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Original Article

Clinical Study of New Tetravalent (Type A, B, E, and F) Botulinum Toxoid Vaccine Derived from M Toxin in Japan

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SUMMARY: Botulinum toxin is the most poisonous substance known, and is believed to be a highly lethal as a biological weapon; researchers of the toxin are exposed to this hazard. Botulinum toxoid vaccines have been produced and used in Japan. However, since clinical studies involving these vaccines were conducted before establishment of the Ethical Guidelines for Clinical Research in Japan, their immunogenicity and safety were not systematically assessed. In this study, we produced a new tetravalent (type A, B, E, and F) botulinum toxoid vaccine, the first ever to be derived from M toxin, and conducted quality control tests with reference to the Minimum Requirements in Japan for adsorbed tetanus toxoid vaccine. Subsequently, a clinical study using the new vaccine in 48 healthy adult volunteers was conducted according to the guidelines in Japan. No clinically serious adverse event was noted. Neutralizing antibody titers for each type of toxin in the participants' sera, 1 month after the 4th injection were more than 0.25 IU/mL, indicating sufficient protection. This study demonstrated that the vaccine has marked immunogenicity and is safe for use in humans.

INTRODUCTION

Clostridium botulinum is classified into 8 types, A, B, C, D, E, F, G, and H, based on immunological characteristics of the botulinum toxins that they produce (1,2). These toxins act on the neuromuscular junction and inhibit the release of acetylcholine from the presynaptic membrane, resulting in muscle relaxation, and ultimately, death from dyspnea in serious cases (3). These toxins are protein complexes, called progenitor toxins, with neurotoxin and nontoxic components produced by the organisms. The progenitor toxins are classified by their molecular weight into 3 forms: LL toxin, L toxin, and M toxin. The M toxin consists of a neurotoxin and a nontoxic component with no hemagglutinin (HA) activity, whilst the L and LL toxins are formed by conjugation of the M toxin with HA (4).

Biological weapons using pathogenic organisms and toxins are generally more accessible than conventional weapons, and can cause marked damage. Therefore, they are more likely to be used in terrorism, which poses a significant threat. Botulinum toxin has actually been used as a biological weapon by terrorists (5). To prevent botulism, the body must possess antibodies that will efficiently neutralize botulinum toxin (6). In Japan, researchers considered active vaccination as an approach against the toxin. Consequently, investigational monovalent (type E) and tetravalent (type A, B, E, and F) botulinum toxoid vaccines were produced in 1969 (7,8). These vaccines were prepared using partially purified toxins. The ratio and amount of various progenitor toxins in these vaccines are not constant due to their culture conditions. In addition, the compositions of these vaccines vary between production lots. These vaccines were used on humans in Japan (7,8). However, the studies were conducted before establishment of the Ethical Guidelines for Clinical Research in Japan, and therefore, not carried out according to the corresponding guidelines or the International Conference on Harmonization (ICH) guideline for Good Clinical Practice (E6(R1)) (9,10). In this clinical study, we produced a new tetravalent botulinum toxoid vaccine derived from each type M toxin. The study was conducted according to the guidelines in Japan, allowing a systematic evaluation of the immunogenicity and safety of the vaccine (9).

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MATERIALS AND METHODS

Production of tetravalent botulinum M toxoid vaccine: *C. botulinum* type A strain 62A, type B strain Okra, type E strain 35396, and type F strain Langeland were used. Each type M toxin was prepared using methods described previously (11). The specific activities of type A, B, E, and F toxins were 3.71 ×

10^6, 9.63×10^6, 3.52×10^6, and 8.77×10^5 LD_{50}/mg, respectively. Formalin was added at 0.2 v/v% in the presence of amino acids, then subsequently, at 0.1 v/v% at daily intervals, up to a final concentration of 0.4 v/v% for each type M toxin. The detoxification of type A toxin proceeded at 37°C for 21 days, and type B, E, and F toxins at 37°C for 17 days. Each formalin-treated toxin was dialyzed to remove formalin against phosphate-buffered saline (PBS). A sample of each toxoid was then administered intraperitoneally (ip) into mice. Each sample was confirmed to be inactive as no animals died or showed specific symptoms of intoxication. These 4 toxoids were combined with aluminum hydroxide gel, and thimerosal was added to be 0.025 mg/mL of each toxoid in final bulk. The final bulk was dispensed at 0.7 mL per a glass vial. The products were stored at 4°C until use.

