Table 4 Scores and summary statistics of TWSTRS-severity Scale (patient 2)

	A						B	C	D	E	F	Total
	A 1	A 2	A 3	A 4	A 5	Total of A 0-12	0-10	0-2	0-3	0-4	0-4	0-35
Numbers of rater	27	27	27	27	27	27	27	27	27	27	27	27
Mean	3.7	1.7	1.0	0.1	0.2	6.6	9.3	1.1	0.9	2.5	3.5	24.0
Standard deviation	0.5	0.7	0.8	0.3	0.4	1.6	1.0	0.4	0.6	0.8	1.3	3.0
Maximum	4	3	2	1	1	9	10	2	2	4	4	30
Minimum	3	0	0	0	0	4	8	0	0	1	0	18
Reference (Mean of 3 raters)	4	2	2	0	0	8	8	1	1	3	4	25

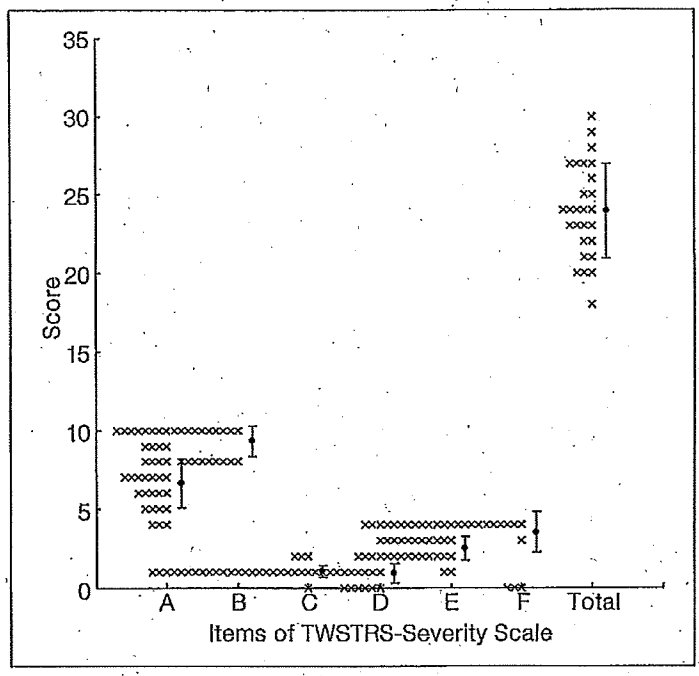

Fig. 2 Score of each rater and summary statistics of the TWSTRS-severity Scale (patient 2)

2．要約統計量の算出

TWSTRS-重症度スケールのスコアを，患者映像別に，Table 3, 4 および Fig. 1, 2 に示した。また，図表には，項目別スコアおよび合計スコアの要約統計量(評価者数，平均値，標準偏差，最大値，最小値) も図示し，さらにエラン社から提供された「正解」も参考として示した。

患者映像 1 の要約統計量を Table 3 および Fig. 1 に示した。その結果，27 人の評価者における TWSTRS-重症度スコアの合計点の平均値は 17.8 点であり，エラン社から提供された「正解」の 18 点とほぼ等しい数値であった。また，A～F の項目ごとの平均値も，エラン社の「正解」とほぼ一致した。

患者映像 2 の要約統計量を Table 4 および Fig. 2 に示した。患者映像 2 における TWSTRS-重症度スコアの合計点の平均値は 24.0 点であり，エラン社から提供された「正解」の 25 点とほぼ等しい数値であった。また，A～F の項目ごとの平均値は，患者映像 1 ほどではないものの，エラン社の「正解」と近い数値であった。

3．評価者間信頼性の検討

TWSTRS-重症度スケールの合計点を用いて，評価者間信頼性の指標として級内相関係数 (Intraclass Correlation Coefficient; ICC) とその 95%信頼区間の下限を算出し，Table 5 に示した。なお，エラン社が実施した信頼性試験 (評価者数：3 人，患者映像数：10 名) の結果も，参考として示した。その結果，ICC=0.745 (95%信

Table 5 Intraclass correlation coefficient of TWSTRS-severity Score

	Intraclass Correlation Coefficient (ICC)	The lower limit of 95% confidence interval
Results of this study	0.745	0.414
Reference	0.763	0.534

頼区間の下限：0.414)となり，エラン社が外国で実施した信頼性試験のICC＝0.763とほぼ同様な値であった。

III. 考　　察

痙性斜頸の多様な症候を統一して評価する目的で，いくつかの評価スケールが発表されている。従来多く用いられたのはTsui評価スケール[9,10]であり，わが国でもA型ボツリヌス毒素の治験の際，これを改良したTsui変法スケールが用いられた[11,12]。Tsui変法スケールは，回旋と前後屈を4段階から5段階とし，体軸の偏倚として側彎も加えられている。しかしながら，これらの評価では痛みなど，しばしば患者の主訴となる症状が評価されないため疾患の重症度を必ずしも反映しているとはいえない。また，患者によっては偏倚として回旋，側屈，前後屈以外に，側方偏倚や前後偏倚といったより複雑な斜頸姿勢を有する場合もある。このような背景のもと，最近の臨床研究ではむしろ，より詳細な評価法であるTWSTRSが推奨されている。今回はこのTWSTRSについて評価者間信頼性を検討した。

TWSTRS評価者間信頼性は，27名の日本人評価者を対象にエラン社が米国で作成したTWSTRS-重症度スケールトレーニングビデオを用いて，本評価のトレーニングおよび評価基準の統一化を行ったうえで実施し，評価者間信頼性の指標としては一般的によく用いられるICC[8]を用いた。ICCは，評価者間の評価の一致性が高い場合には1に近い値をとり，一致性が低くなるにしたがい0に近づく係数である。今回の検討結果，ICC＝0.745（95%信頼区間の下限：0.414）となり，エラン社が外国で実施した信頼性試験のICC＝0.763とほぼ同様な値であった。また，Conskiら[7]が実施したTWSTRSの信頼性試験は，3名の評価者が26名の患者を評価して行われたが，その結果はICC＝0.79であり，substantial (ICC＝0.6-0.8) であるとしている。

以上より，日本においてもTWSTRSの十分な評価者間の信頼性が確認できたと考えられた。また，TWSTRS合計スコアおよび下位項目の平均値がエラン社の基準値と類似していることから，日本と米国の評価の均一性も確認された。

[付記]

TWSTRS評価者間信頼性検討会参加施設および医師名（順不同，敬称略，所属名は2002年7月試験実施当時）

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Quantitative determination of biological activity of botulinum toxins utilizing compound muscle action potentials (CMAP), and comparison of neuromuscular transmission blockage and muscle flaccidity among toxins

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ABSTRACT

The biological activity of various types of botulinum toxin has been evaluated using the mouse intraperitoneal LD_{50} test (ip LD_{50}). This method requires a large number of mice to precisely determine toxin activity, and so has posed a problem with regard to animal welfare. We have used a direct measure of neuromuscular transmission, the compound muscle action potential (CMAP), to evaluate the effect of different types of botulinum neurotoxin (NTX), and we compared the effects of these toxins to evaluate muscle relaxation by employing the digit abduction scoring (DAS) assay.

This method can be used to measure a broad range of toxin activities the day after administration. Types A, C, C/D, and E NTX reduced the CMAP amplitude one day after administration at below 1 ip LD_{50}, an effect that cannot be detected using the mouse ip LD_{50} assay. The method is useful not only for measuring toxin activity, but also for evaluating the characteristics of different types of NTX. The rat CMAP test is straightforward, highly reproducible, and can directly determine the efficacy of toxin preparations through their inhibition of neuromuscular transmission. Thus, this method may be suitable for pharmacology studies and the quality control of toxin preparations.

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1. Introduction

Clostridium botulinum produces toxins that have been classified into 7 serotypes, A–G, based on their immunological characteristics (Sakaguchi, 1983). The toxins act on neuromuscular junctions and induce muscle relaxation by inhibiting acetylcholine release (Sakaguchi, 1983; Jahn and Niemann, 1994). The toxins cause muscle flaccidity, and have recently been utilized to treat spasm in myotonus and dystonia (Jankovic, 2004). Type A and B toxins were approved as drugs for treatment, and are clinically applied. Type C, E, and F toxins have been used in clinical studies (Mezaki et al., 1995; Eleopra et al., 1997, 1998, 2004). The toxins cleave SNARE proteins (i.e., SNAP-25, synaptobrevin, and syntaxin), which fuse to the synaptic vesicle and nerve cell membrane, blocking neuromuscular transmission by

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inhibiting the release of acetylcholine from synaptic vesicles. The different types of toxin are selective for specific SNARE proteins. Type A and E toxins cleave SNAP-25, type B toxin cleaves synaptobrevin II, type C toxin cleaves syntaxin and SNAP-25, and type D, F, and G toxins cleave synaptobrevin I and II. The cleavage sites for each toxin differ, even when toxins cleave the same protein (Montecucco and Schiavo, 1994; Schiavo et al., 2000).

