MEMOIRS

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OF THE

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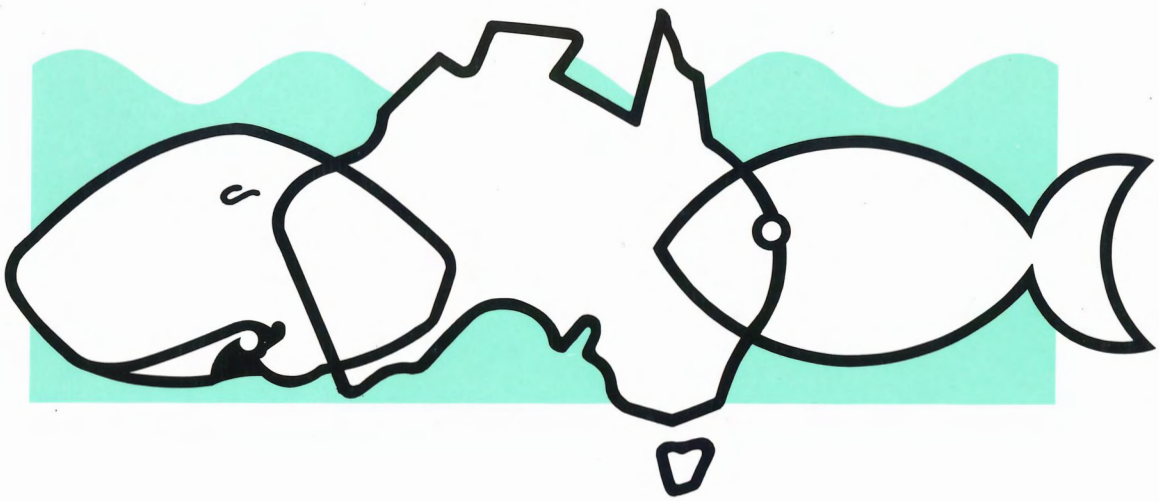

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QUEENSLAND MUSEUM

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Noel in search of the origin of Ciguatera, Flinders Reef, c. 1986.

In Memorian

NOEL CHARLES GILLESPIE

14.12.48 — 29.06.94

Noel died in Brisbane after a 2 year battle with cancer. Noel meant a lot of things to a lot of people. His warmth, generosity and humour set him apart. Noel was a scientist par excellence, a larrikan, a loyal friend, a gifted leader, a loving family man and a real human being, warts and all. He was someone very special. His drive and enthusiasm for life were unique. These qualities never left him. Noel was a pioneer in ciguatera and aquaculture in Australia. Without his clear vision – 'his dream' – for ciguatera research in Australia, this workshop would not have happened. You were our hero and we treasure your memory in our hearts.

Clive, John, Mike and Richard

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Preface

This issue of the *Memoirs of the Queensland Museum* is devoted to the International Workshop on Ciguatera Management that was held on Bribie Island near Brisbane on 12–16 April, 1993. The Workshop was sponsored by the Australian Fisheries Research and Development Corporation and the Queensland Department of Primary Industries (QDPI). Scientists, medical practitioners and fisheries managers with an interest in ciguatera attended the Workshop which focussed on current research having implications for the management of ciguatera.

Fifty six registrants from Japan, USA, France, French Polynesia, New Caledonia, Germany and each of the eastern sea-board states of Australia attended. The Workshop comprised talks, posters and two discussion sessions which specifically addressed (i) the detection of ciguateric fishes and (ii) the management of ciguatera cases. P. Scheuer opened the scientific program with an historical perspective of modern ciguatera research initiated by the late A.H. (Hank) Banner and outlined some of the challenges for the future.

Major themes of the Workshop were: 1,Chemical and immunological aspects of the detection of toxins involved in ciguatera. 2, Pharmacology and treatment of ciguatera. 3, Origin of the toxins involved in ciguatera. 4, Clinical aspects and epidemiology of ciguatera.