Experimental animals: To assess toxicity and neutralizing antibody titer, ddY mice (4 weeks of age, female; Japan SLC, Shizuoka, Japan) were used. Sprague Dawley (SD) rats (5 weeks of age, female; Japan SLC) were used for developing the assay of neutralizing antibody titer, and Hartley guinea pigs (5 weeks of age, female; Japan SLC) were used for tests of freedom from abnormal toxicity, detoxification, and potency. These tests were performed in accordance with the guidelines on handling experimental animals, established by the Japanese Pharmacological Society, and was approved by the Animal Ethics Committee of our universities and institutes. Animals were maintained under controlled light/dark conditions and had free access to food and water.

Quality control tests: The property tests of the vaccine were conducted in accordance with the Minimum Requirements in Japan for adsorbed tetanus toxoid vaccine (12), whilst potency tests (neutralizing antibody titration and toxin challenge tests) were conducted with reference to tests of previous lots of botulinum toxoid vaccine produced in Japan (8). In the property tests, contents of protein, aluminum, formaldehyde, and thimerosal were evaluated, whilst test on pH, sterility, freedom from abnormal toxicity, and detoxification were conducted. The test for freedom from abnormal toxicity was carried out using 2 guinea pigs. Five milliliters of the vaccine was administered ip, and then observed for 7 days. During this period, the guinea pigs showed no abnormal signs, such as weight loss. The detoxification test was conducted with the vaccine kept at 37°C or 4°C for 20 days. Each sample was administered subcutaneously at a dose of 5 mL into 4 guinea pigs. Their body weights were then measured and observed for the presence or absence of paralysis, for 21 days. The potency tests, which involved the use of guinea pigs, was conducted as follows: 2- and 4-fold dilutions of the vaccines with PBS were made. The undiluted and diluted vaccines of each type were administered twice subcutaneously, at a dose of 0.4 mL into 5 guinea pigs with a 2-week interval. Serum samples were taken from the guinea pigs, 5 weeks after the first administration, and pooled according to type and each vaccine dose. The pooled serum samples were used for the neutralizing antibody titration test. A neutralizing antibody titer of these samples higher than 0.1 IU/mL was regarded as having passed the test. In the toxin challenge test for potency, immunized guinea pigs were challenged with 10^4 LD_{50} of each type of toxin and observed for 1 week after toxin administration.

We conducted the quality control tests again, 7 years after production at the end of the clinical study, to assess the stability of the vaccine,

Clinical study: The study was conducted at 5 sites from 2011 to 2014. It was approved by the Medical Ethics, Research Ethics Committee of the universities and institutes and registered with the medical information network clinical trials registry of the university hospital in Japan (unique trial number: UMIN000012905).

Participants of the study were 48 healthy adult volunteers, who participated either in the research into the toxin or its production and were willing to be immunized with the vaccine. The participants were 31 men and 17 women (median age, 42.0 years; range, 22–64 years). Participants with the following were excluded: history of botulism, history of serious adverse events with other vaccines, pregnancy or lactation, history of cardiovascular, renal, liver disorder or hematological disease, history of allergic reaction to food or medicines, and a decision by a physician indicating that the participants is unfit for this study. All the participants provided an informed consent prior to enrolling in the study.

