RAPID AND HIGHLY SENSITIVE ENZYME IMMUNOASSAY FOR QUANTITATIVE DETERMINATION OF TETRODOTOXIN

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SUMMARY: A monoclonal antibody against tetrodotoxin (TTX) was obtained from Balb/c mice immunized with TTX-bovine serum albumin (BSA) conjugate. The monoclonal antibody was highly specific for TTX and had no cross-reaction to tetrodonic acid, which is a TTX derivative, or gonyautoxins, although a minor cross-reaction to anhydro-tetrodotoxin was observed. The monoclonal antibody neutralized the lethal activity of TTX. By using the monoclonal antibody, a rapid and highly sensitive competitive enzyme immunoassay (EIA) for quantitative analysis of TTX was developed. By the competitive EIA system, TTX can be determined quantitatively in about 30 min (90 min are required if the time for preparation of the solid-phase antigen was included), and the working range for quantitative analysis of TTX was 2-100 ng/ml. In recovery tests and examinations of TTX samples, results of the mouse bioassay and EIA analyses correlated well ($r=0.987$). Moreover, it was demonstrated that low concentrations of TTX, which could not be detected by the mouse bioassay, could be determined quantitatively by the competitive EIA.

INTRODUCTION

TTX is one of the most potent neurotoxins and blocks sodium ion channels responsible for nerve and muscle excitability (1,2). TTX is found in various ani-

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mals including puffer fish and some shellfish (3-14) and causes paralytic poisoning and occasional deaths in humans through ingestion (15,16). It has been considered that TTX is originally produced by marine bacteria and subsequently distributed widely in various marine organisms (17-23). It has also been reported that a small amount of TTX is found in cultured puffer fish which are originally nontoxic (24). Therefore, it is very important to monitor closely TTX in edible puffer fish including cultured ones and other marine products including shellfish.

Mouse bioassay and fluorometric high performance liquid chromatography (HPLC) have been the main methods for determination of TTX (25-28). The mouse bioassay has been used as an official method for the quantification of TTX in Japan (25), but it has a low sensitivity. In addition, the bioassay requires also the continuous supply, maintenance and control of mice. On the other hand, the fluorometric HPLC has a high sensitivity (26-28) but an insufficient specificity (29). This method requires cleanup of samples and very expensive instruments (27). In contrast, it is accepted that the competitive EIA has a high specificity and sensitivity and requires no cleanup of samples. Therefore, EIA systems for some marine toxins such as saxitoxin, ciguatoxin and okadaic acid have been developed (30-35). Recently, there have been a few reports on competitive EIA for TTX (36-38); however, these EIA systems for TTX were either of a low sensitivity (36) or required a long time for determination of TTX in comparison with the mouse bioassay (37,38). In these EIA systems, either an antibody against tetrodonic acid, a TTX derivative (36), or that obtained from mice immunized with TTX-keyhole limpet hemocyanin (KLH) conjugate (37,38) was used. However, tetrodonic acid is very different in chemical structure from TTX (Fig. 1) and it has been reported that the antibody obtained from the mice immunized with TTX-KLH conjugate had a low affinity for TTX (39).

Thus, we, using TTX-BSA as an immunogen, attempted to produce a monoclonal antibody with high affinity against TTX and, using the monoclonal antibody, to develop a rapid and highly sensitive competitive EIA for quantitative analysis of TTX.

MATERIALS AND METHODS

TTX: TTX (crystalline 3X) was purchased from Sankyo Co. Ltd. (Tokyo) as the standard for the competitive EIA and from Wako Pure Chemical Industries (Osaka) for preparation of TTX-protein conjugate and recovery tests.

Preparation of TTX-protein conjugate: TTX was conjugated to bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) and ovalbumin (OVA, Sigma) by the method of Johnson et al. (40) with some modifications.

(a) **Preparation of TTX-BSA conjugate.** Five milligrams of BSA was dissolved in 0.4 ml of 0.01 M phosphate buffer (PB, pH 7.6). To this were added 0.25 mg of TTX and 0.1 ml of 2.5% formaldehyde. After incubation for 72 hr at 25 C and then for 12 hr at 4 C, the mixture was loaded onto a PD-10 column (Pharmacia Biotech., Uppsala, Sweden) equilibrated with 0.01 M PB. After eluting the mixture with 0.01 M PB, the protein-containing portion (3.5 ml) was collected.