The biological activity of botulinum toxins has been evaluated using the mouse intraperitoneal (ip) LD_{50} test (Pearce et al., 1994). This method is not an assessment of toxin efficacy, which is the inhibition of neuromuscular transmission, but of lethality due to respiratory muscle paralysis caused by the toxin. In this method, the results vary due to the individual sensitivity of mice against toxins, and many mice are required to ensure sufficient accuracy levels for the quality control of preparations. Therefore, the method has posed a problem with regard to animal welfare. International meetings on alternative methods for animal testing have been held, and replacements for the mouse ip LD_{50} test have been discussed (Straughan, 2006). The 3 Rs (refinement, reduction, and replacement) have been proposed for alternative methods, and alternative *in-vitro*, *ex-vivo*, and *in-vivo* test systems have been investigated. The *in-vitro* test system, ELISA, determining the endopeptidase activity, does not use animals, but the sensitivity is lower than the mouse bioassay (Hallis et al., 1996; Wictome et al., 1999). This method could determine only light-chain activity in many cases, and the inaccurate determination of toxin function has been reported. The *ex-vivo* test system using the mouse phrenic nerve-hemidiaphragm is sensitive, but it requires a skilled technique, and has a low reproducibility (Bigalke et al., 2001; Yoneda et al., 2005). The *in-vivo* test systems, such as the digit abduction scoring (DAS) and local flaccid paralysis assays, use scores for evaluation (Aoki, 2001; Takahashi et al., 1990; Sesardic et al., 1996). The methods of evaluation involved scoring, and so they have a disadvantage in that the obtained data are discrete quantities. As each test systems has disadvantages; no alternative method has been established.

We attached a greater importance to the following point in devising an alternative method to determine the activity of botulinum toxin: The potency of botulinum toxin should be evaluated based on their pharmacological effect of inhibiting neuromuscular transmission, and not based on their lethal activity, as in the mouse ip LD_{50} assay. There are several test systems to evaluate the inhibition of neuromuscular transmission, and we focused on the measurement of the compound muscle action potential (CMAP) used for the diagnosis of various nervous disorders. The CMAP is generated by the contraction of muscle fibers; the microcurrent generated by muscle contraction is amplified and recorded. Botulinum toxin affects nerve endings to suppress neurotransmission. Therefore, by determining the CMAP amplitude, the action of the toxin suppressing the transmission of electric stimulation to the muscle can be shown numerically. CMAP measurement is utilized by the extensor digitorum brevis (EDB) test, which checks the response to the toxin before treatment in patients who might have antibodies to the botulinum toxin (Kessler and Benecke, 1997). It was reported that the CMAP amplitude was measured in the rat gastrocnemius muscle which was injected several times with botulinum toxin (Cichon et al., 1995). The CMAP amplitude decreased as the toxin activity increased, but quantitative determination of the toxin was not carried out.

In this study, based on this previous CMAP study, we investigated the quantification of biological activity (effect of neuromuscular transmission blockage) by different types of botulinum toxin. We also compared toxins with an inhibitory effect on neuromuscular transmission. In addition, we investigated the muscle flaccidity-inducing effect of the toxins, compared CMAP data, and the overall effect of the toxins.

2. Materials and methods

2.1. Preparation of toxin

Botulinum neurotoxin type A, B, C, C/D, D, E, and F (150 kDa, NTX) were prepared using modified culture and purification methods, as previously reported (Sakaguchi et al., 1981). *C. botulinum* type A A2, type B Okra, type C CB-19, type C/D 003-9, type D 1873, type E 35396, and type F Langeland were used. For type A, B, E, and F organisms, PYG medium containing 2% peptone, 0.05% yeast extract, 0.5% glucose, and 0.025% sodium thioglycolate was used. For type C, C/D, and D organisms, a basic medium containing 0.8% glucose, 0.5% starch, 1.0% yeast extract, 1.0% ammonium sulfate, and 0.1% cysteine hydrochloride salt was used, and cooked meat (6 g) and 0.5% calcium carbonate were added to 100 mL of the basic medium. The organisms were cultured by allowing them to stand at 30 or 37 °C for 2–3 days. Culture fluid was purified by acid precipitation, protamine treatment, ion-exchange chromatography, and gel filtration of M toxin. M toxin was adsorbed to 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 protein separation. Each NTX was stored at −70 °C until use.

2.2. Experimental animals

Female ICR/CD-1 mice (4 weeks of age, about 20 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} and CMAP tests, 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 LD_{50} of each NTX was determined following ip injection into mice (Pearce et al., 1994). Seven doses with a dilution factor of 1.25 were used to determine the LD_{50}, and 20 animals were used per dose. Mice were evaluated for the first 96 h after administration, and the LD_{50} was calculated by the probit method.

2.4. CMAP measurements

The different types of NTX were serially diluted with physiological saline containing 0.5% human serum albumin. The following dilutions were prepared: 0.1–300 ip LD_{50}/mL type A, 100–1 × 10^5 ip LD_{50}/mL type B, 1–10,000 ip LD_{50}/mL type C, 0.3–100 ip LD_{50}/mL type C/D; 300–1 × 10^5 ip LD_{50}/mL type D, 1–3000 ip LD_{50}/mL type E, and 10–10,000 ip LD_{50}/mL type F. Rats were anesthetized with an ip injection of pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan). After the eyelid reflex disappeared, the left hind leg was shaved, and 0.1 mL of an NTX dilution was injected into the gastrocnemius muscle of five animals.

The CMAP was measured using a Nicolet Viking Quest (Viasys Healthcare, Tokyo, Japan). Rats were anesthetized and fixed in the prone position. The electrode employed was an alligator clip lead wire (Viasys Healthcare, Tokyo, Japan). The stimulating electrode was placed on the root of the spinal cord, the recording electrode was positioned on the belly muscle of the left hind gastrocnemius muscle, the reference recording electrode was placed on the left hind gastrocnemius tendon, and the earth electrode was positioned on the tail root. Electric stimulation was given at 25 mA for 0.2 ms. The CMAP of anesthetized rats was measured before (0) and 1, 2, 4, 7, and 14 days after administration.

To investigate whether the CMAP amplitude can accurately reflect the inhibition of neuromuscular transmission by botulinum toxin, we evaluated muscle relaxants with different mechanisms of action, nondepolarizing neuromuscular blocking drug, d-tubocurarine (d-Tc, Wako, Osaka, Japan), and a depolarizing neuromuscular blocking drug, succinylcholine (SCC, Wako, Osaka, Japan). d-Tc is an antagonist of nicotinic neuromuscular acetylcholine receptors, and provides muscle relaxation by competitive inhibition of acetylcholine. SCC is binding to the nicotinic acetylcholine receptor, and is opening of the receptor's nicotinic sodium channel; a disorganized depolarization of the motor end plate occurs. SCC is not hydrolyzed by acetylcholinesterase, and occurs to persistent depolarization. The receptor's nicotinic sodium channel is inactivated. When acetylcholine binds to an already depolarized receptor it cannot cause further depolarization. As a result, SCC provides muscle relaxation. So, d-Tc and SCC are relaxed muscle by neuromuscular transmission inhibitory as botulinum toxin. d-Tc and SCC were serially diluted 3-fold to yield 0.1–0.9 and 1–9 mg/mL, respectively. Each drug dilution (0.1 mL) was injected into caudal vein of five animals. Electrodes were attached as described above, and the CMAP amplitude was measured for the left hind leg of each rat at 3 and 2 min after d-Tc and SCC administration, respectively. The rats underwent the insertion of a tracheal tube to maintain respiration after drug administration. The respirator was delivered by SN-480-7 (Shinano manufacturing Co., Tokyo, Japan). Tidal volume was set on the respirator at 2 mL and respiratory frequency at 70 breaths/min.

2.5. Digit abduction scoring assay (DAS assay)

The different types of NTX were compared using the DAS assay (Aoki, 2001), which has been reported to assess the muscle flaccidity-inducing effect of botulinum toxin preparations. In the assay, mice received toxin injection into the gastrocnemius muscle, and the muscle flaccidity-inducing effect of the toxin was determined by the degree of digit abduction. The peak DAS responses were observed on Day 2 or 3 post-injection. The DAS assay was modified for rats, and carried out two days after NTX administration. For negative control, rats were injected with dilution solution (physiological saline containing 0.5% human serum albumin). Rats were suspended by the tail, and the degree of digit abduction in the toxin-treated leg was scored on a 5-point scale by an observer who was masked as to the treatment: score 0 = normal leg extension, and digit abduction equivalent to the contralateral side; score 1 = normal leg extension, but digit abduction differed from the contralateral side or two digits in contact while the other digits completely abducted; score 2 = leg extended, but weak abduction of all digits or three digits in contact; score 3 = leg extended without digit abduction, or leg bent with four digits in contact; score 4 = leg bent with no digit abduction.

2.6. Statistical analysis

The waveforms of a single CMAP were converted to 2000 dots using electromyograph software, and the coordinates of the dots were converted to numbers. The distance between the top and bottom of the waveform was measured as the CMAP amplitude. Statistical analysis for neurotoxin (SAN, ver. 1.07, self made soft) was used to analyze the CMAP amplitude. SAN was created to store raw data of the CMAP amplitude and perform various statistical analyses (i.e., *t*-test, regression analysis, parallel line analysis, and correlation coefficient).

For time course of CMAP amplitude, the MULTTEST procedure of contrast statement was performed using SAS (ver. 9.1).

To determine whether the inhibitory effect on the neuromuscular transmission of each NTX was quantifiable, ip LD_{50} data were plotted versus CMAP amplitudes, and the linearity of the regression line was confirmed by regression analysis.

