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Comparison of the therapeutic indexes of different molecular forms of botulinum toxin type A

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Abstract

Botulinum toxin is produced by *Clostridium botulinum* in three different molecular-weight forms: LL toxin, 900 kDa; L toxin, 500 kDa; and M toxin, 300 kDa. We isolated the M toxin, then compared its muscle-weakening efficacy with those of L+LL toxin and BOTOX® both in vitro and in vivo. The twitch tension of the mouse isolated phrenic nerve-hemidiaphragm was used for the in vitro study. For the in vivo study, grip strength was measured in the toxin-injected legs. Undesirable muscle weakening was evaluated by grip-strength measurement in the contralateral leg. Concentration–response curves for effects on the phrenic nerve-hemidiaphragm showed that M toxin was 10 times more potent than L+LL toxin. The therapeutic index in vivo was 3- to 5-times higher for M toxin than for L+LL toxin or BOTOX®, indicating a greater separation for M toxin between doses with local efficacy and systemic toxicity. These findings indicate that the M toxin preparation may have a better pharmacological profile than the conventional preparation.

Keywords: Botulinum toxin type-A; M toxin; Muscle weakening; Grip strength

1. Introduction

Botulinum toxin is secreted by the anaerobic bacterium *Clostridium botulinum*. It is well established that its principal target is the cholinergic nerve ending at the neuromuscular junction, where inhibition of acetylcholine release by the toxin results in neuromuscular blockade and paralysis (Kao et al., 1976; Simpson, 1981). Botulinum toxin type A (BTX-A) is one of seven different botulinum toxin serotypes, which are referred to alphabetically as types A, B, C_1, D, E, F, and G.

Over the past two decades, BTX-A has become one of the most important therapeutic agents for neurological disorders such as blepharospasm, hemifacial spasm, and cervical dystonia. In addition, BTX-A has recently been applied cosmetically to reduce or eliminate facial wrinkles (Rohrich et al., 2003). BOTOX®–a preparation of BTX-A with a higher safety margin than other commercially available preparations of botulinum toxin (Aoki, 2001; Aoki, 2002)– is the preparation used most widely during the past decade. Disadvantages of treatment with BOTOX® or other BTX-A preparations include (1) short-term complications such as ptosis, dry mouth, and dysphagia, which are the result of undesirable diffusion away from the injection site, and (2) a decrease in efficacy upon repeat injection (Jankovic and Schwartz, 1995; Dutton, 1996; Price et al., 1997).

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Botulinum toxins are produced as protein complexes containing a 150-kDa neurotoxin protein together with one or more nontoxin proteins, (e.g., hemagglutinin or nontoxic nonhemagglutinin protein; Inoue et al., 1996; Chen et al., 1998). The protein complexes, called progenitor toxins, are of 900 kDa (LL toxin), 500 kDa (L toxin), or 300 kDa (M toxin). Conceivably, the differences in molecular size and in the nontoxin constituents of these progenitor toxins might lead to differences in the distribution of the injected toxin and in the immunologic resistance shown by the host, which could in turn lead to different levels of efficacy and/or safety among toxins in clinical use. However, the comparative properties of these progenitor toxins have not been examined.

The present study was designed to assess the clinical usefulness of M toxin, the lowest-molecular-size complex. To this end, we conducted in vitro and in vivo experiments to compare the efficacies of M toxin, L+LL toxin, and BOTOX® [BOTOX® being a product of the type A Hall strain, which produces LL toxin (Henderson et al., 1997)].