(b) **Preparation of TTX-OVA conjugate.** Five milligrams of OVA was dissolved in 0.4 ml of 0.01 M PB. To this were added 0.5 mg of TTX and 0.1 ml of 2.5% formaldehyde. The remaining steps were the same as those described above.

Immunization and somatic cell fusion: TTX-BSA conjugate (1 mg/ml in 0.01 M PB) was used as the immunogen. Six female BALB/c mice (SPF grade, 7 weeks old, Japan SLC, Inc., Shizuoka, Japan) were immunized by ip injection with 0.2 ml of an emulsion prepared from the immunogen with an equal volume of complete Freund's adjuvant. Three, 5, 7 and 9 weeks later, the mice were boosted with 0.2 ml of an emulsion prepared from the immunogen with an equal volume of incomplete Freund's adjuvant in the same manner. Five weeks later, i.e., 4 days prior to cell fusion, the mice received the immunogen alone (0.5 mg/ml in 0.01 M PB, 0.2 ml/mouse) in the same manner. Splenocytes from the immunized mice were mixed with murine myeloma cells (P3-X63-Ag8.U1) at a ratio of 10:1 and fused by the procedure described by Koehler and Milstein (41) with some modifications.

Selection by hypoxanthine aminopterin thymidine medium (HAT medium) and cloning: The fused cells were cultured in HAT medium and screened with the naked eye for cell growth. The culture media were screened for anti-TTX antibody by enzyme immunoassay (EIA) (described below). Hybridoma cell populations secreting anti-TTX antibody were identified by EIA and cloned by limiting dilution on feeder layers of murine thymocytes.

Amplification and purification of hybridoma proteins: Each cloned hybridoma cell line was grown by tissue culture and amplified in the ascites of pristane (2,6,10,14-tetramethyldecanoic acid)-primed BALB/c mice. Murine IgG-containing TTX-specific antibodies were purified from the ascites supernatant by affinity chromatography on a proetin A-agarose gel (AmpureTMPA kit, Amersham International, Buckinghamshire, UK) after salting out with 50%

saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 7.4). Isotyping of the monoclonal antibody was performed with a mouse monoclonal antibody isotyping kit (Amersham International, UK).

Preparation of monoclonal antibody-peroxidase conjugate: F(ab')_2 fragments were prepared by limited proteolysis of purified anti-TTX monoclonal antibody with immobilized pepsin (Sigma). Fab' fragments were prepared by reduction of F(ab')_2 fragments with cysteamine hydrochloride (Wako). Ten milligrams of peroxidase (EIA grade, Boehringer Mannheim, Germany) was dissolved in 1.5 ml of 0.1 M phosphate buffer (pH 6.0). To this was added 0.1 ml of an *N*-(6-maleimidocaproyloxy) succinimide (EMCS, Dojin Laboratories, Kumamoto) solution (31 mg of EMCS in 1 ml of dimethylformamide). After incubation for 60 min at 30 C, this mixture was loaded onto a PD-10 column equilibrated with 0.1 M phosphate buffer (pH 6.0). The mixture was eluted with 0.1 M phosphate buffer (pH 6.0), and the protein-containing portion (2.0 ml) was collected. Fab' fragments (3.2 mg) were dissolved in 1.5 ml of 0.1 M phosphate buffer (pH 6.0) containing 5 mM EDTA. To this was added 0.6 ml of a modified peroxidase solution (5 mg/ml in 0.1 M phosphate buffer, pH 6.0). After incubation for 60 min at 30 C, conjugates were purified from the mixture by affinity chromatography on concanavalin A-sepharose gel (Pharmacia Biotech.).

In vitro neutralization test: To 1 ml of a TTX solution containing 5 mouse units of TTX, 1 ml each of monoclonal antibody solutions at various concentrations (0, 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/ml) was added, which was mixed and incubated for 60 min at 37 C. One milliliter of each mixture containing 2.5 mouse units of TTX, which kills a mouse in about 7 min, was administered intraperitoneally to male ddY mice weighing 20 g. The lethality was observed for 24 hr after administration. In this test, one mouse unit is defined as the amount of TTX that kills a male mouse of ddY strain weighing 20 g in 30 min after ip injection. Also, Dulbecco's phosphate buffered saline without monoclonal antibody was used as a control.