Detection of ciguateric fish

A cost-effective screen for ciguateric fish was recognised as an important management tool able to reduce the adverse effects of ciguatera on public health, fisheries, trade and tourism (R. Lewis, D. Park). CTX-1 was widely considered the major target for screens for ciguateric fish (R. Lewis). Several different approaches to the detection of ciguateric fish were presented. Two approaches measured the interaction between ciguatoxin and the voltage-dependent sodium channel through either (i) the inhibition of brevetoxin binding to sodium channels in a rat brain synaptosome preparation (A.-M. Legrand) or (ii) the cytotoxic effects of ciguatoxin on sodium channel-containing cells pre-exposed to ouabain and veratridine (R. Manger). Both assays were more sensitive than the mouse bioassay and may replace *in vivo* assays in laboratories possessing the specialised equipment required. However, it is unlikely that these approaches, as they stand, could be used for the routine screening of suspect fish prior to consumption.

Antibody-based screens still appear to hold most promise for the cost-effective detection of ciguateric fish (R. Lewis). This approach is the basis of a potential commercial test to detect ciguateric fish being developed by HawaiiChemtect. D. Park presented a summary of the performance of this solid-phase immunobead assay (Ciguatect™) which was claimed to be able to detect most, if not all ciguateric fish. However, the test was reported to be unsuitable for detecting toxins involved in ciguatera if the fish flesh being screened was slightly acidic (pH = ~6.5), a factor that may considerably limit the usefulness of the test. Y. Hokama commented that the test may not work satisfactorily because the solid-phase used in the Ciguatect™ test may not be as efficient at extracting ciguatoxins from fish as the correction fluid used for the solid-phase in the original stick test he developed (apparently the same antibody was used for both tests). When compared with the results of a well conducted mouse bioassay, predictive indices from 5% to 75% were obtained with the Ciguatect™ test in an independent study of ciguateric fish from the Caribbean (R. Dickey). This result suggests that the test may not be responding to the major toxins (as yet unidentified) present in these Caribbean fish. Lack of ready access to samples of pure ciguatoxin or its analogues and an inability to independently validate the levels of ciguatoxins present in the fish samples being screened hinder attempts to validate (or otherwise) the Ciguatect™ test.

Pharmacology and treatment of ciguatera

Major advances are being made on how ciguatoxins cause human poisoning (J. Molgo, E. McLachlan, J. Brock, P. Hamblin, E. Benoit, K. Terao, C. Purcell, M. Capra). This research highlighted the usefullness of ciguatoxin as a tool enabling physiologists to understand physiological processes. The precise mechanism by which mannitol, the treatment of choice for acute ciguatera, acts to relieve the symptoms of ciguatera remains unclear. A double-blind clinical study of the mannitol treatment is being conducted (N. Palafox) but results were not available at the meeting. Clinical experiences with the mannitol therapy (D. Blythe, N. Palafox) continue to be positive and mannitol should remain the treatment of choice for acute ciguatera. Confirmation of the clinical findings would be assisted by the development of an animal model for ciguatera that responds to mannitol.

Clinical aspects and epidemiology of ciguatera

While most of the clinical features of ciguatera are well documented, the long-term effects of ciguatera

and how frequently these occur are poorly understood (T. Ruff). Follow-up research on victims is required to establish the true extent of long-term effects, especially the allergy-like reactions that can last after a single exposure to toxic fish. J. Pearn reported that ciguatera remains typically a poorly recognised and managed disease, despite the introduction of the mannitol therapy. QDPI maintains a database that covers 27 years of ciguatera cases reported in Queensland. An analysis of this database using recently developed statistical modelling approaches revealed major shifts in the species and the nature of the poisoning in Queensland (M. Chaloupka). P. Dalzell provided important insights into how ciguatera could be managed in the Pacific where it can be highly prevalent.
The legal situation with regard to ciguatera was also discussed at the Workshop by J. Payne. Duty of care issues and the Queensland Workplace Health and Safety Act could be pursued for a possible successful court action against suppliers of toxic fish. Legal opinion was that duty of care issues would not be adequately addressed if the problem of ciguatera was not continually monitored and if upto date management options were not being implemented.

