength of the ipsilateral foreleg was about 0 gf (data not shown).

Effect of antitoxin treatment on the contralateral foreleg

To investigate the route of toxin spread to the contralateral muscles excluding axonal transport, we injected antitoxin following toxin administration. As a result, the grip strength of the contralateral foreleg did not decrease in the A2-antitoxin–treated group, and there was no significant difference between A2-antitoxin–treated and vehicle groups (Fig. 7A).

In contrast, the grip strength of the contralateral foreleg markedly decreased in the A1-antitoxin–treated group. This group significantly differed from A1LL-alone ($P = 0.0032$) and vehicle groups ($P = 0.0017$) (Fig. 7A). The neurotomy rats injected with A1-antitoxin were not



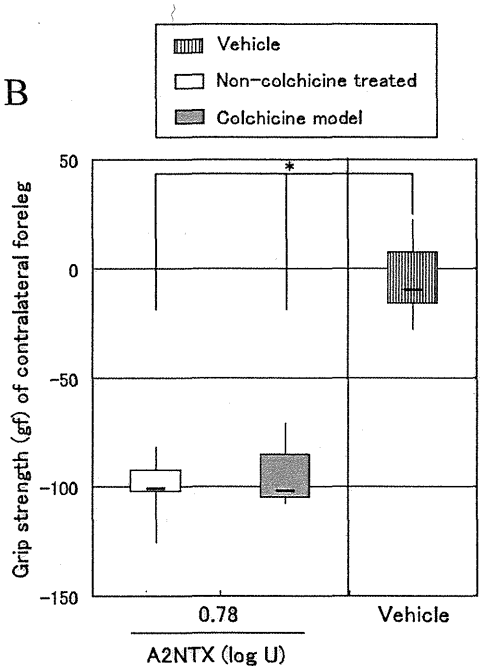
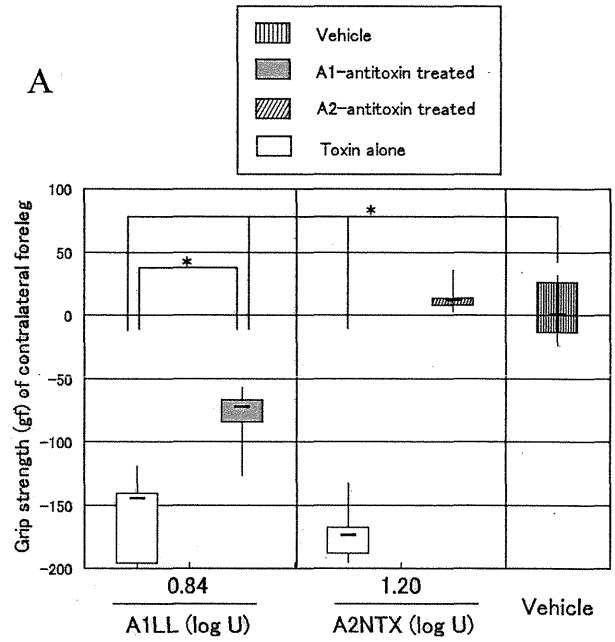


Fig. 6. Change in the grip strength of the contralateral foreleg in neurotomy and colchicine-treated models during the 4 days after A2NTX injection (n = 5; $*P < 0.05$, Tukey's test). A: Rats were denervated at the left brachial plexus and then injected with 0.78 log U of A2NTX into the left foreleg muscles. There was a significant difference between the neurotomy and vehicle groups, but no significant difference between the neurotomy and non-neurotomy groups. B: The left brachial plexus of rats was treated with colchicine, and then the rats received 0.78 log U of A2NTX in the left foreleg. There was a significant difference between the colchicine-treated and vehicle groups, but no significant difference between the colchicine- and non-colchicine–treated groups.