Procedure and application of EIA: EIA was used to select hybridomas that produced antibodies specific to TTX. TTX-OVA or OVA was diluted in 0.05 M sodium bicarbonate buffer (pH 9.6) to a concentration of 3.0 $\mu\text{g/ml}$, and 0.1 ml of the mixture was added to each well of a 96-well microtiter plate (Sumitomo Bakelite Co., Ltd., Tokyo). The plate was incubated for 1 hr at room temperature, and the solution was then removed. After washing three times with Dulbecco's phosphate buffered saline (D-PBS) containing 0.05% Tween 20 (PBS-T), the residual protein-binding sites were blocked with D-PBS containing 20% horse serum for 60 min at room temperature. The plate was washed three times with PBS-T, and 0.1 ml of a hybridoma supernatant solution diluted with PBS-T containing 10% horse serum was then added to each well. After incubation for 1 hr at room temperature and washing five times with PBS-T, 0.1 ml of 1:1,000 diluted goat anti-mouse immunoglobulin-peroxidase conjugate (Sigma) was added to each well. The plate was then incubated for 1 hr at room temperature and washed three times

with PBS-T. After washing, 0.1 ml of a substrate solution (described below) was added to each well. After incubation for 10 min at room temperature, the reaction was stopped with 0.1 ml of 1 N H₂SO₄. The absorbance at 450 nm of the water-soluble reaction products was measured with a microplate-auto reader (Model 550, Japan-Biorad, Tokyo).

The substrate solution was prepared, just before use, by mixing 0.1 ml of a 3,3',5,5'-tetramethylbenzidine (TMBZ, Dojin) solution (10 mg of TMBZ in 1 ml of dimethyl-formamide) and 1.5 µl of 30% hydrogen peroxide in 9.9 ml of 0.05 M citrate buffer (pH 5.5).

Procedure and application of competitive EIA: This assay was used to determine quantitatively TTX and examine the specificity of the monoclonal antibody against TTX. Antigen-coated plates were prepared in the same manner as described in the EIA procedure. After washing, 0.1 ml of TTX standard of increasing concentrations (0-200 ng/ml) or each sample was added to each well, followed by 0.05 ml of monoclonal antibody-peroxidase conjugates (Fab' fragments) at 0.26 µg/ml diluted with phosphate buffered saline (0.03 M phosphate buffer, pH 7.4; 0.41 M NaCl, 8 mM KCl), and the contents of the well were immediately mixed. After incubation for 20 min at room temperature and washing five times with PBS-T, the substrate solution was added to each well. The following steps in the competitive EIA were performed as described in the EIA procedure. The amount of antibody bound to the solid-phase antigen (coated on the plate) was calculated as follows: the absorbance values obtained for TTX standards or samples were divided by the absorbance value of 0 ng/ml of the TTX standard (see below) and multiplied by 100.

Preparation of TTX standards for competitive EIA: First, TTX (0.1 mg/ml) was adjusted to various concentrations (5, 10, 25, 50, 100, 250, 500 and 1,000 ng/ml) in 0.1% acetic acid. The solutions were diluted five-fold with distilled water and then used as TTX standards (final concentrations of TTX standards were 1, 2, 5, 10, 20, 50, 100 and 200 ng/ml). A 0.02% acetic acid solution was used as a control of the TTX standard (0 ng/ml).

Specificity of the monoclonal antibody against TTX: The specificity of the monoclonal antibody against TTX was examined by comparing the concentration of each toxin (TTX, tetrodonic acid, anhydro-terodotoxin or gonyautoxins) that causes 50% inhibition of binding of the antibody to the solid-phase antigen in the competitive EIA. Tetrodonic acid and anhydro-terodotoxin, which are TTX derivatives, were synthesized from TTX by the method of Tsuda et al (42). Gonyautoxins, which were a mixture of gonyautoxin I, II, III and IV, were purchased from Wako.

