FOOD BIOLOGICAL CONTAMINANTS

A Simple Membrane Immunobead Assay for Detecting Ciguatoxin and Related Polyethers from Human Ciguatera Intoxication and Natural Reef Fishes

YOSHITSUGI HOKAMA, WARREN E. TAKENAKA, KRISTI L. NISHIMURA, and JOANNE S.M. EBESU
University of Hawaii, John A. Burns School of Medicine, Department of Pathology, Honolulu, HI 96822
ROBERT BOURKE and PATRICK K. SULLIVAN
Oceanit Laboratories, Inc., 1100 Alakea St, Honolulu, HI 96813

A simple membrane immunobead assay (MIA) for detecting ciguatoxin (CTX) and related polyethers directly from fish tissue is presented. A membrane laminated onto a solid plastic support is immersed with a piece of fish tissue in methanol. The membrane is thoroughly dried and placed into an immunobead suspension containing polystyrene particles coated with monoclonal antibody to CTX (MAb-CTX). Two beads of different diameter and color are used. The color intensity of the membrane is related to the concentration of the toxin bound to the membrane. Twelve of 13 fish implicated in human ciguatera fish poisoning showed borderline or positive responses in the assay. A *Sphyraena barracuda* sample that tested negative with the MIA and was highly toxic with the mouse toxicity bioassay showed only weak CTX-like toxin activity in the guinea pig atrial assay, indicating that the major toxin in the sample was not CTX-like. Examination of 154 routinely caught reef fish from Hawaii, Kosrae, and Kwajalein by MIA found 132 (86%) negative and 8 (5%) positive for CTX, with 14 (9%) giving a borderline response. Fish from Hawaii showed a higher frequency of borderline or positive responses than those from Kosrae and Kwajalein, probably because several species of fish from several islands of Hawaii were tested, whereas only one species from a single area was examined from each of the islands of Kosrae and Kwajalein.

The first successful detection of the low-molecular-weight polyether toxin ciguatoxin (CTX) directly from contaminated fish tissues was accomplished by radioimmunoassay (RIA) with a sheep antibody prepared with purified moray eel CTX conjugated to human serum albumin (HSA) as carrier (1). The RIA was used to screen 5529 *Seriola dumerili* (kahala), a species associated with ciguatera outbreaks, in the State of Hawaii from 1979 through part of 1981 (2). The 15% of fish testing borderline or positive were discarded, and the remaining 85% of fish scoring negative were sold commercially. From these fish, no false-negative responses were obtained, attesting to the RIA's validity. Although the RIA was effective, its complexity encouraged the search for a simpler alternative.

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An enzyme immunoassay (EIA) was developed, with sheep anti-CTX coupled to horseradish peroxidase (2–4). Although less costly than RIA because use of expensive isotope-counting instruments was eliminated, this method was tedious and was abandoned. The next method was the stick-enzyme immunoassay (S-EIA), using sheep anti-CTX and MAb-CTX coupled to horseradish peroxidase. This method has been used extensively for surveys of areas where ciguatera poisoning is endemic and for clinical confirmation of documented ciguatera fish poisoning for the State of Hawaii Department of Health (DOH; 5–11).

Colored polystyrene particles coated with MAb-CTX began to be used in 1990 as markers for direct detection of CTX adsorbed on bamboo paddles coated with organic correction fluid (12). In this solid-phase immunobead assay (SPIA), the correction fluid coat bound both polar and nonpolar components to the bamboo paddle sticks. Numerous documented ciguateric fish from the DOH and reef fish surveyed in Hawaii were assessed with this procedure (13, 14).

The membrane immunobead assay (MIA) presented in this paper is based on the immunological principles used to develop the SPIA (12), using a monoclonal antibody to purified moray eel ciguatoxin (CTX-1; 13, 14), colored polystyrene beads, and a hydrophobic membrane laminated onto a solid plastic support. The polyether toxins bind to the hydrophobic polyvinylidene fluoride membrane and are detected by the MAb-CTX coated onto colored polystyrene beads. The intensity of the color on the membrane relates to the concentration of the toxin (Figure 1).

Experimental

Reagents and Materials

Polyvinylidene fluoride membrane (Biotrace, Gelman Sciences, Ann Arbor, MI) was laminated onto a plastic support

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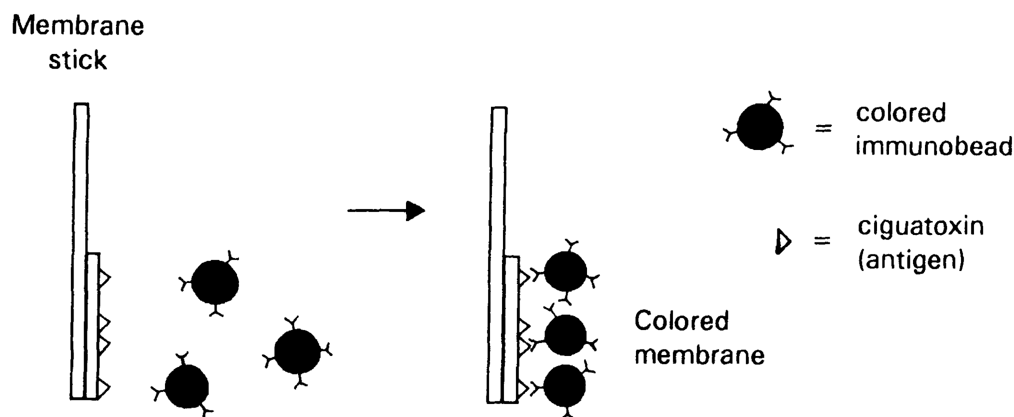