To evaluate the efficacy of each NTX, the regression line of each NTX was calculated for the peak of the effect, as identified above. Regression lines were used to calculate the doses causing 50% (injection site) and 20% (contralateral site) reductions in the CMAP amplitude, and these values were termed the Effective Dose 50 (ED_{50}) and Toxic Dose 20 (TD_{20}), respectively.

For d-Tc and SCh of the data, the Jonckheere–Terpstra trend test was performed using SAS (ver. 9.1).

3. Results

3.1. Dose-dependent effects of the different NTX types on the CMAP amplitude

The CMAP amplitude was measured to compare the inhibition of neuromuscular transmission at the site of toxin administration. In all types of NTX, the CMAP amplitude decreased depending on the concentration of the ip LD_{50}. Following the administration of type B, C, C/D,

and D NTX, the CMAP amplitude in the left hind leg was reduced for four days at all dose, and was recovered thereafter at type B of or more 30 ip LD_{50}, type C and type C/D of all dose and type D of 100–10,000 ip LD_{50}. Following type A, E, and F NTX administration, the CMAP amplitude was reduced for two days and recovered thereafter at type A of 0.03–3 ip LD_{50}, type E of or more 1 ip LD_{50}, and type F of or more 10 ip LD_{50} (Fig. 1). After the administration of type A, B, C, C/D, D, and F NTX, the CMAP amplitude in the contralateral leg was reduced for four days at type A of more 3 ip LD_{50}, type B of 10,000 ip LD_{50}, type C of or more 30 ip LD_{50}, type C/D of or more 3 ip LD_{50}, type D of 100,000 ip LD_{50}, and type F of or more 3000 ip LD_{50}, and subsequently recovered at type A of 10 ip LD_{50}, type B of or more 3000 ip LD_{50}, type C of 300 ip LD_{50}, type C/D of or more 3 ip LD_{50}, type D of 100,000 ip LD_{50}, and type F of 10,000 ip LD_{50}, respectively. Following type E NTX administration, the CMAP amplitude was reduced for two days at or more 100 ip LD_{50} and recovered thereafter at 300 ip LD_{50} (Fig. 2).

3.2. Analysis of the CMAP amplitude after the different NTX types were administered

Type A, C, C/D, and E NTX reduced the CMAP amplitude in the toxin-injected limb below 1 ip LD_{50} one day after administration. Regression analysis revealed that the CMAP amplitude for type A, B, C, C/D, and D of NTX on days 1, 2, 4, 7, and 14 and type E and F of NTX on days 1, 2, and 4 following administration was related to the LD_{50} concentrations dose-dependently, and linearity was noted when the logit-transformed CMAP amplitude was plotted against the log ip LD_{50}. Although each NTX varied regarding its LD_{50} value for the reduction of the CMAP amplitude, the linearity range of all types of NTX was from the minimum ip LD_{50} to about 10^3 fold (Table 1). Parallel line analysis of the regression lines was performed for each NTX on days 1, 2 and 4, followed by Tukey's test. The regression lines for all types of NTX showed parallelism, except for type C NTX (data not shown).

3.3. Inhibitory effect of the different NTX types on neuromuscular transmission

To compare the inhibition of neuromuscular transmission by the different types of NTX, the ED_{50} was defined as the dose that decreased the CMAP amplitude to 50% of the pre-toxin level, and was calculated for the peak effect at each dose. The ED_{50} rank order of NTX was type $D > B > F > E > C > C/D > A$. Type A NTX was most potent to reduce the CMAP amplitude, and type D NTX was lowest potent (Table 2).

3.4. Comparison of diffusion to the contralateral site and safety index of the different types of NTX

To compare diffusion to the contralateral site of the different types of NTX, the CMAP amplitude was measured in the right hind leg. The TD_{20} was defined as the dose that decreased the CMAP amplitude to 20% of the pre-toxin level and was calculated to assess the peak effect at each dose. The TD_{20} value rank order of NTX was type $D > F > B > C > E > A > C/D$. Type C/D NTX was most potent to diffuse to the contralateral site, and type D NTX the lowest potent. The ratio of TD_{20}/ED_{50} was calculated for each NTX, and is expressed as the safety index. The safety index rank order of NTX was type $F > C > D > E > A > C/D > B$. The results revealed that type F NTX showed the widest safety index and was hard to diffuse, and type B NTX the narrowest index and was prone to diffuse (Table 2).

3.5. Changes in CMAP amplitude induced by muscle relaxants (d-Tc and SCh)

To investigate whether the CMAP amplitude can accurately reflect the inhibition of neuromuscular transmission by botulinum toxin, we evaluated muscle relaxants with different mechanisms of action. The average CMAP amplitudes in groups treated with 0.01, 0.03, and 0.09 mg d-Tc were reduced to about 78, 15, and 2% of that in the vehicle group, whereas those in the groups treated with 0.1, 0.3, and 0.9 mg SCh were reduced to about 68, 12, and 1%, respectively. Differences were significant for both d-Tc and SCh ($p < 0.0001$; Jonckheere–Terpstra trend test). The muscle relaxants induced a dose-related decrease in the CMAP amplitude, similar to botulinum toxin, indicating that the CMAP test can be used to evaluate the inhibition of neuromuscular transmission.

3.6. Comparison of muscle flaccidity induced by the different types of NTX

To compare the muscle flaccidity-inducing effects of the different types of NTX, we evaluated them using the DAS assay. When the log of the ip LD_{50} and median DAS score were plotted on the horizontal and vertical axes, respectively, the reaction curves for each NTX showed dose-dependent linearity. The required toxin value rank order of NTX for muscle flaccidity was type $B = D > F > E > A = C = C/D$. Type A, C, and C/D NTX were most potent to exhibit a muscle flaccidity-inducing effect, and type B NTX was lowest potent (Fig. 3). The ip LD_{50} dose necessary to achieve a score of 1 in the DAS assay was greater than that required to reduce the CMAP amplitude.

4. Discussion

The neuromuscular transmission inhibitor (d-Tc and SCC) with different mechanisms of action to the botulinum toxin induced a dose-related decrease in the CMAP amplitude. This result indicated that the CMAP amplitude reflected the inhibition of neuromuscular transmission caused by the muscle flaccidly-inducing effect of the drug. The results generated by the CMAP test are continuous data, whereas those of the mouse ip LD_{50} method and scores are discrete data.

We used the rat CMAP test to determine the toxin activity of different NTX types. Type A and C/D NTX reduced the CMAP amplitude one day after administration at 0.01 and 0.03 ip LD_{50}, respectively. In contrast, 10 ip LD_{50} of type B NTX were needed to reduce the CMAP amplitude. Type A, C, C/D, and E NTX required a dose of 1 ip LD_{50} or below, and the CMAP method was more sensitive than the mouse ip