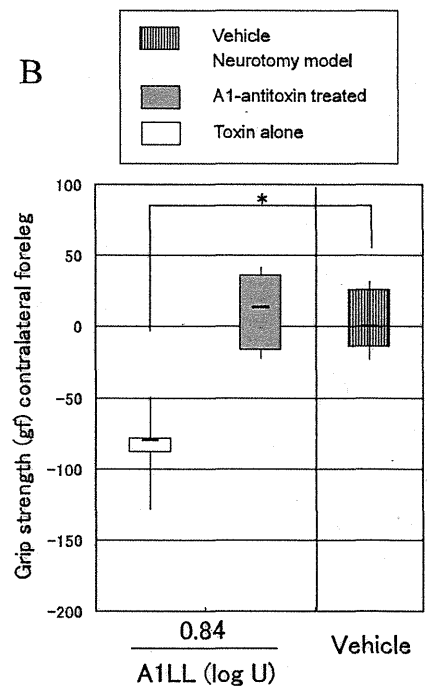


Fig. 7. Change in the grip strength of the contralateral foreleg in the antitoxin-treated rats during the 4 days after toxin injection (n = 5; $*P < 0.05$, Tukey's test). A: Injection of A1LL (0.84 log U) or A2NTX (1.20 log U). In the antitoxin-treated groups, where A1LL- or A2NTX-treated rats were intravenously injected with type A1 or A2 antitoxin, 1 h after toxin administration. The A1-antitoxin–treated group significantly differed from the A1LL-alone and vehicle groups. The grip strength in the A2-antitoxin–treated group did not decrease. B: Injection of A1LL (0.84 log U) in the neurotomy model. The grip strength of the neurotomy model rats that received antitoxin did not decrease.

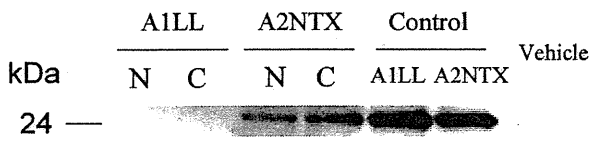


Fig. 8. Immunoblotting for A1LL- or A2NTX-cleaved SNAP-25 in protein extracts from the contralateral foreleg of the neurotomy (N) and colchicine-treated (C) model.

significantly different from the vehicle group ($P = 0.9263$) (Fig. 7B). In the toxin-treated groups (including antitoxin-treated groups), the grip strength of the ipsilateral foreleg was about 0 gf (data not shown).

Immunoblotting

To show the presence of the toxins in the contralateral foreleg, we used immunoblotting to detect cleaved SNAP-25 in the muscle and nerve of the contralateral foreleg as an assay of toxins trafficking. No cleavage of SNAP-25 was observed in the A1LL-administered neurotomy and colchicine-treated models, but SNAP-25 cleavage was observed in all control rats treated with this toxin. All groups given A2NTX showed cleavage of SNAP-25 (Fig. 8). These results indicate that A1LL was not present in the contralateral muscle of these models, but in contrast, A2NTX did diffuse into these muscles.

Discussion

We investigated the route of botulinum toxin spread. The present results clearly indicate that both A1LL and A1NTX decrease the grip strength of the contralateral foreleg via both axonal transport and body fluid, while A2NTX is not transported via nerve pathways but body fluid.

The maximum doses of A1LL and A1NTX that did not reduce the grip strength of the contralateral foreleg were 0 log U (5 U/kg). The dose was 0.70 log U (25 U/kg) for A2NTX. This indicates that A2NTX requires a greater dosage for diffusion to the contralateral muscles than A1LL and A1NTX. A2NTX was 2.2- and 1.5-times more effective in decreasing the rat grip strength than A1LL and A1NTX in the toxin-injected foreleg, respectively, as previously reported (21). Therefore, A2NTX possibly causes a lower incidence of adverse effects than commercial products of botulinum toxin such as A1LL and A1NTX.

Several adverse effects of botulinum toxin preparations have been reported, and an adverse effect involving the relaxation of non-target muscles is a concern. In previous reports, the adverse effects were thought to be caused by the erroneous injection of toxin into non-target muscle or spread due to a high dose of toxin (24, 25). Toxin spread to distant regions is considered to be due to transport via the body fluid or nerves (26 – 28). An RI study showed when labeled toxins were injected into gastrocnemius muscle of rats, the toxins were detected in the sciatic nerve, plasma and contralateral muscle. The toxins were not detected 48 h after injection in the contralateral muscle. More than 70% of the toxins were passed out of the body within 48 h of administration. Another RI study showed the retrograde axonal transport of toxin to the 6th lumbar spinal cord vertebra (L6) within 48 h after injection into the cat gastrocnemius muscle. Because of the elapsed time, it was assumed that the toxin was inactivated during transport (27). However, it has recently been reported that toxin that has entered neurons retains its activity for a prolonged period (16).