Figure 1. The concept of the MIA: Colored immunobeads (colored latex particles) attach to monoclonal antibody to CTX (antigen), *if any, on the membrane portion of the membrane stick.* Positive samples will show a visible color change, whereas negative samples will not. Intensity of color is proportional to concentration of CTX attached to the membrane.

stick (Figure 2). Other materials and chemicals included test tubes (8 mm id, 75 mm length); a punch biopsy tool or sharp razor to obtain fish samples; methanol for soaking fish samples, and a suspension of 0.1% colored beads (0.314 μm diameter, Seradyn, Inc., Indianapolis, IN; 0.124 μm diameter, Bangs Laboratories, Inc., Carmel, IN) coated with MAb-CTX. Standard control for positives consisted of pooled crude extracts of fish implicated in clinically established ciguatera poisonings, as determined by DOH. The presence of CTX in these crude extracts of Hawaiian reef fish implicated in ciguatera poisoning was examined previously by Hokama et al. (15) and Manger et al. (16), using the neuroblastoma cell bioassay. In addition, the presence of CTX at 4 ng/12.5 mg crude extract was established previously by immunoinhibition of MAb-CTX with highly purified CTX (obtained from T. Yasumoto, Tohoku University, Sendai, Japan; 15).

Source of Fish Samples

Toxic fish samples were obtained from DOH, where fish implicated in ciguatera poisoning were submitted by patients with clinical symptoms verified by an attending physician or the DOH Epidemiology Branch staff. Fish were identified according to the handbook by Tinker (17), and both scientific and Hawaiian or common names are presented in tables and text. Inshore reef fish were collected routinely from various areas noted for ciguatera poisoning outbreaks in the State of Hawaii and, in 2 cases, also from the islands of Kosrae and Kwajalein (*Caranx* sp. or papio samples).

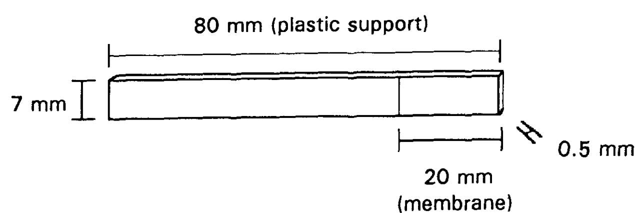

Figure 2. Dimensions of membrane stick for MIA. The membrane portion of the stick is directly laminated onto the longer plastic support.

Fish Extraction

Fish tissues were extracted according to the method of Kimura et al. (18). Tissues were weighed and soaked in acetone (1 + 2.5, w/v; all tissues extracted contained essentially similar moisture contents). After pervaporation under vacuum at 45°–50°C, residues were washed with 20% saline and extracted with chloroform. The chloroform layer was pervaporated under vacuum, and the residue was suspended in hexane–80% methanol (2 + 1). The 80% methanol fraction was separated and pervaporated under vacuum to dryness, and the residue was analyzed by MIA and by the mouse toxicity and guinea pig atrial assays (19). The hexane (nonpolar) fraction was pervaporated under vacuum and stored at –20°C for later use.

Preparation of Monoclonal Antibody to Purified CTX

(**a**) *Source of purified CTX.*—Highly purified CTX was obtained from P.J. Scheuer, University of Hawaii, Department of Chemistry. The toxin was isolated from *Lycodontis (Gymnothorax) javanicus* (moray eel) livers and consisted of 2 interchangeable isomers (20).

(**b**) *Coupling of CTX to HSA.*—Purified CTX (1 μg) was coupled to HSA (1 mg) in 7.5 mL 0.05M phosphate-buffered saline (PBS) by the carbodiimide procedure (1). After exhaustive dialysis against saline at 4°C, the protein conjugate was precipitated with cold acetone at an acetone/conjugate ratio of 4/1. The precipitate was centrifuged, rinsed twice with cold acetone, suspended in saline, and then used to immunize the BALB/C mice. After pervaporation, the residue was analyzed for free CTX. These steps are critical in the immunization process (21).

(**c**) *Immunization of BALB/C mice.*—The method of Hokama et al. (9) was followed. Three 10-week-old BALB/C mice were injected intraperitoneally once a week for 3 consecutive weeks with 0.1 mL CTX/HSA conjugate. A booster was given in the sixth week, 3 days before the animals were sacrificed. Each mouse received a total of 80–100 ng CTX/80–