Mouse bioassay: Mouse bioassay was performed by the method of Kawabata (25). One mouse unit is defined as the amount of TTX that kills a male mouse of ddY strain weighing 20 g in 30 min and is equivalent to 220 ng of TTX. To compare the mouse bioassay with the competitive EIA, the results of the bioassay were expressed in the TTX content.

Preparation of sample: Samples from the muscles of puffer fish were prepared by the 0.1% acetic acid extraction-method (25). To 10 g of homogenized puffer fish muscle, 25 ml of 0.1% acetic acid was added. The mixture was boiled and stirred for 10 min. The extract was cooled and centrifuged at 3,000 rpm for 15 min at 4 C. After collecting the supernatant, aliquots (15 ml) of 0.1% acetic acid were again added to the remaining precipitates. The mixture was then stirred and centrifuged to collect the supernatant. Two supernatants were combined and then filled to 50 ml with 0.1% acetic acid. This solution was used for the mouse bioassay. For the competitive EIA, this solution was diluted five-fold with distilled water. When the samples contained a high level of TTX, these were diluted more than five-fold.

Recovery tests and examinations of TTX samples from puffer fish: To compare the competitive EIA with the mouse bioassay, recovery tests and examinations of TTX samples were carried out. In recovery tests, different quantities (2.5, 5, 10 and 20 µg) of TTX were injected into the muscles (10 g) of three species of puffer fish (*Takifugu rubripes*, *Takifugu xanthopterus* and *Lagocephalus wheeleri*). After keeping for 30 min at room temperature, concentrations of TTX in the muscles were estimated by the mouse bioassay and the competitive EIA. In examinations of TTX samples, the muscles of naturally contaminated puffer fish (six *Takifugu poecilonotus* and seven *Lagocephalus lunaris*) were used. Concentrations of TTX in the muscles were estimated by the mouse bioassay and the competitive EIA, and the results obtained by the mouse bioassay were plotted against those by the competitive EIA.

Takifugu rubripes, a cultured puffer fish, was purchased from a seafood store. *Takifugu xanthopterus*, *Lagocephalus wheeleri* and *Takifugu poecilonotus* were captured offshore Ikitsukishima Island in Nagasaki Prefecture. *Lagocephalus lunaris* was provided by Osaka Quarantine Station, Ministry of Health and Welfare.

RESULTS

Production and Specificity of Monoclonal Antibody against TTX

Because TTX, a hapten, has no immunogenicity, TTX-BSA conjugate was synthesized for the immunization of mice. The other hapten-protein conjugate, TTX-OVA, was synthesized for EIA. For selection of specific antibody-secreting hybridomas, the protein carrier used for EIA must be different from that for mice immunization. The antibodies to the protein carrier were not reactive in EIA. Five culture supernatants in 180 wells of the tissue culture plates showed positive results in EIA (2.8%). Clear competition in the competitive EIA was observed in

Table I. Specificity of the monoclonal antibody against tetrodotoxin

Toxin (mol. wt.)	Concentration of 50% inhibition	Cross-reactivity %
Tetrodotoxin (319)	0.05 nmol/ml (15 ng/ml)	100
Anhydro- tetrodotoxin (301)	0.60 nmol/ml (180 ng/ml)	8.3
Tetrodonic acid (337)	>0.30 μ mol/ml (>100 μ g/ml)	<0.017
Gonyautoxins	>100 μ g/ml	<0.015

The specificity of the monoclonal antibody against TTX was examined by comparing the concentration of each toxin that caused 50% inhibition of binding of the antibody to solid-phase antigen in the competitive EIA. Gonyautoxins are a mixture of gonyautoxin I, II, III and IV.

Fig. 1. The chemical structures of tetrodotoxin, anhydro-tetrodotoxin and tetrodonic acid.