Origin and identification of toxins involved in ciguatera

Gambierdiscus toxicus is now widely accepted as the organism that produces the gambiertoxins which are the precursors to toxins involved in ciguatera. Indeed this organism may be the only source of toxins involved in ciguatera. T. Yasumoto reported the structure for the gambiertoxin GTX-4A (52 epi-GTX-4B), the major gambiertoxin produced by a Rangiroa Atoll strain of *G. toxicus* grown in culture. This study confirms that *G. toxicus* is indeed the origin of the ciguatoxins isolated from fish. Long and short range inverse detected NMR of CTX-1 confirmed the structure originally proposed for ciguatoxin (R. Lewis). The structure of a maitotoxin produced by cultured *G. toxicus* was also presented by T. Yasumoto. Maitotoxin is the largest non-repeating unit compound for which a structure is known. Elucidation of its structure represents a significant milestone in natural product chemistry. Maitotoxin's structure is only distantly related to the ciguatoxins, thereby ending speculation that maitotoxin may be precursor of the ciguatoxins.
The environmental factors that cause the upsurges of ciguatera remain poorly understood (M. Holmes, R. Lewis, Y. Hokama, D. Ichinotsubo, R. Bagnis, U. Kaly). The origin of ciguatera was question by J-P. Vernoux based on studies conducted in French Polynesia. A rapid extraction method for isolating ciguatoxins from *G. toxicus* may assist studies on toxin production in culture (J. Babinchak). Studies on the vectors transferring the gambiertoxins to carnivorous fishes revealed that invertebrates (shrimps) may be involved in some community structures, whereas in other communities herbivorous fish, such as *Ctenochaetus striatus,* may be the key vector involved (R. Lewis).
As well as ciguatera, a range of other toxic algae which produce biotoxins that cause diarrhetic, paralytic, neurotoxic and amnesic shellfish poisoning could become a problem in tropical and sub-tropical waters through ballast water introductions and/or environmental degradation (G. Hallegraeff). These biotoxins have the potential to severely damage fisheries such as the shellfish fisheries in Queensland which are presently unaffected by such toxins.

Future directions for ciguatera research

The Workshop identified at least four areas for further Australian research that could result in improved management of ciguatera and related seafood poisonings. These were in order of priority:
(i) *development of a cost-effective screen for ciguateric fish*: an uncoordinated international effort has to-date failed to develop a useful screening test for ciguateric fish. Results presented at the Workshop indicate that a biosensor could be developed based on ciguatoxins exceptionally high specificity and affinity for the sodium channels found in nerve and muscle tissues.
(ii) *determination of the environmental factors responsible for flare-ups of ciguatera*: little is presently known of the precise environmental factors that result in the proliferation of the toxins that cause ciguatera but human activities including "pollution" have been implicated. The ciguatera 'hot-spot' in Hervey Bay represents an ideal location to pursue studies on the contributing factors.
(iii) *assessment of the potential for other toxic dinoflagellates to enter tropical and sub-tropical waters through ballast-water introductions*: studies are required to determine if toxic dinoflagellate species have already been introduced into tropical waters and to assess the potential for these to bloom and for new ballast water introductions of toxic dinoflagellate cysts.
(iv) *assess the extent and nature of adverse reactions to seafood consumption*: a survey is required to determine the extent and nature of the allergy-like reactions to seafood, including such reactions that follow ciguatera. Such reactions have not been well quantified in Australia but may have a considerable impact on the marketability of seafood.

***Richard J. Lewis**, Chairman of the Workshop, Scientific and Organizing Committees*
Deception Bay; June, 1994.

PRODUCTION OF CIGUATOXINS IN CULTURED *GAMBIERDISCUS TOXICUS*

JOHN A. BABINCHAK, PETER D.R. MOELLER, FRANCES M. VAN DOLAH, PAMELA B. EYO AND JOHN S. RAMSDELL

Babinchak, J.A., Moeller, P.D.R., Van Dolah, F.M., Eyo, P.B. & Ramsdell, J.S. 1994 08 01: Production of ciguatoxins in cultured *Gambierdiscus toxicus*. *Memoirs of the Queensland Museum* **34**(3): 447–453. Brisbane. ISSN 0079-8835.