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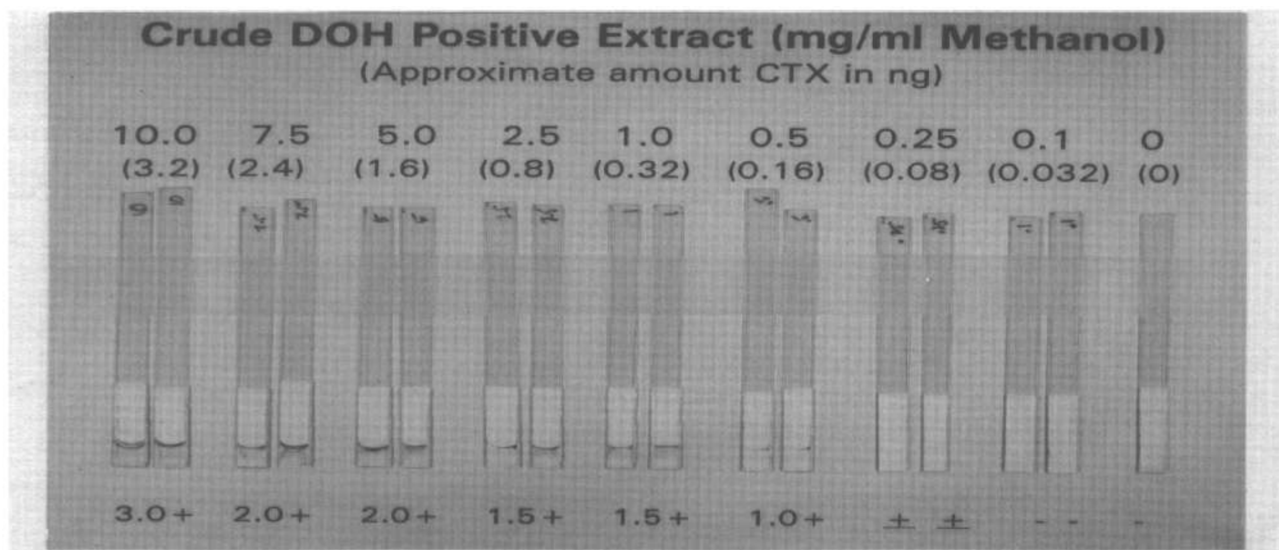

Figure 3. DOH mixed ciguateric fish extracts (0.1–10.0 mg/mL methanol) tested in duplicate by the MIA. Numbers in parentheses are calculated concentrations of CTX in the crude extracts. The symbols –, ±, and + represent relative degrees of activity.

100 μg HSA and 0.2 mL Freund's complete adjuvant (FCA) subcutaneously for each injection of 0.1 mL conjugate.

(**d**) *Fusion step*.—The non-immunoglobulin-synthesizing mouse myeloma cells selected for fusion were those previously reported by Kearney et al. (22), designated PBX63-Ag8.656B. These cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS). Myeloma cells in the logarithmic growth phase were used for fusion.

Sensitized cells from BALB/C mice were fused with myeloma cells by the method of Kennet et al. (23). Essentially, the original method of Kohler and Milstein (24) as described in detail by Schreier et al. (25), was followed. These procedures included hybridoma selection, media preparation, and limiting dilution methods. The original active hybridoma containing reactive anti-CTX was designated 5C8. Analysis of 5C8 with commercial immunofluorescence-labeled goat anti-mouse immunoglobulin (Ig) isotypes (Sigma Chemical Co., St. Louis, MO) demonstrated the following hybrid cells: IgM, 85%; IgG_1, 55%; IgG_{2a}, 14%; IgG_{2b}, 3%; and possibly IgG_3. This result suggested a mixture of clones because the myeloma produced no Igs. Nevertheless, 5C8 supernatants and ammonium sulfate fractions (50% precipitates) were used successfully from 1987 through 1994 in numerous studies using a variety of test procedures (5, 8–10, 12–14, 26).

From 1987 to 1996, subculturing of 5C8 caused gradually diminishing activity of the MAb-CTX. Subsequent isotyping by enzyme-linked immunosorbent assay (ELISA) of different lots of 5C8 (50% ammonium sulfate fractions) showed loss of IgM, IgG_{2a}, and IgG_{2b} isotypes and appearance of IgG_3. The

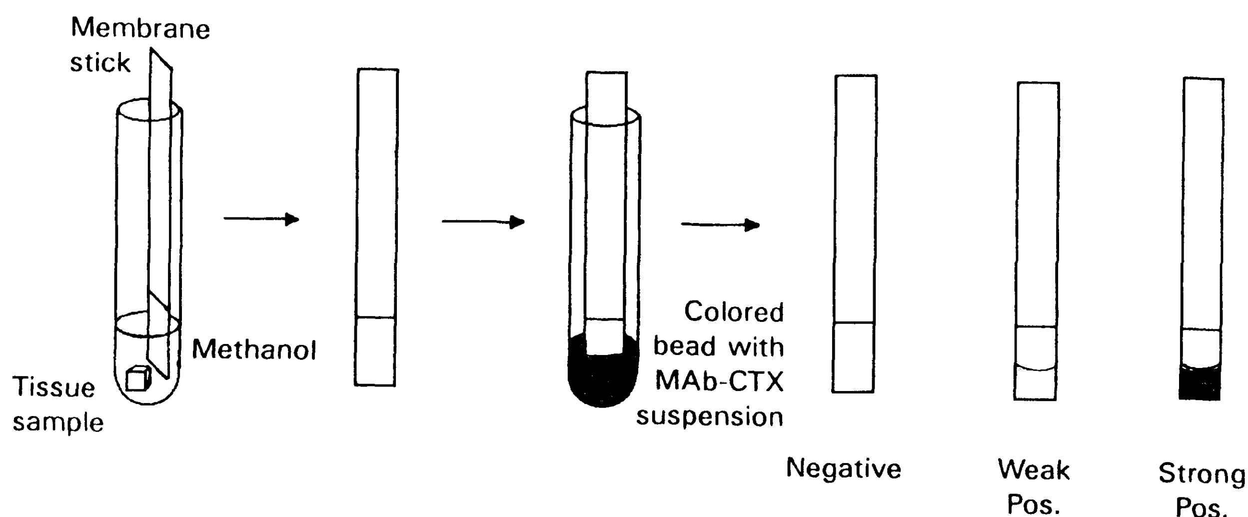

Figure 4. Sequence of steps in the MIA.