In this study, the results indicated that the grip strength of the contralateral foreleg reduced when the toxins were injected at a high dose. We considered it most likely that the toxins moved in the nerves to affect the grip strength of the contralateral foreleg, so we investigated whether the toxins moved to the contralateral foreleg through nerves or another pathway. When we treated the rats with A1LL or A1NTX, the grip strength of the contralateral foreleg decreased in the non-neurotomy and non-colchicine groups, but not in the neurotomy nor left colchicine model rats, indicating that A1LL and A1NTX were transported axonally from the ipsilateral muscle to the spinal cord and acted on the innervated contralateral foreleg muscles. To investigate the route of A1LL and A1NTX to the contralateral side, right colchicine model rats were treated with these toxins. The grip strength of the contralateral foreleg decreased in the non-colchicine groups, but not in the model rats. These results suggested that A1LL and A1NTX were retrogradely transported from the ipsilateral foreleg muscle to the spinal cord, and then the toxin was anterogradely carried to the nerve endings. To show that the toxin moves to the contralateral side, we used detection of cleaved SNAP-25 in contralateral nerve ends as an assay of the toxins trafficking. Botulinum toxin acts at the picogram level in rats. In order to detect the toxins directly in vivo, ^{125}I-labeled toxins would be required since otherwise a high dose exceeding the lethal dose would have to be used (26). However, we used a non-isotope–requiring method in this study that had been reported to be the most sensitive test to monitor the presence of active toxins in vivo, because a single toxin molecule can proteolyse a large number of SNAP-25 target molecules, providing a dramatic amplifying effect (16). In A1LL, we found cleavage SNAP-25 in the control of the contralateral nerve endings, but no SNAP-25 fragments were detected in the neurotomy and colchicine-treated models. This result

indicated that type A1 toxin transported to the contralateral foreleg via a nerve pathway. For A1LL and A1NTX to act on the innervated contralateral foreleg muscles, these toxins must be transported from the injected muscle to the spinal cord and between the ipsilateral and contralateral nerves in the spinal cord. Botulinum toxin binds to receptors on membranes of nerve endings and is introduced into neurons by endocytosis. The light chain of the toxin is translocated into the cytoplasm after acidification of endocytic vesicle. This vesicle was acidized at nerve endings. Then, the light chain cleaves SNARE protein. On the other hand, tetanus toxin, which has a similar structure to botulinum toxin, is also introduced into vesicle by endocytosis. However, the vesicle is not acidized at nerve ending. The tetanus toxin is retrogradely transported in these non-acidifiable vesicles in nerves. Then, the tetanus toxin is moved to other neurons in the spinal cord by transcytosis and acts on central nerves (29). In this study, the results suggest that A1LL and A1NTX were transported from injected muscle to spinal cord and between nerves as a tetanus toxin. When A1 toxin was injected into the hippocampus, the toxin was reported to cleave SNAP-25 in the contralateral side 3 days after injection (16). The two hippocampi are interconnected by commissural connections. The spinal cord also has nerve fibers that connect the two sides as a pathway that crossed extensor reflex follows. A1LL and A1NTX may be transported to the contralateral side via the nerve fibers. The mechanism of A1LL and A1NTX action in the spinal cord is a subject of future investigation.

In the neurotomy model, the grip strength of the contralateral foreleg after the injection of doses in excess of 0.78 log U of A1LL or A1NTX decreased, indicating that these toxins were transported to the contralateral foreleg not only via nerves but also via other pathways when these toxins were injected at high doses. To clarify the other pathways, antitoxin for botulinum toxin was injected at 1 h after the administration of 0.84 log U A1LL to control and neurotomy model rats. The grip strength of the contralateral foreleg in the A1LL-treated rat group without antitoxin decreased to about 150 gf over the 4 days after administration and that of the A1 antitoxin–treated rat group was about 75 gf. The neurotomy model rats injected with antitoxin 1 h after toxin administration did not show any decrease in the grip strength of the contralateral foreleg. These results indicate that the additional A1LL pathway excluding axonal transport is via the body fluid. The grip strength of the A1 antitoxin–treated rat group was reduced to one half of that in the A1LL-treated rat group without antitoxin. The antitoxin neutralizes toxins diffused from toxin-treated muscle to the body fluid; however, it does not neutralize toxins inside nerve cells (axonal transport of toxin). These results clearly indicate that A1LL was transported not only via nerves but also via the body fluid to the contralateral foreleg when a high dose beyond the axonal transport capacity (about 0.80 log U or more) was used.