Participants were injected 3 times with the vaccine at an interval of 1 month between administrations. An additional administration was given 1 year after the first administration. Blood samples were taken before each administration, 1 month after the third administration, and 1 month after the final administration. The investigator explained to the participants, the aim and protocol of the study, and interviewed participants about their background. To diagnose the possibility of an allergic reaction, the participants were intradermally inoculated with 10 μL of the vaccine (intradermal test). The investigator observed the participants for 30 min for local or systemic symptoms, and judged whether or not to be vaccinated. The participants were then inoculated with 0.5 mL of the vaccine, and the subjective (fever and local reactions etc.) and objective symptoms were assessed and recorded for 7 days after each injection.

Safety parameters of the vaccine were assessed based on adverse events and reactions during the study. The adverse events and reactions were coded using the medical dictionary for regulatory activities/Japanese ver. 17.0, defined based on the ICH E2A Guideline for Clinical Safety Data Management (13), and assessed by separating them into immunization and intradermal tests.

The immunogenicity of the vaccine was assessed using enzyme-linked immunosorbent assay (ELISA) and neutralizing antibody titers against botulinum toxins type A, B, E, or F in the obtained sera. ELISA titers were measured as previously reported (8). For ELISA, each type of neurotoxin was used as the capture antigen. The ELISA titers were expressed in the highest dilution factor showing an absorbance higher than twice that of the negative control serum. Neutralizing antibody titers were measured by mouse neutralization test and rat compound muscle action potential (CMAP) assay (8,14). In both tests, each type of standard botulinum antitoxin

(National Institute of Infectious Diseases, Tokyo, Japan) was used as reference. The mouse neutralization test was performed using a previously reported method (8). For rat CMAP assay, each standard antitoxin or human sera and test toxin were mixed to initiate a reaction. A volume of 0.1 mL of the mixtures were administrated into the left gastrocnemius muscle of the rats. The CMAP amplitude of the injection leg was measured 24 h after injection. Neutralizing antibody titers in human sera were calculated from calibration curves of the standard antitoxins.

Statistical analysis: Statistical analysis was performed using SAS (ver. 9.2; SAS Institute, Cary, NC, USA). To compare the presence or absence of a botulinum toxoid vaccination before this study, the neutralizing antibody titers, which were measured by mouse neutralization tests and rat CMAP tests, were analyzed by Fisher's exact test and Student's *t*-test, respectively. The significance level of the two-sided test was set at 5%.

RESULTS

Production of tetravalent botulinum toxoid vaccine and quality control tests: Protein concentrations of each purified type M toxin were 0.8–1.4 mg/mL. The M toxoid vaccine passed all the tests performed in accordance with the Minimum Requirements, and the results of the quality control tests are shown in Table 1. In the potency tests, the neutralizing antibody titers of the vaccine immunized groups for all types were higher than 0.1 IU/mL. The immunized guinea pigs were challenged with each type of toxin, and all animals survived, showing no symptom of toxicity.

To assess the stability of the vaccine, we conducted quality control tests 7 years after its production, the end of the clinical study. The concentrations of protein and thimerosal etc. decreased after the 7-year storage (Table 1).

Clinical study: A flow diagram of the study is shown in Fig. 1. Out of the total number of volunteers enrolled, participants who were non-vaccinated botulinum toxoid vaccine was 37 (77%), 23 (48%) had negative past history, and 38 (79%) had no underlying disease. Moderate adverse effects not considered to be related to the vaccine were recorded in 1 participant each after the 1st and 2nd vaccinations, respectively. These symptoms were hypersensitivity angiitis by Arthus reaction and dermatitis by type II hypersensitivity, respectively. These 2 participants discontinued vaccination based on the investigator's judgment. One participant developed