Table II. In vitro neutralizing activity of the monoclonal antibody to the lethality of tetrodotoxin

Monoclonal antibody concentration (mg/ml)	Survival/Total	% Survival
1.0	5 / 5	100
0.5	5 / 5	100
0.25	5 / 5	100
0.125	2 / 5	40
0.0625	0 / 5	0
0	0 / 5	0

To 1 ml of a TTX solution containing 5 mouse units of TTX, 1 ml of a monoclonal antibody solution of various concentrations (0, 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/ml) was added and the mixture was incubated for 60 min at 37 C. One milliliter of the mixture (containing 2.5 mouse units) was administered intraperitoneally to each of five male ddY mice weighing 20 g. The lethality was determined 24 hr after administration. One mouse unit is defined as the amount of TTX that kills a male mouse of ddY weighing 20 g in 30 min after ip injection.

two of the five wells by using 5 ng of TTX per assay. Each hybridoma-secreting anti-TTX antibody was cloned three times by the limiting dilution method, and two cloned hybridoma cell lines (T1-1 and T2-1) were obtained. The clone (T1-1) that showed the highest affinity to free TTX was selected and used in this study. The class of heavy and light chains of the monoclonal antibody were IgG1 and k, respectively. The specificity of the monoclonal antibody against TTX is shown in Table I. The monoclonal antibody had no cross-reaction to tetrodonic acid, whose chemical structure is shown in Fig. 1 (c), or gonyautoxins under the experimental condition ($<0.017\%$). However, the antibody had only a minor cross-reaction to anhydro-tetrodotoxin, which is very similar in chemical structure to TTX, as shown in Fig. 1 (b) (8.3%). However, affinity between the antibody and anhydro-tetrodotoxin was lower than one-tenth of that between the antibody and TTX.

Table III. Reproducibility of standard curve for tetrodotoxin in the competitive EIA

	TTX standard (ng/ml)	Antibody binding (%)	
		Mean $\pm$ SD	CV (%)
intra-assay	2	85.0 $\pm$ 2.3	2.7
	5	74.1 $\pm$ 2.4	3.2
	10	55.7 $\pm$ 1.9	3.4
	20	43.9 $\pm$ 1.8	4.1
	50	25.6 $\pm$ 2.4	9.4
	100	12.8 $\pm$ 1.2	9.4
inter-assay	2	86.2 $\pm$ 1.2	1.4
	5	75.5 $\pm$ 1.2	1.6
	10	57.0 $\pm$ 1.3	2.3
	20	45.1 $\pm$ 1.4	3.1
	50	26.5 $\pm$ 1.4	5.3
	100	14.1 $\pm$ 1.5	10.6

Antibody binding (%); see Fig. 2. Each antibody binding (%) of intra-assay represents the mean of six assays, and each antibody binding (%) of inter-assay represents the mean of triplicate assays. SD and CV represent standard deviation and coefficient of variation, respectively.

In Vitro Neutralization Test

The results of the neutralization test are shown in Table II. The mice injected with a mixture of a TTX solution and PBS or an antibody solution at a low concentration (0.0625 mg/ml) all died. In contrast, the mice injected with a mixture of TTX solution and an antibody solutions at a high concentration (0.125, 0.25, 0.5 or 1.0 mg/ml) showed from 40 to 100% survival. These results showed that the monoclonal antibody neutralized the lethal activity of TTX.

Competitive EIA

Figure 2 and Table III show standard curves for TTX in the competitive EIA and its reproducibility, respectively. The absorbance values obtained for 0 ng/ml

Fig. 2. Standard curve for tetrodotoxin in competitive EIA. Microtiter wells coated with TTX-OVA were incubated with increasing concentrations of TTX standards (0, 1, 2, 5, 10, 20, 50, 100 and 200 ng/ml) in the presence of a constant amount of monoclonal antibody-peroxidase conjugate (0.26 μ g/ml). Antibody binding (%) was calculated as follows: the absorbance values obtained for TTX standards were divided by the absorbance value of 0 ng/ml of TTX standard and multiplied by 100. Each point represents the mean of six assays.

of TTX standard ranged from 1.60 to 1.70. Linearity of the standard curve for TTX was observed from 2 to 100 ng/ml (Fig. 2). The standard curves for TTX had good reproducibility in a range from 2 to 100 ng/ml since the intra-assay and inter-assay coefficients of variation were less than 9.4 and 10.6%, respectively (Table III). In the competitive EIA, the concentration of toxin causing 10-20% inhibition of binding of the antibody to the solid-phase antigen is generally established as the detection limit of the assay (31,32,34). Therefore, the detection limit for TTX in the competitive EIA was established at 2 ng/ml. The results shown in Fig. 2 and Table III show the working range for quantitative analysis of TTX in the competitive EIA being 2-100 ng/ml. When a sample was prepared by the 0.1% acetic acid-extraction method, the detection limit for TTX in the competitive EIA was regarded as 50 ng/g of the original sample in the calculation.