When A2NTX was injected at more than 0.78 log U, the grip strength of the contralateral foreleg in the neurotomy and colchicine-treated groups decreased to almost the same level as that of the non-neurotomy and non-colchicine–treated groups. In addition, we used immunoblotting to determine if A2NTX was present in the contralateral foreleg. A2NTX cleaved SNAP-25 in the neurotomy and colchicine-treated models. These results indicated that A2NTX was not transported via a nerve pathway to the contralateral foreleg. To clarify the transport mechanism of A2NTX, antitoxin was injected 1 h after administration of the toxin at 1.20 log U. The grip strength of the contralateral foreleg did not show any decrease. This indicates that A2NTX was transported via the body fluid. A1LL diffused to the contralateral muscles via axons at a dosage from 0.30 to 0.84 log U and via both axons and the body fluid at high dosages of more than about 0.80 log U. The dose via the body fluid was almost the same as that of A2NTX (about 0.80 log U or more). Therefore, the doses of A1LL and A2NTX transported via the body fluid might be almost the same (about 0.80 log U or more), and the difference between toxins in the dose diffusing to the contralateral foreleg may have been caused by the presence or absence of the axonal transport of toxins.

A2NTX shares 89% amino acid sequence homology with A1NTX, 95% in light chains, and 87% in heavy chains (6). A1 toxins but not A2NTX underwent axonal transport, and the reason is considered to be a difference in the amino acid sequences between them. Very different amino acid sequences in toxins can be seen in the heavy chains, which are involved in the binding of receptors and translocation of the intraneuronal light chains. We considered the following possible mechanisms for the contralateral transport of these toxins: one hypothesis is that A1 toxin binds to receptors not only of acidifiable endocytic vesicles but also non-acidifiable ones as tetanus toxin–containing vesicles. In contrast, A2NTX may bind to receptors of only acidifiable vesicles. In another hypothesis, both toxins are retrogradely transported to the spinal cord. A1 toxin is moved into the contralateral nerve by transcytosis. In contrast, A2NTX is moved in another way.

In this study, A2NTX caused less muscle flaccidity of non-toxin–treated muscle than A1 toxins (A1LL or A1NTX) as commercial botulinum toxin products. It was suggested that the variation in the amino acid sequence between A1NTX and A2NTX causes the difference in

the spreading pathways. The required dose for spread (adverse effect) to the contralateral side is different according to the pathway.

Acknowledgments

The authors thank Mr. Ayataka Nagano, Mr. Yusuke Ohyama, Ms. Kaori Harada, and Ms. Shiho Itai for providing A2NTX; Ms. Miho Shinmura, Ms. Satomi Munechika, and Ms. Ayumi Tanaka for technical assistance; and Mr. Seiji Matsuo for statistical support.