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Table 1. Analysis of toxic fish implicated in ciguatera poisoning and samples of reef fish by methods prior to the MIA

Procedure	Antibody	Type and No. of fish analyzed	No. of responses (%)		Reference
			Borderline or positive	Negative	
RIA	Sheep anti-CTX	DOH, 46	44 (95.6%)	2 (4.4%)	1, 2, 19
		Routine samples (*S. dumerili*), 5596	824 (14.7%)	4772 (85.3%)	
		Other species, 766	17 (2.2%)	749 (97.8%)	
S-EIA	Sheep anti-CTX	DOH, 16	16 (100%)	0	3
		Routine samples (various species), 574	212 (36.9%)	362 (63.1%)	
	MAb-CTX	DOH, 83	81 (96.8%)	2 (2.4%)	4–11
		Routine samples (*C. strigosus*), 712	392 (55%)	320 (45%)	
		Routine samples (*S. dumerili*), 168	82 (49%)	86 (51%)	
		Other species, 3539	1720 (48.6%)	1819 (51.4%)	
SPIA	MAb-CTX	DOH, 31	30 (96.8%)	1 (3.2%)	12–14
		Routine samples (various species), 482	261 (54.1%)	221 (45.9%)	

first recloning of 5C8, accomplished despite a 24 h electrical blackout, resulted in 6 viable, moderately active clones out of 100 plated. Clone 2E6 was further cloned by limiting dilutions, resulting in 11 (6%) clones with good activity, 11 (6%) with moderate activity, and 168 (88%) with fair or questionable activity. All good and moderately active clones were frozen and stored at –80°C. The 168 poor clones were discarded. One of the highly active clones, designated 1C6, has been cultured further and was used in this present study. Purification using a gel affinity protein G column and isotyping by the ELISA procedure demonstrated equal amounts of IgG_1 and IgG_3 in clone 1C6 (data not shown).

Analyses for sensitivity and specificity were taken from Morgan et al. (27):

$$\text{Sensitivity} = \frac{\text{Pos. (DOH) fish implicated in ciguatera det. by MIA}}{\text{Total no. of positive fish implicated in ciguatera}} \times 100$$

$$\text{Specificity} = \frac{\text{Fish presumably w/o ciguatera toxins, negative by MIA}}{\text{Total no. fish tested, presumed negative for ciguatera}} \times 100$$

(e) *Immunobead preparation.*—Clone 1C6 was selected, and its cultured supernatants were collected and diluted 1:20 with saline in a final suspension of 0.1% colored polystyrene beads in saline. The 1:20 dilution of antibody was selected as the optimal concentration after titration of each batch of mono-

Table 2. MIA of fish samples implicated in ciguatera poisoning, State of Hawaii Department of Health

Species (common name)	Source	MIA result[a]	Remarks
Bodianus bilunulatus (a'awa)	Oahu	+	Cooked sample
Cephalopholis argus (roi)	Oahu	+	Cooked sample
Cephalopholis argus (roi)	Kauai	+	Cooked sample
Sphyraena barracuda (barracuda)	Big Island	–[b]	Cooked sample
Mulloidichthys auriflamma (weke)	Oahu	+	Tissue
Unidentified	Kauai	+	Tissue
Unidentified	Kauai	+	Tissue
Kyphosus cinerascens (nenue)	Oahu	+	Cooked sample
Acanthurus dussumieri (palani)	Maui	+	Cooked sample
Snapper (species unidentified)	Barbados (via New Jersey)	±	Cooked sample
Red Fish (species unidentified)	Texas DOH	+	Tissue
Barracuda (species unidentified)	Texas DOH	+	Tissue
Cephalopholis argus (roi)	Big Island	±[c]	Cooked sample

[a] +, positive; ±, borderline; –, negative.

[b] When tested with the MIA, sample reacted weakly at 7.0 mg/mL in methanol and gave a negative response at 3.5 mg/mL. Results of mouse toxicity assay (dead in 7 min after intraperitoneal injection) suggest presence of palytoxin- or maitotoxin-like toxins. The guinea pig atrial assay suggested the presence of low levels of CTX-like compounds in sample (*see* Figure 3).

[c] One patient ate the same species for 3 consecutive meals, although it is uncertain if the same or 3 different fish were consumed. Three other people ate only one meal of roi with no toxicity reported. A roi from the same catch was tested and found to be negative.