Table IV. Recovery tests of tetrodotoxin in muscles of puffer fish

Detection method	Toxin injected (ng/g)	Determination value (ng/g)	
		Mean $\pm$ SD	CV (%)
Competitive EIA	0	<50	—
	250	251 $\pm$ 39.8	15.9
	500	485 $\pm$ 35.0	7.2
	1000	1025 $\pm$ 115.5	11.3
	2000	2042 $\pm$ 167.8	8.2
Mouse bioassay	0	ND	—
	250	ND	—
	500	ND	—
	1000	ND	—
	2000	1962 $\pm$ 148.0	7.5

Different quantities (2.5, 5, 10 and 20 μ g) of TTX were injected into muscles (10 g) of three species of puffer fish, and then extracted by the 0.1% acetic acid extraction-method. Concentrations of TTX in the samples were estimated by the mouse bioassay and the competitive EIA. When the 0.1% acetic acid extraction-method was used, the detection limits of the competitive EIA and the mouse bioassay were 50 and 1100 ng/g of the original sample, respectively. Each determination value represents the mean of three experiments. SD and CV represent standard deviation and coefficient of variation, respectively. ND is not detectable.

Recovery Tests and Examinations of TTX Samples from Puffer Fish

Table IV shows the results of recovery tests. When the 0.1% acetic acid extraction-method was used, final concentrations of TTX injected in the extracts were 0, 50, 100, 200 and 400 ng/ml. Therefore, by the mouse bioassay, whose detection limit was 220 ng/ml, ranges from 250 to 1,000 (ng/g) of TTX injected were not detected. In contrast, by using the competitive EIA, all ranges of TTX injected were precisely determined (coefficients of variation for determination values in three experiments were less than 15.9%). These results showed that such low concentrations of TTX that could not be detected by the mouse bioassay, were

Fig. 3. Correlation of results for TTX obtained by the mouse bioassay and by the competitive EIA ($n=9$). In examination of the muscles from naturally contaminated puffer fish (six *Takifugu poecilonotus* and seven *Lagocephalus lunaris*), the results of the mouse bioassay were plotted against the results of the competitive EIA. To compare the mouse bioassay with the competitive EIA, the results of the mouse bioassay were expressed as TTX content (one mouse unit is equivalent to 220 ng of TTX). Four samples of *Takifugu poecilonotus* gave positive results only by the competitive EIA. TTX concentrations of the samples estimated by the competitive EIA were 0.25, 0.31, 0.56 and 0.59 $\mu\text{g/g}$, respectively.

determined quantitatively by the competitive EIA. Figure 3 shows correlations of TTX determinations by the competitive EIA and the mouse bioassay. An excellent correlation was observed between the results of the mouse bioassay and EIA analyses ($r=0.987$).