2. Materials and methods

2.1. Purification of toxins

Two *C. botulinum* type A strains, 7I03-H and 62A, were used to obtain toxins. Strain 62A produces all three types of BTX-A progenitor toxin (M, L, and LL), whereas strain 7I03-H produces only M toxin (Tabita et al., 1990). M toxin from strain 7I03-H and a mixture of L and LL toxins from strain 62A were prepared as previously described (Sakaguchi, 1983), with minor modifications. Briefly, the cells were cultured in cooked-meat medium supplemented with glucose 0.3% and starch 0.2% for 2 days at 30 °C. A portion of this culture in cooked-meat medium was inoculated into PYG medium, which consisted of 2% peptone, 0.5% yeast extract, 0.5% glucose, and 0.025% sodium thioglycolate. After incubation for 3 days at 30 °C, the culture was adjusted to pH 3.5 by adding 1.5 M sulfuric acid. The precipitate was collected by centrifugation, and the crude toxin was extracted using 0.2 M phosphate buffer (pH 6.0). The extract was then treated with 2% protamine sulfate, and the precipitate removed by centrifugation. The supernatant was precipitated at a 60% saturation of ammonium sulfate, and the precipitate, collected by centrifugation, was dissolved in 0.05 M acetate buffer containing 0.2 M sodium chloride (pH 4.2). The solution was dialyzed, and the dialyzed material applied to a SP-Sepharose Fast Flow column (Amersham Biosciences, Tokyo, Japan) equilibrated with the same acetate buffer. Then, the toxin was eluted using a linear gradient of sodium chloride concentrations from 0.2 M to 0.7 M. The fractions containing the M toxin and a mixture of L and LL toxins were pooled separately and concentrated by ultrafiltration. The concentrated solutions were subjected to gel-filtration on a column of Sephadex G-200 equilibrated with 0.05 M acetate buffer containing 0.2 M sodium chloride (pH 6.0). Since the chromatography steps did not allow L and LL toxins to resolve into separate fractions, the mixture of L and LL toxins (referred to as "L+LL toxin") was used in the following experiments. Each purified toxin was stored at −80 °C until used.

2.2. Experimental animals

Male ddY mice (20–33 g), purchased from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan), were kept under controlled light/dark conditions with food and water available ad libitum. All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, which has been approved by the Japanese Pharmacological Society.

2.3. Twitch tension assay (in vitro)

The in vitro assay was conducted as previously described (Sugimoto et al., 1992), with minor modifications. Phrenic nerve-hemidiaphragm preparations were isolated from ddY mice, 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 7.2–7.4. The costal margin of the muscle was fixed and the central tendon connected to a tension transducer (TB-651T; Nihon Kohden, Tokyo, Japan) by a string. The preparation was suspended vertically 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 and 10 ms at a frequency of 0.25 Hz. The

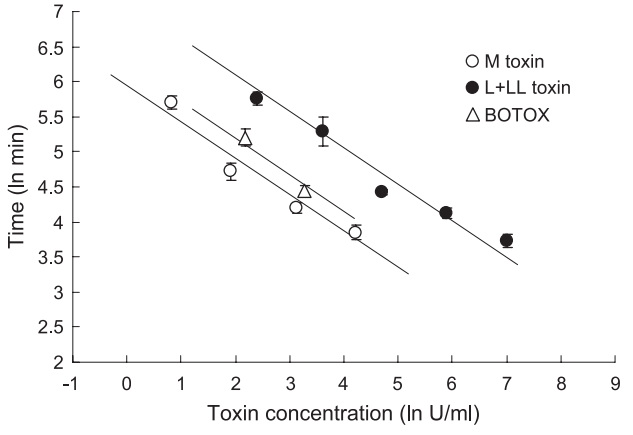

Fig. 1. Concentration–effect curves of three different forms of BTX-A. Mouse phrenic nerve-hemidiaphragm preparations were treated with various concentrations of the preparations of botulinum toxin type A. The response was expressed as the time required for twitch tension to decline to 1/e of that observed immediately before application of toxin. For definition of "U", see Materials and methods. Values are mean±S.E.M., n=3 or 4. Regression lines are drawn with the slope of the line obtained with purified neurotoxin of botulinum type A.

tension transducer was connected to an amplifier (EF-601G; Nihon Kohden), and muscle tension was recorded on paper using a pen recorder (RJG-4124; Nihon Kohden). After the twitch tension had become stable, one of the purified toxins [diluted appropriately with 20 mM Tris–HCl containing 150 mM NaCl and 0.02% bovine serum albumin (pH 7.4)] was applied to the bathing solution. The reduction in twitch tension was recorded, and the time required for twitch tension to decline to 1/e of that observed immediately before toxin application was calculated.

2.4. Estimation of biologic activity of toxins

The biologic activities of the purified toxins and BOTOX® (Allergan, Irvine, CA, USA) were determined by measurement of the mouse intraperitoneal (i.p.) LD_{50} values. In this study, the LD_{50} is given the value "1.0 Unit (U)" (i.e., 1.0 LD_{50}=1.0 U). As in a previous study assaying mouse lethality to estimate the biologic activity of botulinum toxin (Pearce et al., 1994), the LD_{50} was determined using an assay involving 5 doses and 10 animals per group, and the chosen evaluation period was the first 72 h after the i.p. injection.