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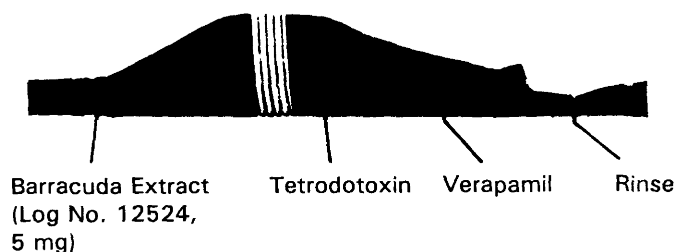
a. *S. barracuda* extract



b. DOH-positive (containing CTX) extract

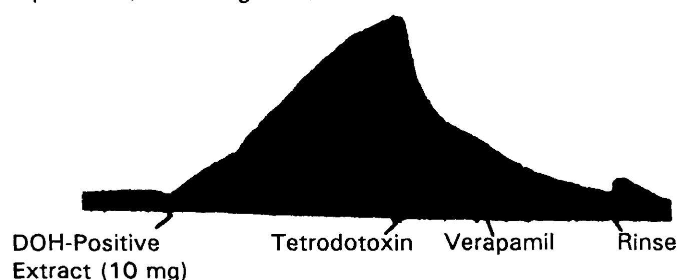


Figure 5. Inotropic patterns from guinea pig atrial assays for (a) *S. barracuda* and (b) DOH positive (containing CTX) extracts with addition of TTX, 10^{-3}M (12.5 µL); verapamil, 10^{-3}M (12.5 µL); and rinse. The number 12524 indicates the identification log book number of the *S. barracuda* extract. The difference in the TTX block is clearly demonstrated in the CTX-containing extract (b).

clone 1C6 cultured supernatants, with the endpoint determined as approximately 1:100 dilution (data not shown). The 1:20 dilution circumvents the "Hook effect" (28) and appears to be able to detect CTX at levels as low as 0.16 ng (*see* Figure 3). Sensitivity can be enhanced by increasing MAb-CTX concentration (1:5 or 1:10 dilutions). Specificity can be enhanced by decreasing MAb-CTX concentration (1:40 or 1:50 dilutions; 29).

Blue (0.314 µm diameter) and red (0.124 µm diameter) polystyrene beads were used in a ratio of 3:1. These bead sizes were selected because larger beads, >0.8 µm diameter, tended to settle readily to the bottom of the vial. Although use of a single color was satisfactory, the combination of 2 colors gave a deeper hue.

Membrane Immunobead Assay

The principle of MIA is similar to that of SPIA: Colored immunobeads will adhere to CTX (antigen), if any, on the membrane portion of the plastic stick previously exposed to the antigen. Positive samples will show visible color changes; negative samples will not.

A fish tissue sample (ca 5 ± 3 mg) cut with a punch biopsy tool or razor blade was placed into a test tube, along with 0.5 mL methanol and a membrane stick. After a 20 min soak, the stick was removed from the test tube and thoroughly air dried for at least 20 min. The completely dried membrane stick was immersed into 0.5 mL latex immunobead suspension, removed after 10 min, rinsed in saline or water, shaken to remove excess liquid, and air dried. Then the membrane was observed for color. Results were scored as follows: negative, no distinct color on the membrane; weakly positive, light blue-purple color on the membrane, with or without a darker band at the meniscus level; or strongly positive, membrane was colored and had more than one dark band at the meniscus level. Fish with weakly positive scores and higher should not be eaten.

Positive control consisted of pooled extracts of fish implicated in ciguatera poisonings, as determined by DOH. Samples contained either high (7.0 mg/mL) or low (3.0 mg/mL) concentrations of DOH fish extract in methanol, equivalent to 2.24 or 0.96 ng CTX/mL methanol, respectively. The relative concentration of CTX in the crude standard extract was previously assessed by immunological inhibition assay using various concentrations of pure CTX added to MAb-CTX conjugated to horseradish peroxidase (MAb-CTX-HRP) and tested against 12.5 mg DOH crude CTX extract. This assay demonstrated that 4 ng pure moray eel CTX added to MAb-CTX-HRP conjugate completely inhibited its interaction with DOH crude pooled extract in the S-EIA (15). The DOH crude extract was calculated to contain 0.32 ng CTX/mg (16). Negative controls were blank membranes used directly or soaked in methanol and dried thoroughly before being immersed into the immunobead solution. No difference was found between these 2 preparations for negative controls. The sequence of steps in the MIA is presented in Figure 4.

Mouse Toxicity Bioassay (MT)

This assay was performed with a standard dose of 100 mg crude extract (80% methanol fraction) suspended in 1 mL 1% Tween-60 in 0.9% saline. Each mouse was injected intraperi-

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toneally, and results were scored as described by Kimura et al. (18). One milliliter 1% Tween-60–saline with no extract was used as a negative control. Values of toxicity indices were as follows: 0–3, animals survived, with clinical symptoms for up to 4 h; 4, animals dead within 24 h after injection; and 5, all animals dead in less than 6 h after injection after experiencing severe clinical symptoms (rapid or slow breathing, paralysis of hind legs, inactivity, slow respiration; 18).