References

1 Jankovic J. Botulinum toxin in clinical practice. J Neurol Neurosurg Psychiatry. 2004;75:951–957.
2 Sadick NS. Botulinum toxin type B. Dermatol Surg. 2003; 29:348–351.
3 Takeda M, Araki I, Mochizuki T, Nakagomi H, Kobayashi H, Sawada N, et al. The forefront for novel therapeutic agents based on the pathophysiology of lower urinary tract dysfunction: pathophysiology of voiding dysfunction and pharmacological therapy. J Pharmacol Sci. 2010;112:121–127.
4 Sakaguchi G. Clostridium botulinum toxins. Pharmacol Ther. 1983;19:165–194.
5 Montecucco C, Schiavo G, Rossetto O. The mechanism of action of tetanus and botulinum neurotoxins. Arch Toxicol Suppl. 1996;18:342–354.
6 Arndt JW, Jacobson MJ, Abola EE, Forsyth CM, Tepp WH, Marks JD, et al. A structural perspective of the sequence variability within botulinum neurotoxin subtypes A1-A4. J Mol Biol. 2006;362:733–742.
7 Dover N, Barash JR, Arnon SS. Novel Clostridium botulinum toxin gene arrangement with subtype A5 and partial subtype B3 botulinum neurotoxin genes. J Clin Microbiol. 2009;47:2349–2350.
8 Dressler D, Benecke R. Pharmacology of therapeutic botulinum toxin preparations. Disabil Rehabil. 2007;29:1761–1768.
9 Borodic GE, Joseph M, Fay L, Cozzolino D, Ferrante RJ. Botulinum A toxin for the treatment of spasmodix torticollis: dysphagia and regional toxin spread. Head Neck. 1990;12:392–399.
10 Camargo CHF, Teive HAG, Becker N. Cervical dystonia: clinical and therapeutic feature in 85 patients. Arq Neuropsiquiatr. 2008; 66:15–21.
11 Coté TR, Mohan AK, Polder JA, Walton MK, Braun MM. Botulinum toxin type A injections: adverse events reported to the US Food and Drug Administration in therapeutic and cosmetic cases. J Am Acad Dermatol. 2005;53:407–415.
12 Mancini F, Sandrini G, Moglia A, Nappi G, Pacchetti C. A randomized, double-blind, dose-ranging study to evaluate efficacy and safety of three doses of botulinum toxin type A (Botox) for the treatment of spastic foot. Neurol Sci. 2005;26:26–31.
13 Willis AW, Crowner B, Brunstrom JE, Kissel A, Racett BA. High dose botulinum toxin A for treatment of lower extremity hypertonicity in children with cerebral palsy. Dev Med Child Neurol. 2007;49:818–822.
14 Goldstein EM. Safety of high-dose botulinum toxin type A therapy for the treatment of pediatric spasticity. J Child Neurol. 2006;21:189–192.
15 Kuehn BM. FDA requires black box warnings on labeling for botulinum toxin products. JAMA. 2009;301:2316.
16 Antonucci F, Rossi C, Gianfranceschi L, Rossetto O, Caleo M. Long-distance retrograde effects of botulinum neurotoxin A. J Neurosci. 2008;28:3689–3696.
17 Sakaguchi G, Ohishi I, Kozaki S. Purification and oral toxicities of Clostridium botulinum progenitor toxins. In: Lewis GE, editor. Biomedical aspects of botulism. New York: Academic Press; 1981. p. 21–34.
18 Pearce LB, Borodic GE, First ER, MacCallum RD. Measurement of botulinum toxin activity: evaluation of the lethality assay. Toxicol Appl Pharmacol. 1994;128:69–77.
19 Meyer OA, Tilson HA, Byrd WC, Riley MT. A method for the routine assessment of fore- and hindlimb grip strength of rats and mice. Neurobehav Toxicol. 1979;1:233–236.
20 James KAC, Bray JJ, Morgan IG, Austin L. The effect of colchicine on the transport of axonal protein in the chicken. Biochem J. 1970;177:767–771.
21 Torii Y, Kiyota N, Sugimoto N, Mori Y, Goto Y, Harakawa T, et al. Comparison of effects of botulinum toxin subtype A1 and A2 using twitch tension assay and rat grip strength test. Toxicon. 2011;57:93–99.
22 Jones RGA, Corbel MJ, Sesardic D. A review of WHO international standards for botulinum antitoxins. Biologicals. 2006;34: 223–226.
23 Hallis B, James BAF, Shone CC. Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities. 1996;34:1934–1938.
24 Aoki KR. Botulinum neurotoxin serotypes A and B preparations have different safety margins in preclinical models of muscle weakening efficacy and systemic safety. Toxicon. 2002;40: 923–928.
25 Dressler D. Clinical features of antibody-induced complete secondary failure of botulinum toxin therapy. Eur Neurol. 2002; 48:26–29.
26 Tang-Liu DD, Aoki KR, Dolly JO, de Paiva A, Houchen TL, Chasseaud LF, et al. Intramuscular injection of 125I-botulinum neurotoxin-complex versus 125I-botulinum-free neurotoxin: time course of tissue distribution. Toxicon. 2003;42:461–469.
27 Wiegand H, Erdmann G, Wellhöner HH. 125I-labelled botulinum A neurotoxin: pharmacokinetics in cats after intramuscular injection. Naunyn Schmiedebergs Arch Pharmacol. 1976;292:161–165.
28 Dressler D, Adib Saberi F. Botulinum toxin: mechanisms of action. Eur Neurol. 2005;53:3–9.
29 Lalli G, Bohnert S, Deinhardt K, Verastegui C, Schiavo G. The journey of tetanus and botulinum neurotoxins in neurons. Trends Microbiol. 2003;11:431–437.