DISCUSSION

Initially, we attempted to improve the preparation method of immunogen to obtain a monoclonal antibody with a high specificity against TTX. In previous studies, TTX-KLH conjugate was used as an immunogen (37,38). However, there was a report stating that it was difficult to produce the antibody with a high affinity for TTX in mice immunized with TTX-KLH conjugate (39). Actually, the antibody obtained from the mice immunized with TTX-KLH conjugate prepared in our preliminary study had no reaction to free TTX (data not shown). Therefore, in this study, TTX-BSA conjugate was used as an immunogen to produce the antibody against TTX. In addition, in this study, an immunogen binding a larger quantity of TTX than that in the previous studies (37,38) was used for the immunization. It appears that the application of this immunogen is one of the reasons why we succeeded to obtain a monoclonal antibody with a high specificity against TTX. TTX (Fig. 1, a) an Na^+ channel blocker, shows toxicity by attaching to an Na^+ channel with three points of the guanidinium group and the hydroxyls at C9 and C10 (1). Anhydro-tetrodotoxin (Fig. 1, b), in which C4-OH and C9-OH are joined by an oxygen bridge, has a low affinity to the Na^+ channel and little toxicity (toxicity is at least a 100 times less than that of TTX) (1,2,4,42). Tetrodonic acid (Fig. 1, c), in which both C9-OH and C10-OH are modified, has neither affinity to the Na^+ channel nor the toxicity (1,2,4,42). The monoclonal antibody produced in this study had only a minor cross-reaction to anhydro-tetrodotoxin, but had no cross-reaction to tetrodonic acid (Table I). In addition, this antibody neutralized the toxicity of TTX (Table II). The guanidinium group was used as a site to conjugate TTX to protein in this study (40). Therefore, it is regarded that this antibody reacts with the C9-OH and C10-OH of TTX as in the previous study (38). The antibody in the previous study also had only a minor cross-reaction to tetrodonic acid except for anhydro-tetrodotoxin (38). Consequently, this antibody is more specific against TTX than that in the previous study.

Next, using this monoclonal antibody, we attempted to develop a rapid and highly sensitive competitive EIA system for quantitative analysis of TTX. The EIA system for TTX reported previously had an extremely low sensitivity because an antibody against tetrodonic acid, a TTX derivative, was used (36). More recently, the EIA system reported by Matsumura et al. (37,38) had a high sensitivity, but required a long time to determine TTX since the avidin-biotin system was used to improve a sensitivity of the assay. In contrast, the EIA system developed

in this study had a sufficient sensitivity without the avidin-biotin system, since the antibody with high affinity against TTX was used (the working range for the quantitative analysis of TTX was 2-100 ng/ml). In addition, the extra steps in the assay could be omitted by use of the antibody-peroxidase conjugate. Therefore, in the EIA system developed in this study, TTX could be determined quantitatively and sensitively in about 30 min (90 min was required if the time for preparation of the solid-phase antigen is included). If this EIA system is used, a rapid response to an outbreak of food poisoning and the determination of small amounts of TTX in leftovers will be possible. Moreover, this EIA system, which uses the antibody that had no or minor cross-reaction to TTX derivatives, is useful to determine the exact toxicity of puffer fish, since other TTX derivatives found in puffer fish show no or slight toxicity (42,43). The toxicity of puffer fish is different depending on the organ and species, and the consumption of the muscles of 21 species, the skins of 11 species and the tests of 18 species of puffer fish are allowed in Japan. Puffer fish whole are generally marketed, but the muscles, the skins and the tests of puffer fish have often been marketed separately. In the latter case, it is very difficult to identify the toxicity by morphological features. Therefore, it is necessary to monitor closely TTX in seafoods derived from puffer fish. At present, the methods for monitoring TTX are mainly mouse bioassay and fluorometric HPLC (25-28). The mouse bioassay is the official method (25), and TTX can be simply determined in a short time by the bioassay; however, the bioassay has a low sensitivity (its detection limit is 220 ng/ml) (25). In this study, four samples of *Takifugu poecilonotus* were negative in the mouse bioassay, while they were positive in the competitive EIA (Fig. 3). It has recently been reported that a small amount of TTX, which could not be detected by the mouse bioassay, was found in a cultured puffer fish which originally was nontoxic (24). Therefore, it is considered that TTX in the samples must be monitored by a more sensitive method than the mouse bioassay. The fluorometric HPLC is highly sensitive (26-28), but not sufficiently specific (29). In addition, this method requires cleanup of samples and very expensive instruments (27). For the above reasons, it appears that the newly developed EIA system is more useful for the rapid and sensitive monitoring of TTX in seafoods derived from puffer fish and other marine products than the mouse bioassay or the fluorometric HPLC.

It is considered that TTX is originally produced by bacteria, and there are many reports on TTX-producing bacteria (17-23). However, reexamination of possible TTX-producing bacteria is necessary because the existence of such bacteria is being challenged (29). For such studies, it is thought that the antibody ob-

tained in this study would extremely be useful. Moreover, this antibody with a high specificity to TTX can be used widely in TTX-related studies.

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