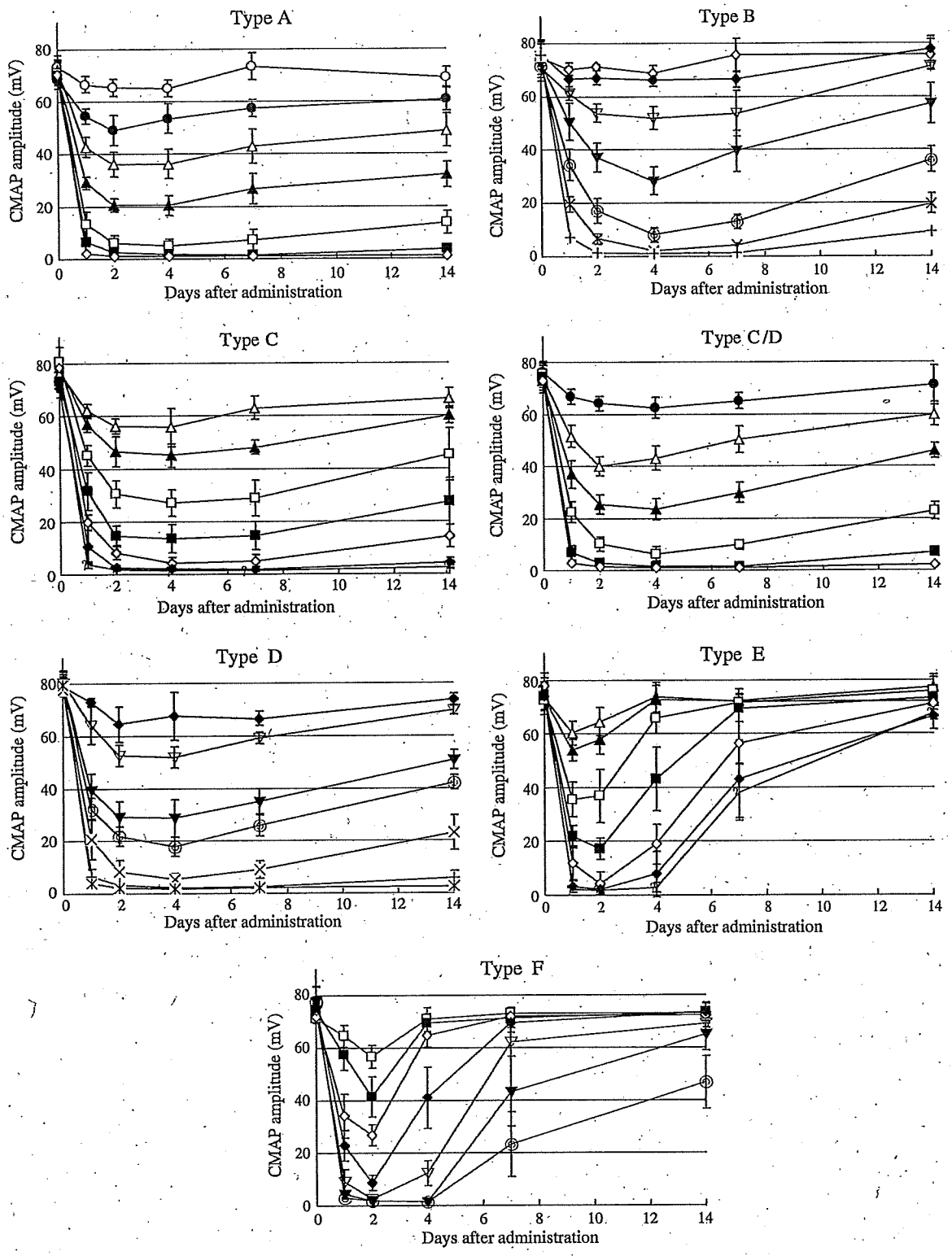

Fig. 1. Dose–response of the CMAP amplitude with the different NTX types at the injection site. Rats received botulinum toxin into left gastrocnemius muscle (○: 0.01 ip LD_{50}; ●: 0.03 ip LD_{50}; △: 0.1 ip LD_{50}; ▲: 0.3 ip LD_{50}; □: 1 ip LD_{50}; ■: 3 ip LD_{50}; ◇: 10 ip LD_{50}; ◆: 30 ip LD_{50}; ▽: 100 ip LD_{50}; ▼: 300 ip LD_{50}; ⊙: 1000 ip LD_{50}; ×: 3000 ip LD_{50}; +: 10,000 ip LD_{50} and •: 30,000 ip LD_{50}). CMAP amplitude was measured for the left hind leg of each rat at before and 1, 2, 4, 7, and 14 days after administration. Each point is the mean ± SD, $n = 5$.

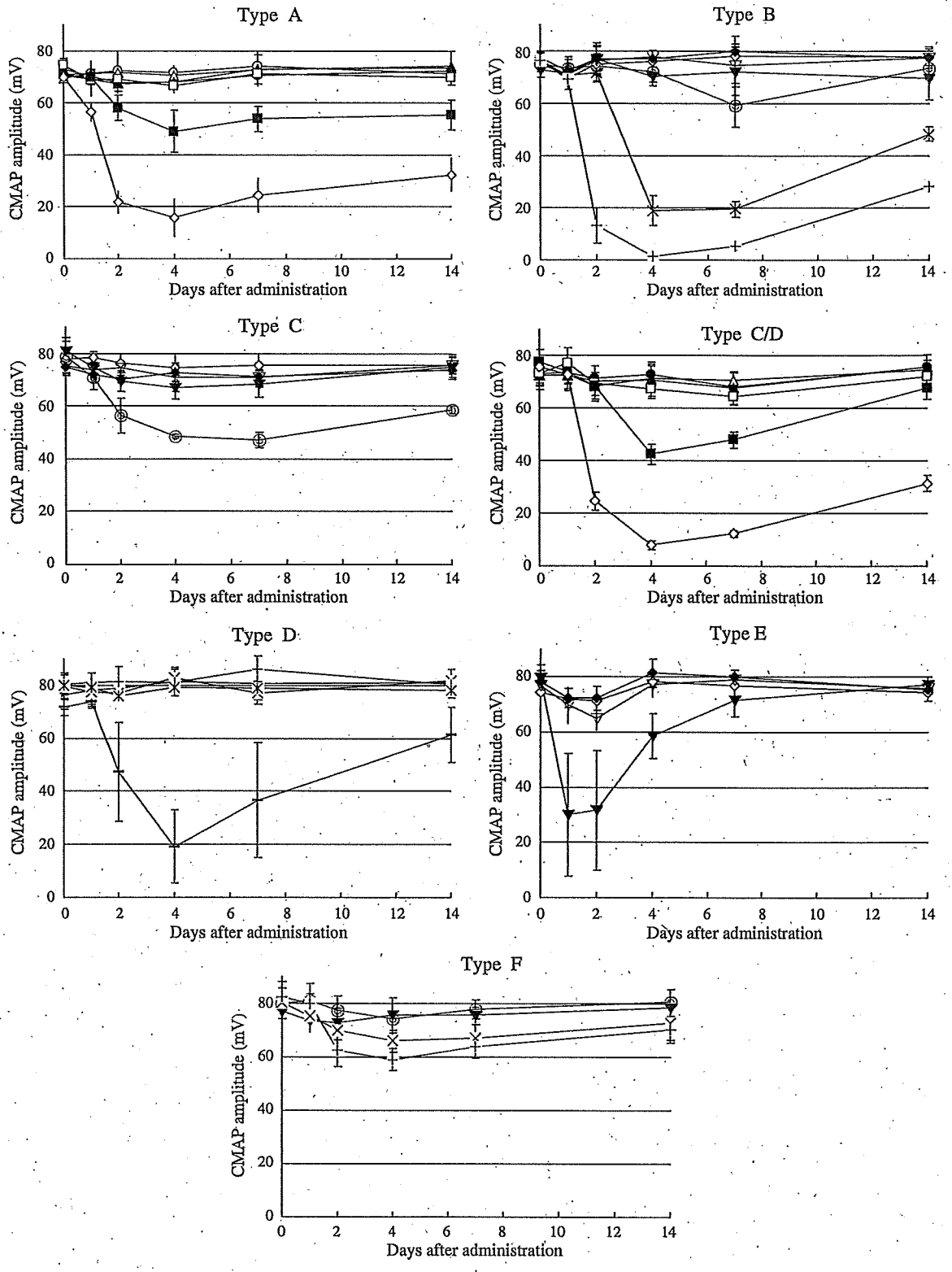

Fig. 2. Dose–response of the CMAP amplitude with the different NTX types at the contralateral site. Rats received botulinum toxin into left gastrocnemius muscle (○: 0.01 ip LD_{50}; ●: 0.03 ip LD_{50}; △: 0.1 ip LD_{50}; ▲: 0.3 ip LD_{50}; □: 1 ip LD_{50}; ■: 3 ip LD_{50}; ◇: 10 ip LD_{50}; ◆: 30 ip LD_{50}; ▽: 100 ip LD_{50}; ▼: 300 ip LD_{50}; ⊙: 1000 ip LD_{50}; ×: 3000 ip LD_{50}; +: 10,000 ip LD_{50}; •: 30,000 ip LD_{50} and –: 100,000 ip LD_{50}). CMAP amplitude was measured for the right hind leg of each rat at before and 1, 2, 4, 7, and 14 days after administration. Each point is the mean ± SD, $n = 5$.

Table 1
Linearity ranges on regression analysis of the CMAP amplitude after the administration of type A to F NTX.

Type	Linearity range (ip LD_{50})				
	1 day (R^2)[a]	2 day (R^2)	4 day (R^2)	7 day (R^2)	14 day (R^2)
A	0.01–30 (0.979)	0.01–10 (0.971)	0.01–10 (0.966)	0.01–10 (0.954)	0.01–10 (0.959)
B	10–10,000 (0.932)	10–10,000 (0.954)	10–10,000 (0.949)	10–10,000 (0.950)	10–10,000 (0.915)
C	0.1–100 (0.957)	0.1–30 (0.957)	0.1–30 (0.955)	0.1–30 (0.968)	0.1–100 (0.946)
C/D	0.03–10 (0.964)	0.03–10 (0.980)	0.03–3 (0.968)	0.03–3 (0.962)	0.03–10 (0.963)
D	30–30,000 (0.942)	30–30,000 (0.953)	30–10,000 (0.958)	30–10,000 (0.949)	30–30,000 (0.937)
E	0.1–30 (0.930)	0.1–30 (0.928)	0.3–100 (0.915)	–	–
F	1–300 (09.37)	1–300 (0.954)	10–300 (0.956)	–	–

[a] R^2: multiple correlation coefficient.

Table 2
ED_{50}, TD_{20}, and safety index values for type A to F NTX.

Type	ED_{50}[a] (ip LD_{50})	TD_{20}[b] (ip LD_{50})	Safety index (TD_{20}/ED_{50})
A	0.09	1.57	18
B	167	1226	7
C	0.54	385	718
C/D	0.13	1.38	11
D	206	36,433	177
E	0.85	50	59
F	4.67	3772	808

[a] ED_{50}, dose which caused a 50% reduction of the CMAP amplitude.
[b] TD_{20}, dose which caused a 20% reduction of the CMAP amplitude.

LD_{50} assay. However, in type B, D, and F NTX, the CMAP method was less sensitive. These results suggest that mice and rats show a different sensitivity to toxins. The results indicated an advantage whereby the method can be used to measure a broad range of toxin activities on the day following administration.