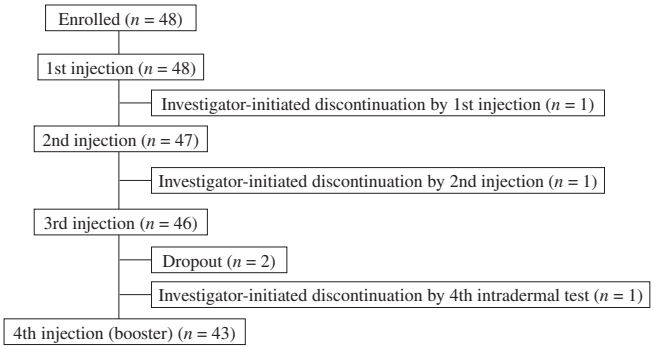

Fig. 1. Summary of clinical study using tetravalent botulinum M toxoid vaccine. A total of 48 participants were enrolled. Three participants discontinued vaccination based on the investigator's judgment. Two participants withdrew for personal reasons (working conditions and resignation). Thus, a total of 43 participants completed the study.

symptoms of allergy (anaphylaxis) after an intradermal test before the 4th immunization, and discontinued vaccination based on the investigator's judgment. Two participants withdrew for personal reasons (working conditions and resignation). Thus, a total of 43 participants completed the study.

Adverse events in the clinical study comprised 30 reactions in102 cases, although they were all mild (Table 2). Adverse events after 1st, 2nd, 3rd, and 4th injection were 34, 36, 16, and 16 cases, respectively. Adverse reactions to vaccination comprised 13 reactions in a total of 64 cases, all cases being mild (Table 2). Adverse reactions after 1st, 2nd, 3rd, and 4th injection were 21, 20, 11, and 12 cases, respectively. The rate of occurrence of adverse reaction to events was 62.75%. Adverse events of the intradermal tests comprised 8 reactions in 68 cases, although they were all mild (Table 2).

The immunogenicity of the vaccine was assessed by ELISA and neutralizing antibody titers, against each type of botulinum toxin in the sera of participants (Fig.2 and 3). ELISA titers are shown in factorials of 2 and increased depending on the number of doses. A preliminary ELISA test was conducted using some positive control sera since ELISA using human sera often show non-specific reaction. In the test, the lowest titer of the positive control sera was 6. Therefore, ELISA titers were not re-measured in cases < 6, but were treated as 5. Average ELISA titers of type A, B, E, and F in the participants' sera before 1st injection were 5.3, 6.1, 5.6, and 6.0, and that of 1 month after the 3rd injection were 8.0, 11.0, 8.7, and 11.1, respectively. The titers, after 1 year of 1st injection (before 4th injection),

Table 1. Properties of tetravalent botulinum M toxoid vaccine: quality control test

Item	Just after production	7 years after production	Criteria
Protein (mg/mL)	0.118	0.096	No criteria
pH	6.52	6.69	5.4 – 7.4
Aluminum (mg/mL)	0.20	0.19	< 0.5
Formaldehyde (%)	0.0005	0.0003	< 0.01
Thimerosal (%)	0.00088	0.00007	< 0.012
Sterility test	Passed	Passed	Absence of viable microorganisms
Test for freedom from abnormal toxicity	Passed	Passed	No animals show any abnormal signs
Detoxification test (4°C, 37°C)	Passed	Passed	No animals show toxic symptoms or other abnormal signs
Potency test (anititoxin titer test)	Passed	Passed	> 0.1 IU/mL (all immunized groups)
Potency test (toxin challenge test)	Passed	Passed	No immunized animals were challenged with toxin die

Table 2. Number of adverse events and reactions after immunization and adverse events after intradermal test during the clinical study

	Symptom	Immunization								Intradermal test
		Adverse event				Adverse reaction				Adverse event
		1st	2nd	3rd	4th	1st	2nd	3rd	4th	
Local symptoms	Injection site pain	6	5	3	4	6	5	3	4	1
	Injection site erythema	4	3	2	4	4	3	2	4	41
	Injection site swelling	2	4	2	2	2	4	1	2	4
	Injection site hemorrhage	5		1	1	5			1	1
	Injection site pruritus		4	2			4	2		18
	Injection site discomfort	1	1	1		1	1	1		
	Hemorrhage subcutaneous	1	1			1	1			
	Injection site induration		1		1		1			1
	Injection site rash	1				1				
	Injection site discoloration			1				1		1
	Injection site warmth									1
Systematic symptoms	Upper respiratory tract inflammation[1)]	1	3		1					
	Nasopharyngitis	1	3	1						
	Fatigue	2	2			1	1			
	Headache	2		1						
	Pyrexia	1	1		1				1	
	Oropharyngeal pain		2							
	Cough	1	1							
	Blepharospasm	1	1							
	Rash		1	1						
	Drug eruption			1				1		
	Other systematic symptoms (10 symptoms)	5	3		2					
subtotal		34	36	16	16	21	20	11	12	
Total		102				64				68