2.5. Measurement of grip strength (in vivo)

To evaluate in vivo efficacy, each mouse received a single intramuscular injection of one of the toxins (0.3, 1, 3, 10, or 30 U/kg) or vehicle into the right gastrocnemius muscle at a volume of 250 μl/kg. Injections were made using a 30-gauge needle fitted to a sterile 25 μl Hamilton syringe. Grip strength was measured in the right hind leg of

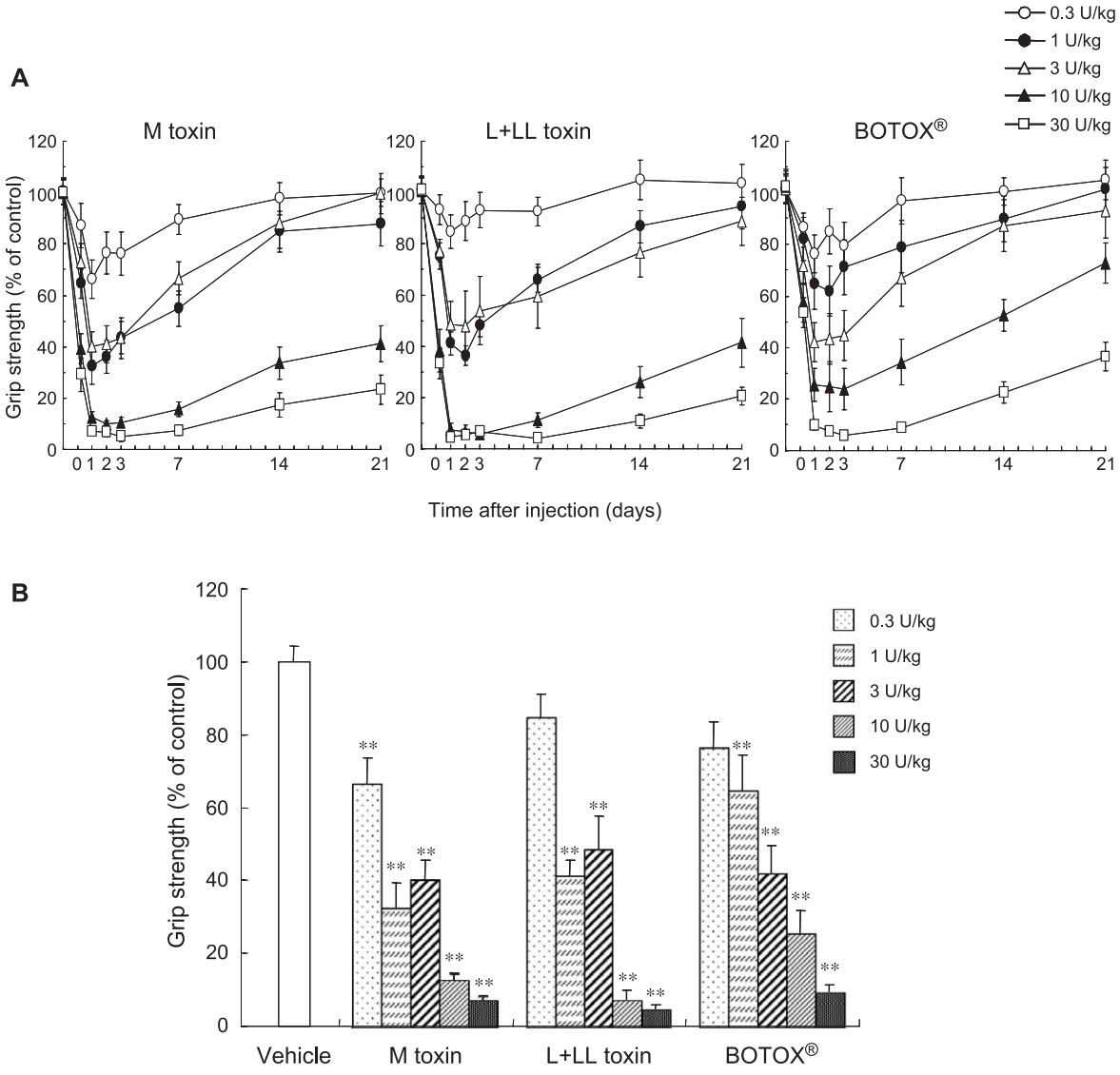

Fig. 2. Grip strength as a function of time after injection in mice. Mice received 250 μl/kg of M toxin, L+LL toxin, or BOTOX® (each at 0.3, 1, 3, 10, or 30 U/kg) into the right gastrocnemius muscle. Grip strength was measured for the right hind leg of each mouse at 0.25, 1, 2, 3, 7, 14, and 21 days after the injection (A). Data of the grip strength at 1 day after the injection were extracted and statistical analysis was made (B). Values are mean±S.E.M., n=10. **P<0.01 vs. vehicle (Dunnett's test).

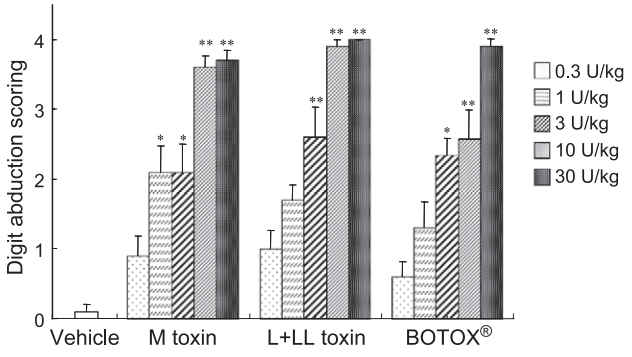

Fig. 3. Effects of three preparations of botulinum toxin type A on digit abduction in mice at 1 day after the injection. Mice received 250 μl/kg of M toxin, L+LL toxin, or BOTOX® (each at 0.3, 1, 3, 10, or 30 U/kg) into the right gastrocnemius muscle. Values are mean±S.E.M., n=10. *P<0.05 and **P<0.01 vs. vehicle (Dunnett's test).

each mouse using a Grip Strength Meter (Neuroscience, Osaka, Japan) at 0.25, 1, 2, 3, 7, 14, and 21 days after the injection. In addition, a digit abduction scoring method was conducted as previously described (Aoki, 2001). In this method, the varying degrees of digit abduction were scored on a five-point scale (0=normal to 4=maximal reduction in digit abduction and leg extension) by an observer who was masked as to the treatment.

To evaluate the extent of undesirable systemic diffusion of each toxin, each mouse received a single intramuscular injection of a given toxin (10, 25, 50, 75, or 100 U/kg) or vehicle into the right quadriceps muscle at a volume of 500 μl/kg. The grip strength in the contralateral left hind leg of each mouse was measured at 1, 2, 3, 7, and 14 days after the injection.