The rat CMAP test is useful not only for measuring toxin activity, but also for evaluating the characteristics of the neuromuscular transmission-inhibitory effect of different NTX types. To assess whether the neuromuscular transmission-inhibitory effect was correlated with the muscle flaccidity-inducing effect, the latter effect of the different NTX types was compared using the DAS assay. The neuromuscular transmission-inhibitory effect (ED_{50}) using the CMAP test was compared to that using the DAS assay, and all types of NTX showed a correlation between the effect of the inhibition of neuromuscular transmission and the potency of muscular flaccidly, except type C NTX. The ED_{50} of type C NTX showed a higher dose than that of type A and C/D NTX; however, the effect of flaccid muscle paralysis was the same. The toxins cleave SNARE proteins (i.e., SNAP-25, synaptobrevin, and syntaxin), which fuse to the synaptic vesicle and nerve cell membrane, blocking neuromuscular transmission by inhibiting the release of acetylcholine from synaptic vesicles. This suggested the possibility that the muscle-relaxing effect of type C NTX is caused not only by the inhibition of neuromuscular transmission through the cleavage of SNARE proteins, but also by other action mechanisms (i.e., the effect on the muscle).

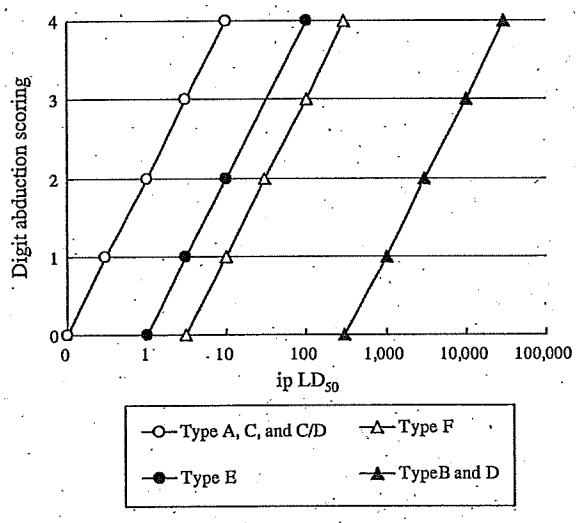

Fig. 3. Comparison of the muscle flaccidity-inducing effect of type A to F NTX on digit abduction in rats two days after administration. The scores indicate the median of each dose, $n=5$.

Assuming that the findings can be extrapolated to humans, type A and C NTX might show a higher efficacy and safety than other types of NTX as muscle relaxants. Type A NTX showed the strongest effect on the inhibition of neuromuscular transmission and muscle flaccidity, having longer-lasting effects than type B NTX. Type A NTX showed a higher sensitivity than the other types of NTX in humans, and so might be the most suitable as a muscle relaxant. However, the results suggested that type A NTX has the disadvantage that it is prone to diffuse compared to the other types, except for type B and C/D NTX. Type C NTX might be the most suitable for relaxing a particular muscle, because it showed a potent muscle flaccidity-inducing effect and diffuses less than the other types. A dose inhalation toxicity study in monkeys showed equivalent effects between type A and C NTX (LeClaire and Pitt, 2005). In a clinical study, it was reported that type C had a muscle-relaxing effect equivalent to that of type A NTX (Eleopra et al., 1997; Eleopra et al. 2004).

Recently, type C, E, and F toxins have been tried as treatments for various diseases. The inhibitory effect of type E NTX on neuromuscular transmission was the fourth strongest after type A, C, and C/D NTX. The safety index of type E NTX was ranked fourth after type F, C, and D, and the duration of the effect of type E NTX was the shortest. It was reported that the muscular flaccidity-inducing effect of type E and F toxins was of a short duration in humans, similar to the result in this study. Since an effect of type E toxin on the central nervous system was reported (Bozzi

et al., 2006), the toxin may be of use in diseases of the central nervous system. In the future, the other NTX except types A and B may be applied to treatment, and they might be approved as new drugs.

In this study, the rat CMAP test was able to quantify the toxin activity of types A–F toxin. This method is applicable to different types of botulinum toxin preparation which might be marketed, and aids in quality control. This method is useful for evaluating the pharmacological effects of muscle relaxants.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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Quantitative determination of the biological activity of botulinum toxin type A by measuring the compound muscle action potential (CMAP) in rats

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ABSTRACT

Quantitative determination of the biological activity of botulinum toxin type A usually depends on the LD_{50} method after intraperitoneal injection into mice. This method requires a large number of mice to determine the toxic activity at a high level of precision and 3–4 days to obtain the results. Techniques to replace the LD_{50} method have been attempted at various institutes. As a substitute for this method, by directly measuring the inhibition of neuromuscular transmission after the administration of a toxin, a method to quantitatively assess the toxin's activity by determining the compound muscle action potential (CMAP) was examined. Toxin solutions were injected into the rat gastrocnemius muscle, and that of the CMAP amplitude was determined over time. The CMAP amplitude decreased over 4 days after the injection of the toxin, and then slowly recovered. A dose-response relationship was noted for each dose, and a linear relation was observed between 0.01 and 30 U on the 1st day. From these results, we propose the CMAP as a substitute for the LD_{50} method to examine the activity of toxin products as it is simple and reliable, reduces the number of experimental animals required, and lowers pain levels.

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1. Introduction

Clostridium botulinum produces toxins that have been classified into 7 serotypes, A, B, C, D, E, F, and G, based on their immunological characteristics. The toxins act on neuromuscular junctions and induce muscle relaxation by inhibiting acetylcholine release (Sakaguchi, 1983). Type A and B toxin preparations have been utilized as a treatment for spasm in myotonus and dystonia (Jankovic, 2004; Sadick, 2003).

The standard method to determine the biological activity of botulinum toxin is the lethal dose 50% (LD_{50}) test involving intraperitoneal (ip) injection into mice, and many mice are required to ensure sufficient accuracy levels for the quality control of preparations, which has posed a problem with regard to animal welfare (Pearce et al., 1994). International meetings on alternative methods for animal testing have been held, and replacements for the mouse ip LD_{50} test have been discussed (Straughan, 2006).

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The 3 Rs (refinement, reduction, and replacement) have been proposed for alterative methods, and alternative *in-vitro*, *ex-vivo*, and *in-vivo* test systems have been investigated. The *in-vitro* test system, ELISA, determining the endopeptidase activity, does not use animals, but the sensitivity is lower than the mouse bioassay (Hallis et al., 1996; Wictome et al., 1999). This method could determine only light-chain activity in many cases, and the inaccurate determination of toxin function has been reported. The *ex-vivo* test system using the mouse phrenic nerve-hemidiaphragm is sensitive, but it requires skilled techniques, and has a low reproducibility (Yoneda et al., 2005; Bigalke et al., 2001). The *in-vivo* test systems, such as the digit abduction scoring (DAS) assay and local flaccid paralysis assay, use scores for evaluation (Aoki, 2001; Takahashi et al., 1990; Sesardic et al., 1996). When an evaluation lies between two scores, judgment is difficult. As each test system has both advantages and disadvantages, no alternative method has been established.

We attached a greater importance to the following point in devising an alternative method to determine the activity of botulinum toxin: the potency of botulinum toxin preparations should be evaluated based on their pharmacological effect of inhibiting neuromuscular transmission, and not based on their lethal activity, as in the mouse ip LD_{50} assay. There are several test systems to evaluate the inhibition of neuromuscular transmission, and we focused on the measurement of the compound muscle action potential (CMAP) used for the diagnosis of various nervous disorders. The CMAP is generated by the contraction of muscle fibers; the microcurrent generated by muscle contraction is amplified and recorded. Botulinum toxin affects nerve endings to suppress neurotransmission. Therefore, by determining the CMAP amplitude, the action of the toxin suppressing the transmission of electric stimulation to the muscle can be shown numerically. CMAP measurement is utilized by the extensor digitorum brevis (EDB) test, which checks the response to the toxin before treatment in patients who might have antibodies to the botulinum toxin (Kessler and Benecke, 1997). It was reported that the CMAP amplitude was measured in the rat gastrocnemius muscle which was injected several times with botulinum toxin (Cichon et al., 1995). The CMAP amplitude decreased as the toxin activity increased, but quantitative determination of the toxin was not carried out. Based on this previous CMAP study, we investigated a botulinum toxin quantification method, aiming at replacing the mouse ip LD_{50} assay.

2. Materials and methods

2.1. Toxin preparation

Botulinum neurotoxin type A (150 kDa, NTX) was prepared using modified culture and purification methods, as previously reported (Sakaguchi et al., 1981). *C. botulinum* type A strain A2 was cultured in PYG medium containing 2% peptone, 0.5% yeast extract, 0.5% glucose, and 0.025% sodium thioglycolate in a glass bottle. After incubation for 3 days at 30 °C, the culture fluid 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 with 0.2 M phosphate buffer (pH 6.0). The extract contained nucleic acids, which were removed by treating with 2% protamine sulfate, and the precipitate was removed by centrifugation. The supernatant was precipitated at a 60% saturation of ammonium sulfate, and the precipitate, collected by centrifugation, was dialyzed against 0.05 M acetate buffer containing 0.2 M sodium chloride (pH 4.2). The dialyzed material was applied to an SP-Sephadex column equilibrated with the same buffer. Then, M toxin was eluted using a linear gradient of sodium chloride concentrations from 0.2 to 0.7 M. M toxin was dialyzed against 10 mM phosphate buffer (pH 7.5), adsorbed onto a DEAE sepharose column equilibrated with the same buffer, and eluted with a 0–0.3 M NaCl gradient in the buffer to separate NTX and nontoxic proteins. The NTX was concentrated to 1 mg/mL using the YM-10 membrane (Millipore, Tokyo, Japan), dialyzed against 10 mM phosphate buffer (pH 7.5), and stored at −70 °C until use.