[1)]: Upper respiratory tract inflammation of participants were diagnosed by doctors belong to regional hospital during 7 days after each vaccination.

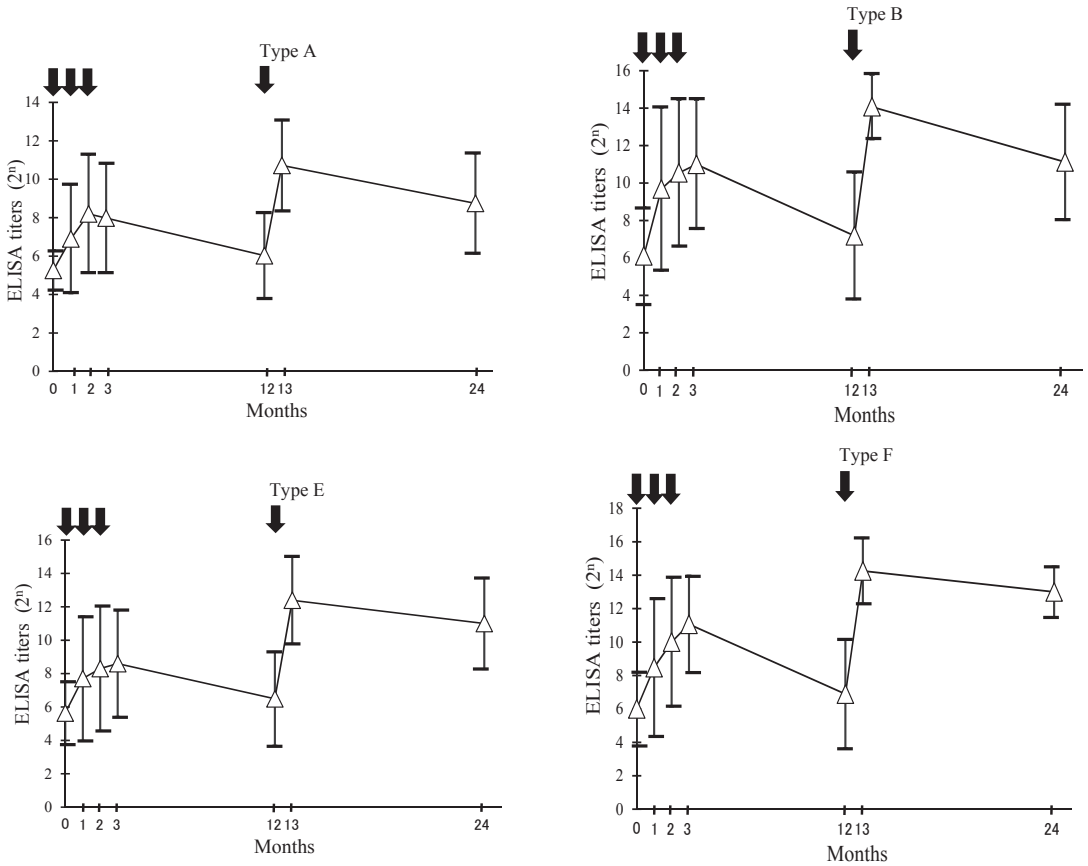

Fig. 2. Time course of ELISA titers in all participants' sera. △, average of ELISA titers (error bar shows standard deviation [SD]), and ↓, vaccinations. Titers at 1st injection (n = 48), 2nd injection (n = 47), 3rd injection (n = 46), 1 month after 3rd injection (n = 46), 4th injection (n = 46), 1 month after 4th injection (n = 43), and 1 year after 4th injection (n = 8), were examined.