In another experiment, mice were injected with one of the toxins (0.015 U) into the right gastrocnemius muscle, and the grip strength of the right hind leg was measured. Then, 28 days after the first injection, they were injected again into the same muscle at the same dose, and their grip strength was measured again in the same manner.

2.6. ED_{50}, TD_{20}, and therapeutic index

Grip-strength data are expressed as a percentage of vehicle. Then, the values obtained for peak muscle-weakening effects at each dose were entered into regression equations that were used to calculate the doses at which 50% (ipsilateral to the injection site) and 20% (contralateral) reductions occurred to determine local efficacy and undesirable systemic muscle weakening, and these values were termed Effective Dose 50 (ED_{50}) and Toxic Dose 20 (TD_{20}), respectively (see Discussion). The ratio TD_{20}/ED_{50} was calculated for each toxin, and is expressed as the therapeutic index.

2.7. Statistical analysis

Results for grip strength are expressed as mean±S.E.M. Data were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test or a Student's t-test, with a significant difference being accepted at P<0.05.

3. Results

3.1. Effects of toxins on twitch tension (in vitro)

The preparations of botulinum toxin each caused an attenuation with time of the twitch tension elicited in diaphragm by the electrical stimulation of the phrenic nerve in vitro. The kinetics of the attenuation were well fitted to an exponential curve, except for the initial phase. Hence, the time for the twitch tension to decline to 1/e of that observed immediately before the toxin application was used as an index of the tissue response to the toxin. Comparison of the linear range of the concentration–response relationship revealed that M toxin was roughly 10- and 2- times more potent than L+LL toxin and BOTOX®, respectively, in its neuromuscular blocking activity (Fig. 1).