For the test control, commercial progenitor LL toxin (900 kDa, BOTOX®, Allergan Inc., U.S., LL hereafter) was used.

2.2. Experimental animals

Female ICR/CD-1 mice (4 weeks of age, about 20 g) for the LD_{50} test and female S/D rats (8 weeks of age, about 200 g) for the CMAP test were purchased from Charles River Laboratories Japan (Tokyo, Japan). The animal room was maintained under controlled light/dark conditions, and animals were given free access to feed and water. This study was performed in accordance with the guidelines concerning experimental animals established by the Japanese Pharmacological Society, and approved by the Internal Animal Ethics Committee.

2.3. Mouse ip LD_{50} assay

The toxin activity of NTX and LL was determined by the mouse intraperitoneal LD_{50} method, defining one mouse ip LD_{50} = 1 unit (U) (Pearce et al., 1994). 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.

2.4. Toxin administration

Each toxin was serially diluted with physiological saline containing 0.5 w/v% human serum albumin, and 0.1–300 U/mL solutions were prepared. Rats were anesthetized by an intraperitoneal injection of about 40 mg/kg pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan). After the eyelid reflex had disappeared, the hind leg was shaved, and 0.1 mL of the toxin dilution was injected into the left hind gastrocnemius muscle using a 30-G insulin syringe (Becton Dickinson, Tokyo, Japan).

2.5. CMAP measurement

The CMAP was measured using Nicolet Viking Quest (Viasys Healthcare, Tokyo, Japan). Rats were anaesthetized and fixed in the prone position. The electrode used was an alligator clip lead wire (Viasys Healthcare, Tokyo, Japan),

attached to the skin. The electrode attachment sites are shown in Fig. 1. The stimulating electrode (cathode) was placed on the skin over the fourth lumbar vertebra. The stimulating electrode (anode) was placed at 2 cm from the cathode on the spinal column. The recording electrode was placed on the belly muscle of the left hind gastrocnemius muscle, the reference recording electrode on the left hind gastrocnemius tendon, and the earth electrode on the tail root. Electric stimulation was loaded at 25 mA for 0.2 ms, and the CMAP was obtained. The CMAP was measured before (0) and 1, 2, 4, 7, and 14 days after administration.

2.6. Statistical analysis

For analysis of the CMAP amplitude, Statistical Analysis for Neurotoxin (SAN, ver. 1.05) was used. The waveform of one CMAP was converted to 2000 dots using the software supplied with the electromyograph, and the coordinates of the dots were converted to numbers. The distance between the top and bottom of the waveform was calculated using SAN as the CMAP amplitude. SAN was created to store raw data of the CMAP amplitude and perform various statistical analyses (*t*-test, one-way and two-way layout ANOVA, regression analysis, parallel line analysis, and correlation coefficient).

For the confirmation of CMAP quantifiability, logarithm LD_{50} doses on horizontal and logit-transformed CMAP amplitudes on vertical axes were plotted, respectively, and the linearity of the regression line was confirmed by regression analysis.

3. Results

3.1. Toxin activity

The toxin activity of NTX and LL was 93,000 ± 565 U and 92.4 ± 1.48 U, respectively.

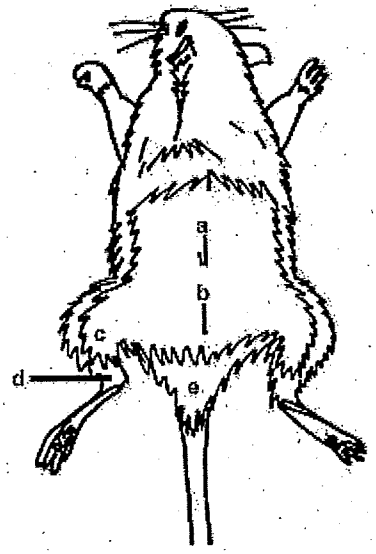

Fig. 1. CMAP measurement method. a. Stimulating electrode (+); b. Stimulating electrode (–); c. Recording electrode (–) and injection site; d. Recording electrode (+); e. Grounding electrode.

3.2. Dose-responsiveness of the CMAP

On time-course measurement of the CMAP amplitude after NTX administration, the left hind leg CMAP amplitude decreased until the 4th day, and then slowly recovered after the 7th day. Limb paralysis was observed after the administration of 3 U or more. It was found that the CMAP amplitude decreased depending on the concentration of LD_{50} doses (Fig. 2). These results were subjected to regression analysis, and linearity was detected over a range of 0.01–30 U on day 1 ($R^2 = 0.979$) (Fig. 3), and 0.01–10 U on days 2, 4, 7, and 14 ($R^2 = 0.971$ on day 2, $R^2 = 0.966$ on day 4, $R^2 = 0.954$ on day 7, and $R^2 = 0.959$ on day 14, respectively).

To investigate whether other molecular forms of the toxin show a different dose-responsiveness regarding NTX, the CMAP amplitude was measured in the same experiment using LL, and the linearity of the regression line between the CMAP amplitude and potency was verified. Parallel line analysis of the regression lines of NTX and LL on the 1st day after administration indicated that the regression lines of toxins were parallel to each other. No significant difference was noted between the two lines, and the relative potency of LL in relation to NTX was 0.965 (95% confidence interval: 0.791–1.178). Accordingly, the same calibration curve could be used for the evaluation of type A preparations, NTX and LL (Fig. 3).

3.3. Reproducibility of the CMAP test

Reliability is important for substance-measurement methods, and reproducibility is essential. To confirm the reproducibility, the CMAP amplitude was measured 3 times using the same procedure on different days and by two measurers. The reactions after administration were similar. The regression lines of the dose-response curve of NTX and LL on the 1st day after administration were analyzed by two measurers, and were parallel to each other. The relative potency of LL as assessed by the two measurers was 0.958 (95% confidence interval: 0.763–1.203), showing that there was no significant difference in the dose-response regression lines for LL obtained by them (data not shown). This result suggested that the CMAP test regarding the response to toxin activity was reproducible.

3.4. Time-course CMAP amplitude of botulinum toxin-treated muscle

We investigated whether time-course reactions of the CMAP amplitude could be fitted to an equation, and found that the amplitude after administration conformed to the following experimental equation: $y = a - b(\log(x)) + C(\log(x)\log(x))$ (the equation represents a physical phenomenon). Using this equation, the days showing a maximum reduction of the CMAP amplitude, recovery to the pre-treatment condition, 50% recovery, and 50% amplitude reduction can be calculated. For example, the days after the administration of 0.1 U NTX shown in Table 1 were predicted using this equation.

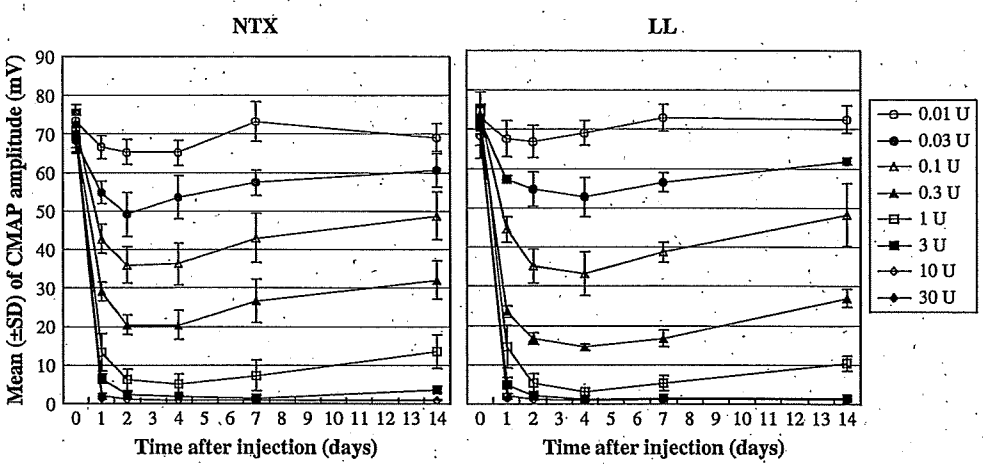

Fig. 2. Dose-response of the CMAP amplitude of NTX and LL. Each point is the mean ± SD ($n = 5$).

4. Discussion

The mouse ip LD_{50} method, a quantitative method for determining the biological activity of botulinum toxin now widely used, is based on lethality in mice, but the method requires 3–4 days to make a judgment and many animals to obtain reliable results. Various alternative methods have been investigated for the quantification of botulinum toxin, but all have advantages and disadvantages, and so an appropriate replacement test is still awaited.