CASE REPORT

Gait and posture assessments of a patient treated with deep brain stimulation in dystonia using three-dimensional motion analysis systems

Shigetaka Nakao[1], Koji Komatsu[2], Waka Sakai[3], Michiharu Kashihara[1], Yuki Masuda[2], Koji Nishikawa[2], Tetsuya Okahisa[2], Shin Kondo[2], Toshifumi Osawa[2], Ryuji Kaji[4], Satoshi Gotoh[4], Shinji Nagahiro[5], Shinjiro Takata[1], and Natsuo Yasui[1]

[1]*Department of Orthopedics, Institute of Health Biosciences, the University of Tokushima Graduate School,* [2]*Division of Rehabilitation,* [3]*Department of Neurology, Tokushima University Hospital,* [4]*Department of Clinical Neuroscience, and* [5]*Department of Neurosurgery, Institute of Health Biosciences, the University of Tokushima Graduate School, Tokushima, Japan*

Abstract : Kinesiologic analysis of gait disorders, postural instabilities and abnormal movements is quite difficult to assess objectively by clinical observation, such as by specific scale and video recordings. In this study, we reported one of the aspects of the usefulness of three-dimensional motion analysis (Vicon Systems, Oxford, United Kingdom), which can measure inclusive data of movement disorders and substitute for conventional assessments. A 49-year-old man who had various dystonic symptoms, mainly on his left side of the body, responded well to deep brain stimulation (DBS). The examination quantified how the involuntary movements or other symptoms with dystonia changed before and after treatments. J. Med. Invest. 58 : 264-272, August, 2011

Keywords **:** *motion analysis, dystonia, DBS*

INTRODUCTION

Involuntary movements of dystonia interfere with normal posture and gait. Abnormal posture and gait disturbance are associated with frequent falls and restrict activities of daily living (ADL), which can reduce the quality of life (QOL). Deep brain stimulation (DBS) is a safe and successful therapeutic option for patients with gait disturbance in Parkinson's disease and dystonia. Globus Pallidus internus (GPi) DBS is effective for the treatment of generalized dystonia (1). GPi DBS for X-linked dystonia improves 67.9-80.6% of symptoms (2-5).

Functional neurological impairment has been measured by specific observational analysis, such as a rating scale, video monitoring, conventional three-dimensional kinematic recordings using multiple videos, electromyography and electro-goniometry for the analysis of gait, a force plate for the analysis of postural stability, and multiaxial accelerometers and gyroscopes for the analysis of movement disorders. Conventional three-dimensional kinematic recordings are complicated and time consuming, and it is difficult to extract data (6). The Vicon system is a simplified three-dimensional motion analysis system that integrates conventional approaches. Vicon motion measurements have been used in clinical and research laboratories combined with

Received for publication May 12, 2011 ; accepted June 28, 2011.

Address correspondence and reprint requests to Shigetaka Nakao, Department of Orthopedics, Institute of Health Biosciences, the University of Tokushima Graduate School, Kuramoto-cho, Tokushima 770-8503, Japan and Fax : +81-88-633-9313.

an MX camera, which captures three-dimensional optical marker-based technology, to provide inclusive motion data in detail. We used the Vicon system to assess a patient with generalized dystonia before and after the implantation of GPi DBS electrodes. In this paper, we present the outcome of a case of dystonia treated with DBS to show its therapeutic efficacy to improve postural alignment and gait disturbance.