Guinea Pig Atrial Assay

The in vitro guinea pig atrial assay used in this study is that of Miyahara et al. (19). A guinea pig weighing 350–400 g was anesthetized with isofluorane (Anaquest, Inc., Liberty Corner, NJ). The whole heart was removed quickly and placed in Krebs–bicarbonate buffer aerated with O_2. The right and left atria were separated and placed into an electrophysiograph apparatus. The physiobath final volume was 25 mL. Fish extracts at concentrations of 5–10 mg/100 μL methanol were added to the physiobath in which the atrial tissues were attached to electrodes suspended in the bath. After atrial beats were stabilized to obtain a baseline, the effect of the added extracts on the beating atria were recorded by a polygraph recorder. The inotropic responses induced by the fish extracts were examined for inhibition by 12.5 μL 10^{-3}M tetrodotoxin (TTX), a sodium channel blocker, and 12.5 μL 10^{-3}M verapamil, a calcium channel blocker.

Results

Data for fish implicated in ciguatera poisonings obtained by RIA, S-EIA, and SPIA are shown in Table 1. The data include both fish implicated in ciguatera poisoning as compiled by the DOH and routinely tested Hawaiian reef fish generally associated with ciguatera poisoning in Hawaii. The major species tested included *Ctenochaetus strigosus* (kole) and *Seriola dumerili* (kahala). Ciguatera poisoning in patients was clinically documented by DOH physicians or epidemiologists. Of 176 ciguatera-implicated fish tested with these assays, 171 (97.2%) gave borderline or positive responses and 5 (2.8%) gave negative (false-negative) responses. The procedures demonstrated a high degree of sensitivity with both sheep anti-CTX and MAb-CTX. On the other hand, examination of Hawaiian reef fish of unknown toxicity by S-EIA and SPIA yielded only fair or moderate specificity with the MAb-CTX (45.0–51.4%), due in part to nonspecific binding to the coated bamboo stick. This fair specificity has limited the commercial use of these tests. The sheep anti-CTX gave the best specificity (85.3–97.8%) and sensitivity (95.6%) in the RIA assessment of *S. dumerili* and other reef fish. The need for higher specificity led to a search for a better solid-phase medium for binding CTX and related polyethers. During this search, several synthetic membranes were examined. A hydrophobic membrane proved to be the best.

Data in Table 2 represent an MIA assessment of fish recently (1996–1997) involved in clinically documented ciguatera poisoning cases. Of 13 samples examined, 10 were positive and 2 were borderline, containing approximately 60–160 pg CTX or related toxin/g extract. This result represents a sensitivity of 92.3%, with a false-negative response of 7.7%. Extract from a *Sphyraena barracuda* sample from the island of Hawaii gave a negative response in the MIA. However, further examination of the extract by mouse toxicity and guinea pig atrial assays demonstrated it to contain a potent toxin unlike CTX. A classical CTX pattern is strong inhibited by TTX, but the *S. barracuda* pattern showed only a slight TTX effect (Figure 5). Nevertheless, the extract at a concentration of 7.0 mg/mL methanol showed a weak response in the MIA and a 100 mg dose killed a mouse in 7 min. The patient who consumed part of this fish demonstrated classical ciguatera symptoms (26). Taken to-

Table 3. MIA of routine catches of fish from the State of Hawaii and other Pacific Islands

Species	Source	Total No. of fish	No. of responses (%)		
			Negative	Borderline	Positive
Caranx sp. (papio/ulua)	Hawaii	26	20 (77.0)	2 (7.6)	4 (15.4)
Caranx sp. (papio)	Kwajalein	82	78 (95.0)	4 (5.0)	0 (0.0)
Caranx sp. (papio)	Kosrae	20	18 (90.0)	0 (0.0)	2 (2.0)
Ctenochaetus trigosus (kole)	Midway	3	3	0	0
Mugil cephalus (mullet)	Hawaii	3	3	0	0
Mulloidichthys auriflamma (weke)	Hawaii	5	1	4	0
Seriola dumerili (kahala)	Hawaii	2	1	0	1
Aphareus furca (wahanui)	Hawaii	3	2	1	0
Mulloidichthys pflugeri (weke ula)	Hawaii	2	0	2	0
Bodianus bilinulatus (a'awa)	Hawaii	2	2	0	0
Aprion virescens (uku)	Maui	2	2	0	0
Cephalopholis argus (roi)	Oahu	1	0	0	1
Sphyraena helleri (barracuda)	Oahu	1	0	1	0
Priacanthus meeki (aweoweo)	Oahu	1	1	0	0
Acanthurus triostegus (manini)	Oahu	1	1	0	0
Total		154	132 (86.0)	14 (9.0)	8 (5.0)