3.2. Grip-strength measurement for efficacy test (in vivo)

All three types of BTX-A reduced the grip strength in the toxin-injected leg. The tested toxins had roughly dose-related

Table 1
ED_{50}, TD_{20}, and therapeutic index values for three types of BTX-A (M toxin, L+LL toxin, and BOTOX®)

Toxin	ED_{50} (U/kg)[a]		TD_{20} (U/kg)[b]		Therapeutic index[c]	Ratio[d]
	Value	95% confidence interval	Value	95% confidence interval		
M toxin	0.57	0.28–0.93	37.3	22.5–76.4	65.4	5.1
L+LL toxin	0.88	0.54–1.27	17.0	13.3–20.6	19.3	1.5
BOTOX®	1.27	0.73–1.94	16.2	9.4–22.4	12.8	1

[a] ED_{50}, dose at which 50% reduction occurred in evaluation of efficacy.
[b] TD_{20}, dose at which 20% reduction occurred in evaluation safety.
[c] Therapeutic index, TD_{20}/ED_{50}.
[d] Ratio, therapeutic index for the relevant toxin/therapeutic index for BOTOX®.

effects in terms of the magnitude and duration of the muscle weakness they induced, with their maximal effects being seen at 1–3 days after the injection (Fig. 2A). Fig. 2B summarized the muscle-weakening effects of these toxins at 1 day after the injection. M toxin had a statistically significant muscle-weakening effect at a dose of 0.3 U/kg or more, whereas L+LL toxin and BOTOX® had no significant effect at 0.3 U/kg. When we evaluated the muscle-weakening effects using a digit abduction scoring method, a statistically significant effect was not observed at a dose of 0.3 or 1 U/kg of L+LL toxin or BOTOX®, whereas M toxin had a significant effect at 1 U/kg or more (Fig. 3). Although the differences were small, the ED_{50} value obtained for M toxin (calculated from the grip-strength data) was the lowest among the toxins tested (Table 1).

3.3. Grip-strength measurement for safety test (in vivo)

The safety of each toxin was evaluated by measuring grip strength in the contralateral, toxin-untreated leg (left hind leg) after injection into the intramuscular right quadriceps muscle. Using this method, any undesired muscle-weakening effect induced by toxin that had leaked from the injected muscle can be evaluated. Fig. 4 shows the time-course of changes in grip strength in the contralateral hind leg after toxin injection. Because large numbers of mice died after intramuscular injection of 100 U/kg of some toxins, data for this dose are not shown in Fig. 4 and were not used to calculate the TD_{20} values shown in Table 1. Maximal effects were obtained at 2 or 3 days after the injection, and grip strength recovered within 2 weeks. The dose-related muscle-weakening effects were comparable between L+LL toxin and BOTOX® (Fig. 4), and these toxins showed greater toxicity than M toxin (at 100 U/kg, 9 mice and 7 mice died following injection of L+LL toxin and BOTOX®, respectively, whereas only 1 mouse died following M toxin injection). The TD_{20} value was higher for M toxin than for L+LL toxin or BOTOX® (Table 1). The higher TD_{20} value indicates a weaker systemic effect of M toxin than of the other toxins.

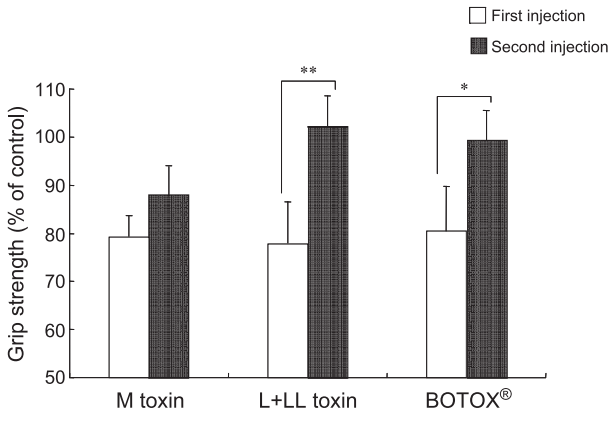

Fig. 5. Reduced effect on grip strength upon repeat treatment with each preparation of botulinum toxin type A. Mice received M toxin, L+LL toxin, or BOTOX® (0.015 U) into the right gastrocnemius muscle. Twenty-eight days after the first injection, the same toxins were injected again in the same manner. Grip strength at 1 day after the first or second injection is shown. Values are mean±S.E.M., n=11 or 12. *P<0.05 and **P<0.01 vs. first injection.