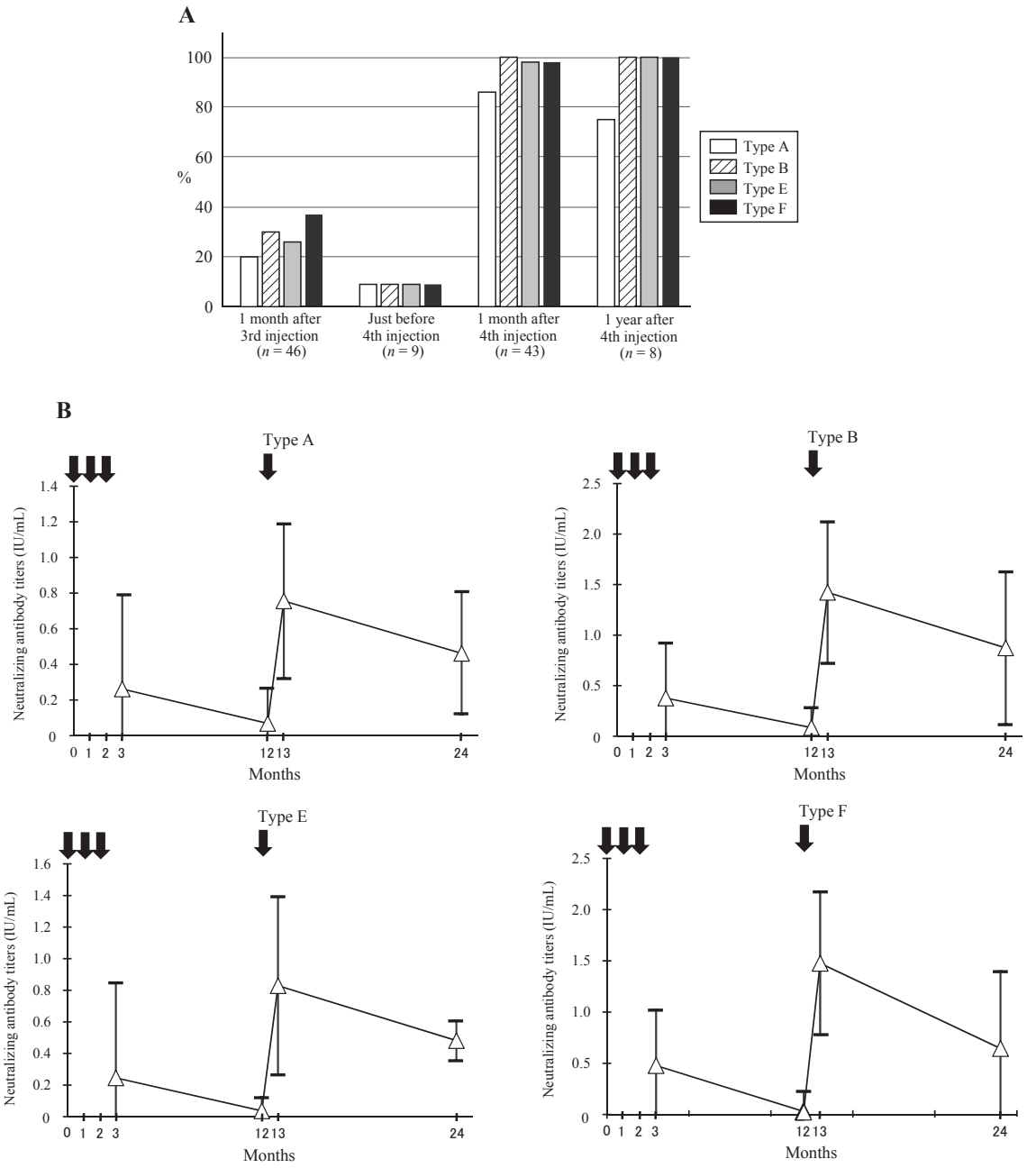

Fig. 3. (A) Percentage of neutralizing antibody titers over 0.25 IU/mL in participants' sera. (B) Time course of neutralizing antibody titers in all participants' sera. △, average of neutralizing antibody titers (error bar shows SD), and ↓, vaccinations. Titers at 3rd injection (n = 46), 1 month after 3rd injection (n = 46), 4th injection (n = 46), 1 month after 4th injection (n = 43), and 1 year after 4th injection (n = 8), were examined.

decreased to almost the same level as it was before immunization (type A, 6.0; B, 7.2; E, 6.5; and F, 6.9). The titers, after 1 month after the 4th injection increased up to higher than that of 1 month after the 3rd injection (type A, 10.7; B, 14.1; E, 12.4; and F, 14.3). The titers of 8 participants at only one study site were measured 1 year after the 4th injection, and 7 of them had no previously botulinum vaccination before the study. The titers of type A, B, E, and F in the sera of 8 participants were 8.8, 11.1, 11.0, and 13.0, respectively. The titers of the 7 participants who had not been previously vaccinated were also at the same level (type A, 8.7; B, 11.3; E, 10.7; and F, 13.1). To evaluate persistence of the titers, the titers of 1 month and 1 year after the 4th injection were analyzed using the Student's t-test. In type E and F, there was no significant difference between the titers of 1 month and 1 year after the 4th injection (p = 0.174 and 0.090 in type E and F, respectively). However, there was a significant difference between the titers in type A and B (p = 0.009 and 0.0003, respectively).

In the mouse neutralization test, the rates of participants with neutralizing antibody against each type of toxin of more than 0.25 IU/mL were 20–37% at 1 month after the 3rd injection, 84–100% at 1 month after the 4th injection, and 75–100% at 1 year after the 4th injection (Fig. 3A). To investigate the association