CASE REPORT

Patient presentation

A 49-year-old Filipino male, native to the island of Panay, was admitted to our hospital to treat dystonia. He had been previously diagnosed with X-linked dystonia-parkinsonism (DYT3 ; "lubag" disease) (7, 8). He first presented with involuntary movements of his bilateral halluxes at the age of 41. He later developed involuntary movements of his upper and lower limbs, cervical dystonia and spinal torsion causing gait disturbance and abnormal posture at the age of 43.

His ambulation progressively worsened. The baseline United Dystonia Rating Scale (UDRS) score performed at our institution was 64/5/112, and his Burk-Fahn-Mardsden Scale was 57/120 (9). He was found to have dystonic symptoms mainly on the left side of his body with a retracted head, twisted trunk and involuntary movements of his upper limbs. He also had difficulties with vocalization, swallowing and eye opening. His dystonia was very disabling, greatly affecting his quality of life (QOL).

Experimental design

Motion analysis using the Vicon system was conducted to record kinematic and kinetic data during static posture and dynamic walking. The patient was evaluated in three-dimensional static posture and consecutive gait using the Vicon MX system (Vicon Motion Systems, Oxford, United Kingdom). Kinematic data were collected at 120 Hz using a passive eight-camera system (Vicon MX T20 ; Vicon Motion Systems). Kinetic parameters were recorded at 120 Hz using a four-embedded ground force platform (AMTI, model OR-06 ; Advanced Mechanical Technology, Watertown, MA). Kinematic and kinetic systems were synchronized for simultaneous collection. Nexus 1.4 software (Vicon Motion Systems) derived the kinematic and kinetic parameters of standing for thirty seconds and five times walking on a ten-meter walkway.

Markers (14-mm diameter) which reflected infrared rays were placed on landmarks of the whole body following the Plug-in-Gait model (Fig. 1, Vicon Motion Systems). Eight MX cameras captured the motion of markers and Nexus 1.4 processed the motion data as stick images from marker positions in three dimensions. Nexus 1.4 extracted c3d data (three-dimensional coordinated data of model) extracted from the plug-in-gait model parameters. Polygon 3.1 software (Vicon Motion Systems) simulated the human motion (skeletal model) expressed by the rigid body of the plug-in-gait model from c3d data. In addition, Medicaptures (Winpod, Balma, France) were used to record the distribution of foot pressure and the tracks of length (LNG) by postural sway of the center of mass (COM) in static posture, which showed the stability of static balance. He was evaluated before and after DBS (16 days after DBS) using Vicon. He was examined using various parameters such as neck angle (the angles between the head relative to the thorax) for cervical dystonia and spine angle (the angles between the thorax relative to the pelvis) for spinal torsion, COM changes for the stability of dynamic balance, patterns of ground reaction force (the force exchanged between the foot and the ground while walking) for symmetrical motion of limbs, and gait parameters (cadence, speed, step

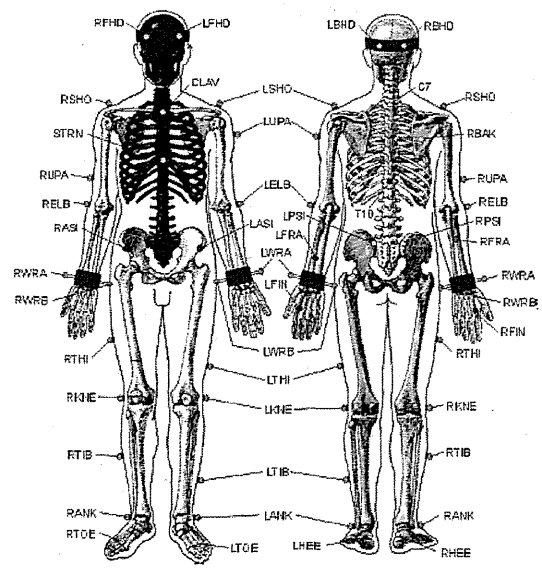


Figure 1. Plug-in gait marker placement
Thirty-five reflective markers were placed on landmark of the whole body. The distribution of markers consisted of head (four), trunk (nine), upper limbs (fourteen) and lower limbs (ten).