3.4. Therapeutic index

The TD_{20}/ED_{50} ratio for each toxin was taken as the therapeutic index (see Table 1). The therapeutic index was higher for M toxin than for L+LL toxin or BOTOX®, and the therapeutic indexes for M and L+LL toxins were 5.1- and 1.5-times, respectively, higher than that of BOTOX®. This

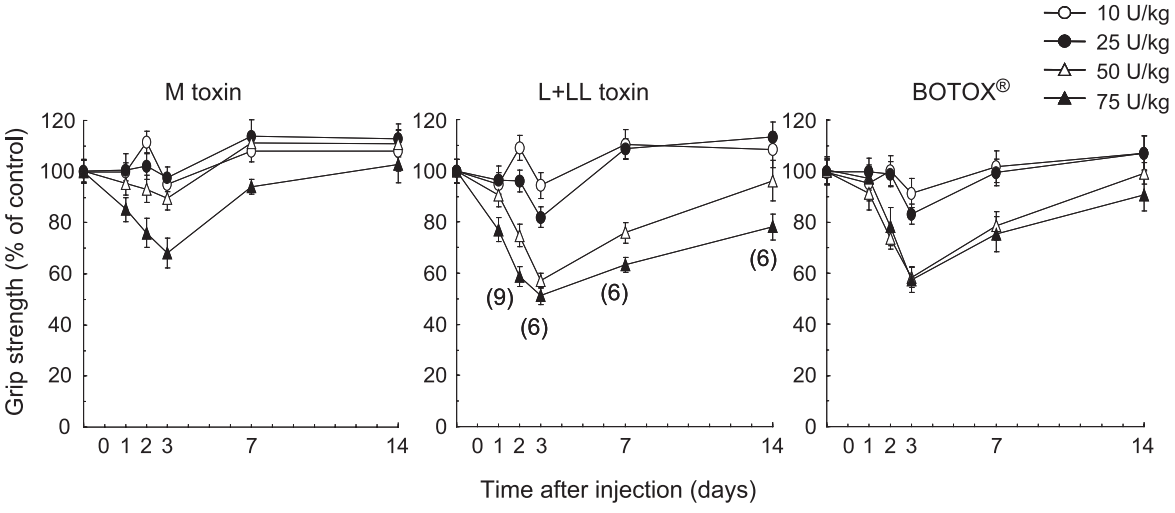

Fig. 4. Grip strength in contralateral leg as a function of time after injection in mice. Mice received 500 μl/kg of M toxin, L+LL toxin, or BOTOX® (each at 10, 25, 50, or 75 U/kg) into the right quadriceps muscle. Left grip strength was measured in each mouse at 1, 2, 3, 7, and 14 days after the injection. Values are mean±S.E.M. (n=10, except for 75 U/kg of L+LL toxin, where numbers in parenthesis indicate numbers of surviving animals).

indicates that the separation between the locally effective and systemic toxic doses is greater for M toxin than for the other toxins (following intramuscular injection).

3.5. Reduced muscle-weakening effect upon repeated injection

Fig. 5 shows grip strength at 1 day after the first and second injections of toxins (0.015 U). Since the first toxin injection might cause a body-weight loss as a systemic side effect, we did not adopt dose per body weight, instead the absolute numbers of units of a given toxin were kept the same for the two injections. The difference in muscle-weakening effect between the first and second injections reached statistical significance for both L+LL toxin and BOTOX®, but not for M toxin.

4. Discussion

In the present in vitro experiment, M toxin displayed 10- and 2-times higher inhibitory activity towards neuromuscular transmission than L+LL toxin and BOTOX®, respectively. The binding of a toxin to its receptor and a subsequent endocytosis are thought to occur after a dissociation of 150-kDa neurotoxin protein from the progenitor–toxin complex. This might be thought to indicate that the dissociation rate is faster for M toxin than for L/LL toxin, and that the difference in efficacy between these toxins in the in vitro experiment is due to the difference in their dissociation rates.