Production of ciguatoxin congeners (CTX) from mass cultured dinoflagellates appears to be the only source of CTX that has the potential of providing sufficient quantities of purified toxins for studies on biosynthesis, structural analysis, pharmacology, biotransformation and detection. Established *Gambierdiscus toxicus* clones and recent isolates from Tahiti, Guam and Grand Cayman Island were mass cultured and toxins separated by step-wise elution on a silica gel column. CTX was identified by its binding competition with [^{3}H]-brevetoxin for sodium channel receptor sites in rat synaptosomes. MTX was identified by its ability to induce calcium flux activity in rat pituitary cells. Although the silica gel column separated CTX from MTX, general toxicity of the CTX congeners decreased after separation. A sample of partially purified CTX from *G. toxicus* clone MQ2 was used as a standard for evaluating the assays.

John A. Babinchak, Peter D.R. Moeller, Frances M. Van Dolah, Pamela B. Eyo and John S. Ramsdell. National Marine Fisheries Service, P.O. Box 12607, Charleston, SC 29412 USA; 28 March, 1994.

Two classes of cyclic polyethers produced by the epiphytic dinoflagellate, *Gambierdiscus toxicus,* are non-polar ciguatoxins (CTX), the principal toxins causing ciguatera, and polar maitotoxins (MTX). CTX was first isolated from the Pacific red snapper (Scheuer et al.,1967) and later purified from moray eel liver and viscera. Multiple congeners of CTX (CTX-1, CTX-2 and CTX-3) have been identified in finfish (Lewis et al.,1991), CTX-1 being identical to CTX from moray eel liver. Several CTX congeners have been identified in wild and cultured *G. toxicus* (Legrand et al.,1992; Holmes et al.,1991; Satake et al.,1993) although none identical to that found in the moray eel. Based on their structural relationships, Lewis et al. (1991) proposed that CTX-1 and CTX-2 in finfish represent oxidation products of two different toxins in *G. toxicus*.

Even in the moray eel, which is the most toxic species, CTX content is extremely low, usually only several ppb in whole bodies (Murata et al.,1990). Thus purification of large quantities of CTX from finfish has not been successful. However, obtaining large quantities of the dinoflagellate CTX congeners is limited only by the ability to identify and mass culture a *G. toxicus* clone capable of producing high levels of these toxins. While MTX levels greatly exceed those of CTX, strain-dependent differences in the composition of toxins produced by cultured *G. toxicus* have been described (Holmes et al.,1991). Toxin profiles appeared to be stable, suggesting a genetic basis for the production of different toxin profiles. However, no isolate has yet produced substantial quantities of CTX in culture, with reported yields less than one mouse unit per million cells (Holmes et al.,1991; Satake et al.,1993).

We proposed to screen a collection of 44 *G. toxicus* clones from 12 geographically distinct locations worldwide for their production of CTX in mass culture. The screening of clones has traditionally been a laborious task, requiring the use of mouse bioassay to identify toxins. Since CTX and MTX elicit similar symptoms in the mouse bioassay, the identification of CTX versus MTX in dinoflagellate extracts has required extensive purification. Our laboratory is capable of distinguishing and quantifying CTX and MTX in crude extracts of *G. toxicus* using a battery of established bioassays. These include a rapid, high-throughput *in vitro* cytotoxicity assay for total toxicity, and a Ca^{2+} flux assay as well as receptor binding competition between CTX and [^{3}H]-brevetoxin to distinguish MTX from CTX (Van Dolah et al., in press). Maitotoxin causes an increase in Ca^{2+} permeability, possibly through voltage dependent Ca^{2+} channels (Takahashi et al.,1982; Gusovsky & Daly,1990). Ciguatoxin promotes Na^+ channel opening by binding to site 5 on the voltage dependent sodium channel (Lewis & Endean,1984; Bidard et al.,1984). This site is also recognized by the brevetoxins, a related class of dinoflagellate polyether toxins that can be displaced by CTX (Lombet et al.,1987;

Baden, 1989). The objective of this study was to develop a rapid, single column technique to separate CTX from MTX in crude dinoflagellate extracts. This would expedite identifying *G. toxicus* clones which produce high levels of CTX congeners in mass culture.