Table 3. Association between history of botulinum vaccination and neutralizing antibody titers 1 month after 4th injection

Vaccination history	Neutralizing antibody titer (IU/mL)							
	Type A		Type B		Type E		Type F	
	> 0.25	≤ 0.25	> 0.25	≤ 0.25	> 0.25	≤ 0.25	> 0.25	≤ 0.25
Yes	6	3	9	0	8	1	8	1
No	31	3	34	0	34	0	34	0
p value[1)]	0.095		NA		0.209		0.209	

[1)]: Fisher's exact test.
NA, not applicable.

between the history of botulinum vaccination and increase in neutralizing antibody titer, the presence or absence of a botulinum toxoid vaccination history of the neutralizing antibody titers, 1 month after 4th injection were analyzed by Fisher's exact test. There was no significant difference between the presence and absence of the vaccination history groups (p = 0.095, not applicable, 0.209, and 0.209, in type A, B, E, and F, respectively) (Table 3).

The neutralizing antibody titer in the sera of the participants was quantitatively measured using the rat CMAP method (Fig. 3B). The average of type A, B, E, and F titers, 1 month after the 3rd injection were 0.262, 0.376, 0.243, and 0.478 IU/mL, respectively. The titers, 1 year after the 1st injection, decreased to between 6% and 26% of that of the titer at 1 month after the 3rd injection (type A, 0.069; type B, 0.084; type E, 0.037; and type F, 0.030 IU/mL). Titers recorded 1 month after the 4th injection were 0.756, 1.422, 0.829, and 1.476 IU/mL, respectively. The titers, 1 year after the 4th injection were maintained at almost 50% of titers of 1 month after the 4th injection (type A, 0.462; B, 0.873; E, 0.481; and F, 0.644 IU/mL).

The presence or absence of a botulinum toxoid vaccination history of the neutralizing antibody titers 1 month after 4th injection were analyzed using Student's *t*-test. There was no significant difference between the presence and absence of the vaccination history groups (p = 0.242, 0.299, 0.834, and 0.396 in type A, B, E, and F, respectively).