We investigated the possibility of using the rat CMAP test as a substitute for the mouse ip LD_{50} assay, and identified a dose-response relationship with the mouse ip LD_{50} dose (toxin activity) at 1, 2, 4, 7, and 14 days after injection and the capability of measuring the duration of the toxin's effect. This method was very sensitive, facilitating measurement down to a sensitivity of 0.01 U, which is not possible using the mouse ip LD_{50} assay, and a regression line within a broad range of 0.01–30 U was obtained 1 day after administration. The toxin activity could be measured

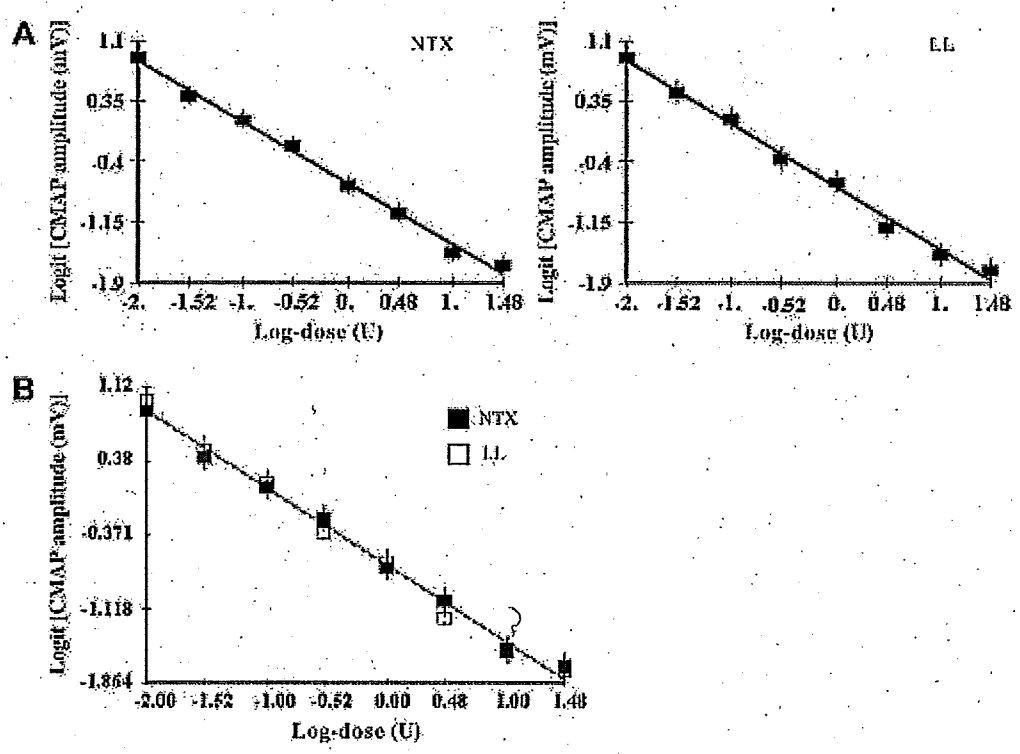

Fig. 3. Regression analysis of the CMAP amplitude of NTX and LL on the 1st day after administration (A), and parallel line analysis of the regression line of NTX and LL on the 1st day after administration (B). Each point is the mean ± 95% confidence interval ($n = 5$).

Table 1
Days after administration of 0.1 U NTX predicted from the response curve.

	Day
Day showing maximum reduction	4.7
Day showing recovery to pre-treatment condition	32.0
Day showing 50% recovery	13.3
Day showing 50% amplitude reduction	1.3

on the day following administration, which is a major advantage. In addition, the number of experimental animals required can be markedly reduced because of the broad range and its high-level precision. Furthermore, the results obtained by the LD_{50} method and score represent a discrete quantity, while those generated by the CMAP test represent a continuous quantity. Thus, statistical processing is applicable, and the quantity of information obtainable is large.

Analysis using SAN clarified that the time-course reactions of the CMAP amplitude fitted an equation. By applying this equation, the CMAP method may be used for not only quantification of the toxin but also the evaluation of its various pharmacological effects. Time-course changes in the amplitude can be predicted by substituting several measurement data in the equation. For example, the duration of an effect of the toxin can be measured with data at minimum measurement points, which may reduce the number of experimental animals needed.

Results determined by several operators or on different days showed satisfactory intermediate-level precision, suggesting that the method is very suitable for the comparison of several toxin preparations and quality control. The difference in the muscle-relaxing effect between equal units of BOTOX® and Dysport® has been problematic in clinical practice (Rosales et al., 2006). To overcome this problem, whether an international standard for the toxin could be established was discussed, and investigated using the mouse ip LD_{50} assay at several laboratories. However, the possibility of its establishment is questionable because the results showed large variation. It was also investigated using other test methods, but the outcome was the same. As each test system has both advantages and disadvantages, no system for evaluating toxin function is both easy to perform and highly reproducible. On the other hand, the rat CMAP test is easy to conduct, highly reproducible, and can directly determine the effects of toxin preparations by assessing the inhibition of neuromuscular transmission. Thus, it is suitable for the quality control and evaluation of toxin preparations. The rat CMAP test is also cheaper to conduct than the mouse ip LD_{50} test.

We propose that the CMAP test with the characteristics described above is an alternative method for the quantification of botulinum toxin.

Acknowledgment

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Short communication

Quantification of potency of neutralizing antibodies to botulinum toxin using compound muscle action potential (CMAP)

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ABSTRACT

We evaluated a method for quantifying botulinum toxin-neutralizing antibodies which utilizes the CMAP. This method can be used just one day after administration, and the detection sensitivity was higher than that of the mouse neutralization test. The CMAP neutralization test detected neutralizing antibodies in patients who were resistant to treatment with the botulinum LL toxin. These results indicate that the CMAP neutralization test is useful for detecting low levels of antitoxin.

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Botulinum toxins have recently been developed and used in the treatment of blepharospasm, spasmodic torticollis, dystonia, pain, and urological disorders (Jankovic, 2004; Truong and Jost, 2006; Casale and Tugnoli, 2008). The toxins show a high-level efficacy at very low doses, and are widely used in medical treatment. Resistance to the toxin was reported in some patients who received repeat high-dose (>100 mouse ip LD_{50} per injection cycle) toxin therapy over a long period of time (Borodic et al., 1996; Sesardic et al., 2004; Dressler, 2004). This reduced therapeutic response was reported to lead to the development of neutralizing antibodies in the patients. The potency of neutralizing antibodies in the serum of patients is measured using the mouse neutralization test (Hatheway et al., 1984). This method is based on the LD_{50} assay, which, in turn, is based on the number of surviving mice typically 96 h after the intraperitoneal (ip) injection of a fixed lethal dose of toxin premixed with different amounts of antitoxin. The antitoxin titer in the sample is expressed relative to that of the standard botulinum antitoxin. The detection limit of this method is reported to be about 10–100 mU/mL (Sesardic et al., 2004; Byrne et al., 1998). A reduced therapeutic response in certain patients was reported to be caused by minute amounts of antibodies which could not be detected by the mouse neutralization test (Sesardic et al., 2004). To detect neutralizing antibody presence in patients, a highly sensitive assay for neutralizing antibodies is needed. We reported quantitative of biological activity of botulinum toxin using the compound muscle action potential (CMAP) (Torii et al., submitted for

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publication). CMAP measurement is utilized by the extensor digitorum brevis (EDB) test, which qualitatively assesses the response to the toxin before treatment in patients who might possess antibodies against the botulinum toxin (Kessler and Benecke, 1997). Based on the EDB test, we investigated a highly sensitive quantification method for botulinum toxin-neutralizing antibodies in animal and human sera. In addition, we investigated whether the CMAP neutralization test was able to detect antitoxin in patient's serum, and we compared detection capability of this method, mouse neutralization test and ELISA.

Botulinum neurotoxin types A, B, E, and F (150 kDa, NTX) were cultured and purified using a previously reported method (Sakaguchi et al., 1981; Torii et al., submitted for publication). Equine-derived Japanese standard botulinum antitoxin types A, B, E, and F (National Institute of Infectious Diseases, Tokyo, Japan) were each used as a standard. One unit (U) of corresponding type of standard botulinum antitoxin neutralizes 10,000 mouse intraperitoneally (ip) LD_{50} of toxin types A, B, F or 1000 mouse ip LD_{50} of toxin type E (Jones et al., 2006). The sera of seven patients resistant to treatment with the botulinum LL toxin (BOTOX®, Allergan, Irvine, U.S.) were collected after informed consent was obtained. For the negative control, sera were collected from one volunteer who did not receive any treatment of botulinum toxin. For the positive control, sera were collected from one volunteer who had been immunized three times with the botulinum tetravalent (A, B, E, and F) toxoid. CMAP neutralization test was performed using a modification of a previously report method of CMAP test using female S/D rats (8 weeks of age, Charles River Laboratories Japan, Yokohama, Japan) (Torii et al., submitted for publication). Modification was using mixture which botulinum antitoxins and test toxins in place of toxins. Mixtures were prepared as follows: the standard botulinum antitoxin and various sera were serially diluted with physiological saline containing 0.5% human serum albumin. The test toxins comprised NTX of each type, at quantities whereby the CMAP amplitudes on day 1 after injection were decreased to one quarter of those before administration (type A: 10 mouse ip LD_{50}/mL, B: 60,000 mouse ip LD_{50}/mL, E: 60 mouse ip LD_{50}/mL, F: 600 mouse ip LD_{50}/mL). The type A of test toxin dose also was set at 1 mouse ip LD_{50}/mL to increase the measurement sensitivity. Equal volumes of the antitoxin or serum and test toxin were mixed and reacted for 1 h at room temperature. The anesthetized rats were injected 0.1 mL of a mixture into the left gastrocnemius muscle. The CMAP amplitude of the left hind leg was measured before (0) and 24 h after injection. The mouse neutralization test was performed using a previously reported method using female ICR/CD-1 mice (4 weeks of age, Charles River Laboratories Japan, Yokohama, Japan) (Torii et al., 2002). ELISA was performed using a modification of a previously reported method (Torii et al., 2002). Modifications were buffer using Tris Buffer containing 0.15 M NaCl, secondary antibody using peroxidase-conjugated goat anti-human IgG, IgA and IgM (Sigma, Tokyo, Japan) and substrate using TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories Inc, Gaithersburg, U.S.). ELISA titers were expressed in multiples of absorbance of the negative control, and antibodies were considered to be detected by ELISA when the absorbance of the sample was more than twice that of the negative control. To determine whether the neutralizing antibody of each type was quantifiable, antibody potencies were plotted versus CMAP amplitudes, and the linearity of the regression line was confirmed by regression analysis using Statistical Analysis for Neurotoxin (SAN, ver. 2.1, self made soft). To determine the antibody titers of patients' sera, the amplitude data of standard botulinum antitoxin were calculated by regression analysis, and the regression line was used as the calibration curve using SAN.