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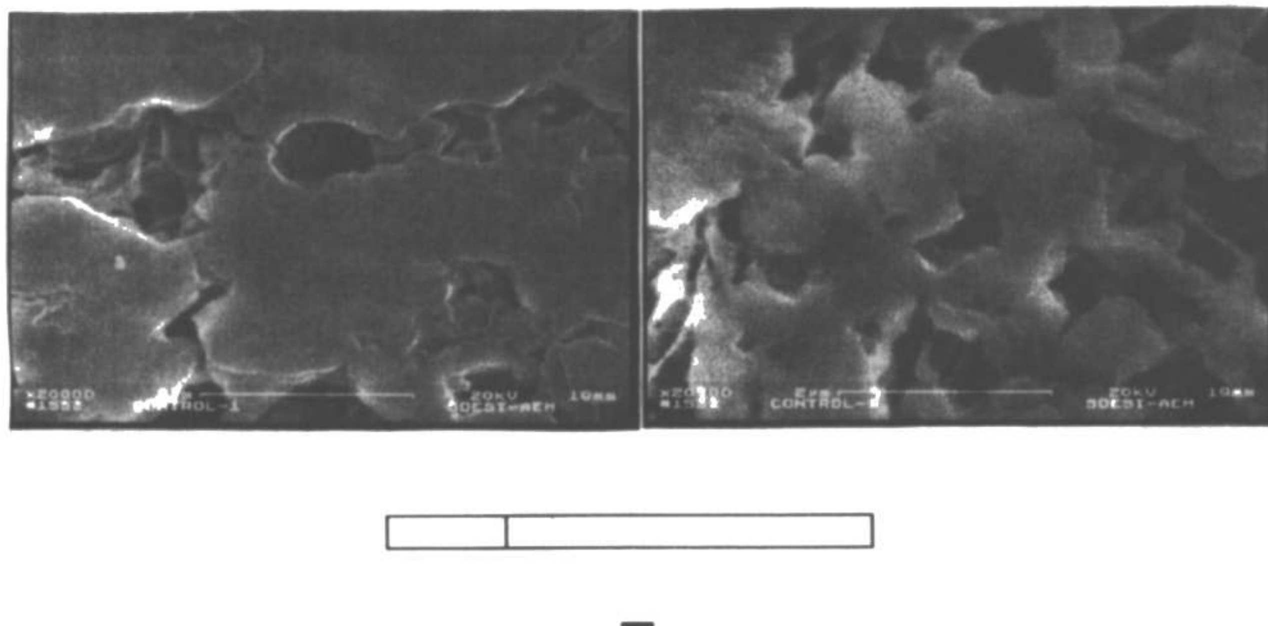

Figure 6. SEM photographs of a negative (blank) pattern (20 000×) showing no beads and porosity of the membrane.

gether, these data suggest the presence of palytoxin, maitotoxin, or as yet undetermined toxins in this barracuda, along with low levels of CTX-like compounds.

Results of MIA analyses of various fish species are presented in Table 3. Samples of the *Caranx* sp. (jack or papio) from Hawaii show a higher percentage of borderline and positive responses (23%) than those from Kwajalein and Kosrae (0 and 10%, respectively). In Hawaii, *Caranx* sp. is one of the major targets of recreational fishermen and is the leading cause of ciguatera poisoning in nearly all of the islands in the State of Hawaii. Of 154 samples tested with MIA, 132 (85.7%) were negative, 14 (9.1%) were borderline, and 8 (5.2%) were positive. The specificity (85.7%) is within acceptable levels for biological assays of this nature. All fish giving negative responses were consumed safely, without any reports of ciguatera poisoning; in other words no false-negative responses were recorded.

Figures 3, 6, and 7 present actual MIA results. Figure 3 represents titration of the standard pooled DOH positive extract. The data show MIA results for extracts tested at different concentrations and the approximate calculated CTX concentrations. The minimal detection point was 0.08 ng or 80 pg CTX in the crude DOH mixture. Figures 6 and 7 show scanning electron microscope (SEM) analysis (20 000×) of a negative and a positive membrane stick used to obtain the MIA data shown in Figure 3. The SEM photos are compatible with the concept illustrated in Figure 1. Note the 2 different sizes of the beads (0.314 and 0.124 μm diameter) visible in Figure 7 and the porosity of the membrane. The negative membrane shows no binding of beads of either size in the absence of CTX. Of 179 blank membrane controls analyzed, 97.2% produced no color and 2.8% produced a slight color (borderline).

Discussion

The new MIA was compared with previously developed immunological test procedures using bamboo sticks coated with correction fluid (30). The S-EIA used bamboo skewers with a correction fluid coat and MAb-CTX conjugated to HRP with appropriate substrate as the detection system. The SPIA used a correction fluid-coated bamboo paddle and MAb-CTX adhering to colored polystyrene beads to detect CTXs. The new MIA system consists of a solid-phase hydrophobic synthetic membrane laminated onto a solid plastic support. The sensitized membrane is dipped into an aqueous suspension of mixed polystyrene beads of 2 different colors and diameters bound to MAb-CTX, and the intensity of the resulting color on the membrane is assessed.

This new procedure was used to assess clinically implicated DOH ciguatera fish samples, a variety of routinely caught fish samples from endemic reef areas of Hawaii, and a number of *Caranx* sp. from Kwajalein and Kosrae. The results show good detection of clinically implicated DOH ciguatera fish with one exception: a *S. barracuda* tissue showed no activity in the MIA, but the extract was highly toxic in the mouse toxicity assay and showed a response in the MIA at a higher dose (7 mg). Analysis of the extract in the guinea pig atrial assay suggested its major toxic component to be other than CTX.