The present in vivo biologic evaluations revealed some distinct advantages of M toxin over L+LL toxin or BOTOX®. Although all the toxins examined displayed dose-dependent muscle-weakening effects and similar durations of action, they differed in their efficacy and safety profiles, as illustrated by the lower ED_{50} and higher TD_{20} values obtained for M toxin. The small difference in ED_{50} values among the three toxins largely resulted from the difference in their muscle-weakening efficacies at lower doses (0.3 or 1 U/kg), because the ED_{50} values for M toxin, L+LL toxin, and BOTOX® were 0.57, 0.88, and 1.27 U/kg, respectively, values at the lower end of the dose-range employed in the present study.

In most mice, the grip strength of the contralateral legs was reduced by less than 50% of the value obtained for the vehicle-treated group (Fig. 4); hence, the TD_{20} value was adopted as the index of an undesirable muscle-weakening effect. Treatment with botulinum toxin carries with it a number of complications, the commonest of which are related to acute local effects, including weakening of the levator muscle resulting in ptosis, the corneal consequences of lagophthalmos, facial numbness, and dysphagia (Dutton, 1996). Therefore, it was important to evaluate the safety profile of M toxin, even though we had to use TD_{20} rather than the intramuscular LD_{50} value, which is generally used to determine therapeutic indexes. In the present study, the therapeutic index was 3- to 5-times higher for M toxin than for L+LL toxin or BOTOX®. This difference may result from differences in the constituent molecules. A study investigating the molecular composition of progenitor toxins suggested (i) that M toxin is formed by a conjugation of the 150-kDa neurotoxin with nontoxic nonhemagglutinin protein, while L toxin is formed by a conjugation of M toxin with hemagglutinin, and that LL toxin is a dimer of L toxin, and (ii) that therefore L and LL toxins each have hemagglutinin activity, but M toxin does not (Inoue et al., 1996). Nontoxin components, including hemagglutinin, have been reported to increase a given toxin's oral toxicity by protecting it from the low pH of the stomach and from intestinal protease (Sharma and Singh, 1998). HA1, which is one of the subcomponents of hemagglutinin, has been suggested to perform the important role of aiding the internalization of a given toxin into the bloodstream by binding to oligosaccharides lining the intestine (Fujinaga et al., 1997; Inoue et al., 1999). This is supported by the finding that anti-HA1 monoclonal antibodies inhibit the binding of botulinum type C neurotoxin to the epithelial cells of the intestine, and abolish or reduce its oral toxicity in mice (Mahmut et al., 2002). Although there have been no reports of the effect of hemagglutinin on toxin kinetics after its intramuscular injection, our results indicate that toxins that contain hemagglutinin may transfer from muscle tissue to bloodstream more easily, which may explain the higher ED_{50} and lower TD_{20} values obtained for L+LL toxin and BOTOX® than for M toxin. It is interesting to note that toxins which have each biological activity (mouse i.p. LD_{50} value) have different therapeutic indexes. Our results suggest that i.p. LD_{50} value could not be enough information for predicting efficacy and safety in administering intramuscularly in clinical uses.

One of the greatest concerns about the clinical use of BTX-A is the relatively weak effect obtained upon repeat administration, which is thought to be caused by the formation of a neutralizing antibody (Comella, 2002). In the present study, grip strength was significantly reduced upon a repeat injection of L+LL toxin or BOTOX® compared with that observed upon the first injection. In contrast, the difference in the effects of M toxin between the first and second injections was smaller, and the two effects were not significantly different from each other. The molecular weight of M toxin is lower than that of L or LL toxin, and therefore immunologic resistance against M toxin may be weaker than those against the L and LL toxins. Be that as it may, this result at least suggests that the frequency with which a nonresponse occurs to a repeat injection may be minimized by using M toxin for clinical purposes.

In summary, our in vitro experiment suggests that inhibitory activity against muscle-nerve transmission may be greater for M toxin than for L+LL toxin or BOTOX®. Moreover, our in vivo study showed that compared to BOTOX®, M toxin has a higher therapeutic index and is

likely to be associated with a lower frequency of loss of efficacy upon repeat injection. The different therapeutic indexes found in the present study between M toxin and L/LL toxin suggest that these toxins differ in the extent to which they are translocated away from the intramuscular injection site.

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