The CMAP amplitude of each mixture of antitoxin and test toxin (types A, B, E, and F) decreased along with the antitoxin titer. For types A and E, regression analysis was performed by plotting the logarithmic values of the CMAP amplitude and antitoxin titer on the vertical and horizontal axes, respectively, and linearity was noted within a range of 3–100 mU/mL in type A ($R^2 = 0.983$) and 1–50 mU/mL in type E ($R^2 = 0.989$). For types B and F, the CMAP amplitudes were plotted on the vertical axis, and the log values of the antitoxin titer on the horizontal axis, and linearity was noted within a range of 25–100 mU/mL in type B ($R^2 = 0.953$) and 3–50 mU/mL in type F ($R^2 = 0.974$) (Fig. 1). To increase the measurement sensitivity, the test toxin dose was set at 1 mouse ip LD_{50}/mL in type A, and linearity was noted within a range of 1–6 mU/mL (data not shown). This method can be used to measure a broad range of neutralizing antibodies titers the day after administration. In this study, the CMAP neutralization test demonstrated six advantages over the standard technique. 1) The CMAP is more sensitive than the mouse neutralization test. 2) The CMAP neutralization test incorporates a concise procedure. 3) The CMAP neutralization test can determine the neutralizing antibody titer within 24 h; whereas, the mouse neutralization test requires 4 days to obtain the same results. 4) The CMAP neutralization test is highly reproducible. 5) Only 20–30 animals are used in the CMAP neutralization test, whereas more than 100 animals are necessary in a single mouse neutralization test. In addition, the rats are anesthetized during the test, and the amounts of injected test toxin do not completely block neuromuscular transmission nor paralyze the muscles. 6) The CMAP amplitude values obtained by the CMAP neutralization test are a continuous quantity. Taken together, these advantages indicate that this method is a useful substitute for the mouse neutralization test.

We then investigated whether minute amounts of neutralizing antitoxin present in patients' sera could be detected by the CMAP neutralization test. The neutralization antibodies in seven patients who showed a reduced therapeutic effect after repeated treatment with type A botulinum LL toxin were determined by the CMAP neutralization test, mouse neutralization test, and ELISA. Using the CMAP neutralization test, all sera showed a neutralizing antibody level of 3–50 mU/mL (Table 1). This suggests that the cause of the reduced therapeutic effect was the production of neutralizing antibody against botulinum LL toxin. In contrast, the mouse neutralization test detected neutralizing antibodies in sera from 1

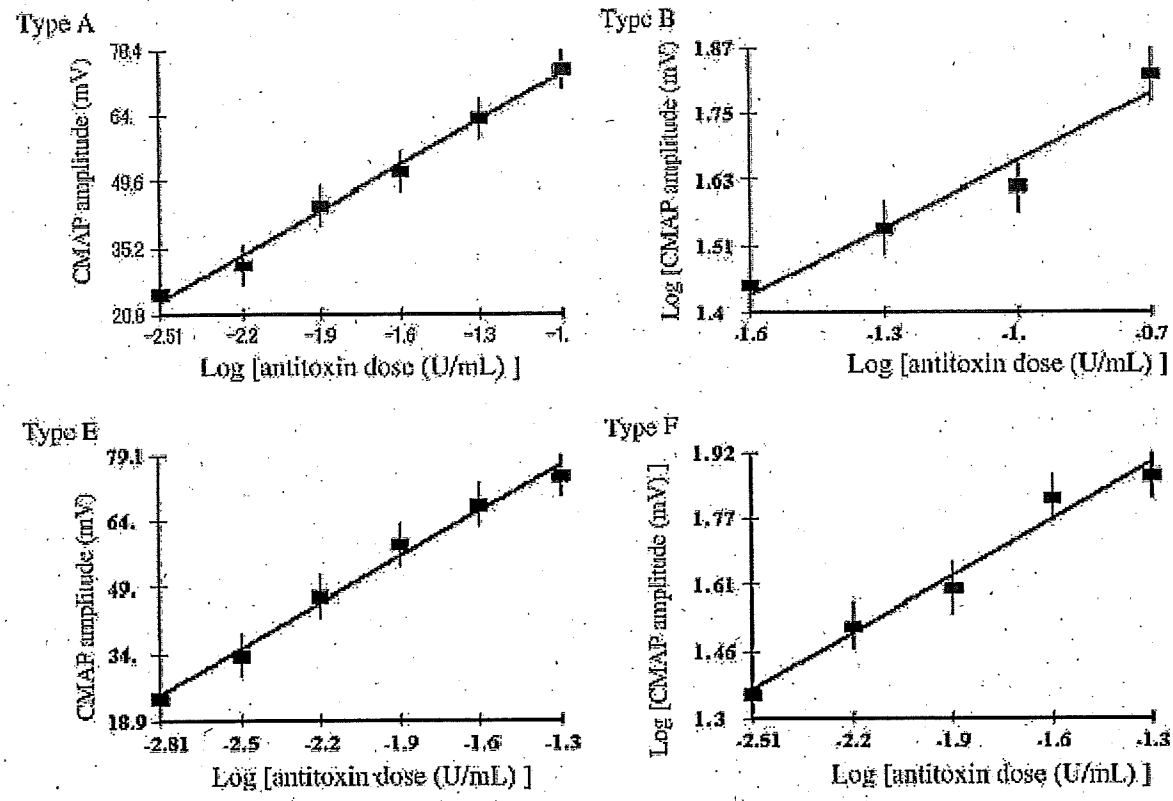

Fig. 1. Dose–response of CMAP amplitude of the test toxin mixed with standard botulinum antitoxin of each type. Rats received mixture of the antitoxin and test toxin into left gastrocnemius muscle. CMAP amplitude was measured for the left hind leg of each rat on day 1 after administration. Each point is the mean ± 95% confidence intervals, $n = 5$.

patient, showing a lower sensitivity than the CMAP neutralization test. ELISA detected neutralizing antibody presence in sera from 2 patients; however, ELISA titers were not correlated with the potency of neutralizing antibodies. This was because ELISA detected all antibodies (including non-neutralizing antibodies) against type A toxin. No antibodies were detected by these methods in the negative control. The antibody titers in serum No. 7 and the positive control detected using the CMAP and mouse neutralization tests showed similar values. Serum No. 7 was also antibody-positive on ELISA (Table 1). Comparing ELISA and the CMAP neutralization test, the correlation coefficient between the two assays was $R^2 = 0.056$ in all sera. No correlation could be identified between these titers.

Table 1
Antibody titers of sera from patients and volunteers measured using the mouse neutralization test, CMAP neutralization test, and ELISA.

Serum No.	Mouse neutralization test (mU/mL)	CMAP neutralization test (mU/mL)	ELISA[a]
1	<100	3	<2
2	<100	4	6
3	<100	4	<2
4	<100	4	<2
5	<100	4	<2
6	<100	5	<2
7	ca)100	50	3
8 (Positive control)	200	190	13
9 (Negative control)	<100	<1	<2

[a] ELISA titer expressed in multiples of the measurement from the negative control value.

As mentioned above, the CMAP neutralization test can be used for the detection of neutralizing antibodies in patients who have received treatment with type A botulinum toxin. Botulinum toxin preparations for treatment are used for various diseases in many patients. Since the therapeutic dose of botulinum toxin is very low, its therapeutic effect may be lost by only minute amounts of antibodies. For patients who show antibody presence, it may be necessary to treat them with increasing toxin doses or to change toxin types. The CMAP neutralization test may be useful to assist in such a diagnosis.

The CMAP neutralization test is capable of detecting minute amounts of neutralizing antibodies, not only against type A toxin, but also against types B, E, and F. Type A and B toxins have already been approved as formulations for the treatment of various disorders, and are currently being used in clinics. However, the effects of type E and F toxins have only recently begun to be investigated in clinical studies, and, in the future, these toxins may therefore be approved as new drugs (Mezaki et al., 1995; Eleopra et al., 1998, 2004). Thus, the ability of the CMAP test to also detect small amounts of neutralizing antibodies against type E and F toxins may be potentially useful for such toxin formulations to be developed in the future. This method is clinically applicable and useful as the measurement is

simple and straight forward, using electromyographs installed at clinical sites.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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