DISCUSSION

Botulinum toxoid vaccine is produced using partially purified toxins containing various progenitor toxins with HA, in Japan (7,8). The toxins do not have constant ratios and amounts of various progenitor toxins due to culture conditions. To produce a fixed composition of a vaccine and reduce side effects at the time of vaccination, we produced a new tetravalent botulinum toxoid vaccine derived from each type M toxin not containing HA. Since the manufacturing of botulinum M toxoid vaccine has not been approved anywhere in the world, there is no standard quality criteria for a vaccine. Therefore, we considered botulinum toxin to be similar in structure and activity to tetanus toxin. As a result, quality control tests of the vaccine were conducted with reference to the Minimum Requirements for adsorbed tetanus toxoid vaccine and that of previous lots of botulinum toxoid vaccine produced in Japan (7,8,12). In the quality control tests, the measured values of the vaccine were within the requirements and yielded similar results to previous products. Botulinum toxoid vaccine is required at the time of crises as terrorism, therefore, the vaccine needs to be stored for a longer period than general vaccines (5). However, liquid vaccines are generally effective for 2–3 years. To assess the stability of the vaccine, we conducted quality control tests up to 7 years after production. The concentrations of protein and thimerosal were decreased after 7-year storage, although the measurement value of each test was within the requirements even after 7 years, suggesting a preservation of the properties and potency of the vaccine until at least 7 years after production. We will investigate the stability of this vaccine in the future.

This was the first clinical study to be conducted according to the Ethical Guidelines for Clinical Research in Japan, using the botulinum toxoid vaccine. Regarding safety of the vaccine, the adverse reactions that were caused by the vaccine were all mild symptoms. The adverse events that were not caused by the vaccine comprised 2 cases classified as moderate. These cases were dermatitis and hypersensitivity vasculitis caused by type II and Arthus reactions, respectively. The participants with the type II reaction had been treated for dermatitis prior to the study. Therefore, it was considered that the dermatitis was not directly caused by the immunization. The case of hypersensitivity vasculitis was considered as not caused by vaccine antigen because the patient did not show an increased antibody titer against botulinum toxin. The adverse events after the intradermal tests comprised 8 symptoms in 68 cases, but all were mild. As a result, the incidence and severity of the adverse reactions and events after vaccination were at the same level as those of commercially available toxoid vaccine products containing aluminum adjuvants such as diphtheria-pertussis-tetanus toxoid vaccine (15–18).

Concerning the immunogenicity of the vaccine, average of the participants' neutralizing antibody titers against each type of toxin were more than 0.5 and 0.25 IU/mL, 1 month and 1 year after the 4th injection, respectively. Type A titers of participants were slightly lower than other types. However, specific activity of type A toxin was no different from other types, and the cause of that was unknown. The Centers for Disease Control and Prevention (CDC) reported that antibody titers against toxins over 0.02 IU/mL may assure sufficient protection and recommend a titer higher than 0.25 IU/mL for those at high risk (19). More than 86% of the participants' neutralizing antibody titers were increased above 0.25 IU/mL, and all the participants' titers were more than 0.1 IU/mL, 1 month after the 4th injection. Furthermore, more than 75% of the

participants neutralizing antibody titers were kept above 0.25 IU/mL, with all the participants' titers recording more than 0.02 IU/mL, 1 year after the 4th injection. The participants showed continuously high titers of antibodies after the 4th injection. The vaccine has the ability to induce neutralizing antibody above the level necessary for protection for those at high risk. This study has demonstrated that the vaccine may provide immunity against botulinum toxin for researchers and counter terrorism of bioweapons.

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Conflict of interest Y. Torii was an employee of KAKETSUKEN. K. Morokuma, Y. Horikawa, and A. Ginnaga are employees of KAKETSUKEN. T. Kohda and S. Kozaki received research grant from KAKETSUKEN.

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