Comparison of the *Caranx* sp. from Hawaii, Kwajalein, and Kosrae showed those from Hawaii to have a higher percentage of toxicity (23%) than the samples from Kwajalein (5%) and Kosrae (10%). In part, this may be attributed to the fact that the *Caranx* sp. from Hawaii were obtained from several areas on different islands, whereas the Kwajalein and Kosrae samples were from one area on a single island. Other groups of fish samples from Hawaii were too small to evaluate properly. But overall, the MIA shows reasonable CTX detection levels, with

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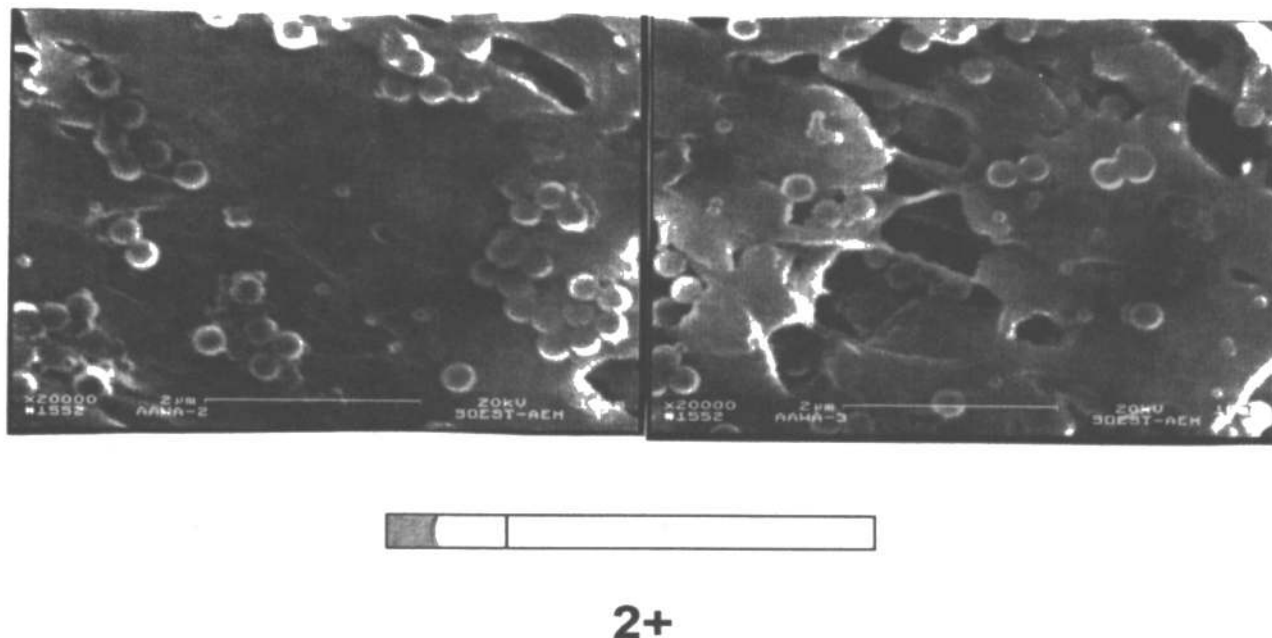

Figure 7. SEM photographs of a 2+ positive pattern (20 000×) showing membrane porosity and binding of both large (0.314 μm diameter) and small (0.124 μm diameter) beads scattered throughout the porous membrane.

86% of samples giving a negative response, 9% giving a borderline response, and 5% giving a positive response. Variability due to nonspecific binding of immunobeads to the membrane is minimized by use of the hydrophobic membrane. Only 2.8% of 179 blank membranes tested showed a borderline reaction. The concept of the MIA as presented is corroborated by SEM data showing both large blue and small red beads on the porous membrane. No beads were found on the membrane when no color was observed, supporting the hypothesis that in an aqueous suspension the immunobeads do not bind to the hydrophobic membrane. The sensitivity achieved with the small number of toxic samples submitted to DOH was 92.3%, and the specificity for unknown reef fish was 85.7%. These values are within acceptable ranges for a biological test system.

The sensitivities of older procedures (RIA, S-EIA, and SPIA) ranged from 95.6 to 97.2%, with specificities close to 50%. This low specificity suggests nonspecific binding of the antibody conjugate and the antibody bead. The antibody bead tended to bind to the bamboo paddles nonspecifically in 22 (10.6%) of 207 blank sticks examined. On the other hand, MIA blanks showed only 2.8% nonspecific binding. The low specificity with correction fluid-coated bamboo paddles needs further study if these procedures are to be used for commercial ventures. However, the SPIA has been useful for recreational fishermen in Hawaii.

With the exception of okadaic acid, cross-reaction studies of the MAb-CTX with other polyethers are limited (4, 15). In accordance with the homology principle of Landsteiner (29), MAb-CTX reacted best with CTX, its homologous antigen (epitope), at the 1 ng level. By comparison, 5 ng okadaic acid was necessary for similar activity. Other related polyethers, brevetoxin, palytoxin, maitotoxin, and congeners of okadaic acid require levels of 50 ng or more to react similarly with the MAb-CTX.

During development of the MIA, several factors critical to obtaining accurate and repeatable results were noted. First, the membrane portion of the membrane stick must not be touched, because touching it may cause false-positive reactions. Second, the membrane stick must be soaked in the methanol/fish sample suspension for at least 20 min for optimal results. Third, both the stick and the test tube must be completely dry before the latex immunobead suspension is added to the test tube. Failure to do so could also cause false-positive reactions. Finally, to prevent false-positive results, the membrane stick should not be soaked in the latex immunobead suspension for more than 10 min. If these points are followed, the MIA is a simple and reliable method for detecting CTX and related polyethers in fish.

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