MATERIALS AND METHODS

STOCK CULTURES

Isolation procedure for clonal cultures followed Babinchak et al. (1986). Stock cultures of clonal strains of *Gambierdiscus toxicus* (Table 1) were maintained at 27°C under an illumination of 30–40$\mu EM^{-2}S^{-1}$ and a 16:8 hour light:dark cycle without aeration. Illumination was provided from above by a 50:50 mixture of Cool White (North American Phillips Lighting Corp.) and Vita-Lite (Duro-Test Corp.) fluorescent bulbs. K medium (Keller et al., 1987), an enriched seawater medium used in all culturing, was modified by eliminating $CuSO_4$, Tris buffer, silica and using ES vitamin concentrations (Guillard & Keller, 1984). Seawater was collected from a saltwater well in Vero Beach, FL, (Florida Institute of Technology field station). The seawater (35–36 ‰) was filtered through 0.45μm cartridge filters into sanitized polycarbonate carboys and refrigerated in the dark. The seawater was autoclaved in 10 litre borosilicate glass bottles. The vitamin mixtures and enrichments for K medium were prepared in concentrated stocks, filter-sterilized and autoclaved respectively, and stored frozen. The enrichment and vitamins were added aseptically to autoclaved seawater which was then used immediately for culturing. *G. toxicus* clones were harvested by filtration through 12μm polycarbonate membranes, washed 3 times with sterile seawater and inoculated at 200–300 cells/ml into 2.8 litre Fernbach flasks containing 1 litre of medium. Stock cultures of *G. toxicus* were harvested for transfers and mass culture inoculum at 10–14 days.

MASS CULTURE

Twenty-nine clones (Table 1) were selected for mass culture. Micro-carrier spinner flasks (Bellco Glass, Inc.), designed for suspension cell culture systems, were selected as mass culture vessels. The 8 litre model was of a design, wide mouth with two access ports, and weight that could be easily handled and cleaned. The maximal working volume of these flasks for dinoflagellate culture was increased to 12 litre. Magnetic stirring units (Bellco Glass, Inc.), designed for gentle agitation, maintained a stirring speed of 20 RPM. Shelving units accommodated 6 culture vessels and stirrers and provided illumination from above with 4ft Vita-Lite fluorescent bulbs, and from behind with 2ft Cool White fluorescent bulbs at 40$\mu EM^{-2}S^{-1}$ using a 16:8 hour light:dark cycle. Two shelving units were installed in each of two walk-in environmental rooms (1800 cu. ft. and 600 cu. ft.). These rooms were maintained at 27°C and provided space for 24 culture vessels. Vessels were inoculated at a concentration of 500-1000 washed cells/ml. Air was supplied at 1 litre min^{-1} by Whisper 800 aquarium air pumps (Willinger Bros., Inc.) and filtered through AQ microfiber disposable filter tubes, (Balston Filter Products). Aeration was initiated after 10–14 days incubation and bubbled into the culture vessels through sterile plastic air diffusers, (Lee's Aquarium Products).

HARVESTING PROCEDURE

Harvesting the micro-carrier spinner flasks required minimal handling of the vessels. After 21–28 days of incubation, the stirring and aeration apparatus were removed, the flask swirled and a 10ml sample taken for determining cell counts. The cells were allowed to settle and the supernatant removed with a peristaltic pump and filtered through a 142mm stainless filter holder (43μm polyester membrane, Spectrum Medical Industries, Inc.). The settled cells from all the vessels were combined and collected on a 43μm polyester membrane in a 90mm glass filter holder which produced a cake of wet cells. The cells were stored frozen at -20 or -90°C until extracted for toxin content.

CELL COUNTS

Cell counts on individual micro-carrier flasks were determined at harvest using natural chlorophyll autofluorescence and direct epifluorescence microscopy. Duplicate 0.5ml volumes of each micro-carrier sample were collected on 5μm black polycarbonate membranes and observed at 320x on a video display monitor. A Leitz Dialux 20 microscope was used, equipped with a 150W xenon lamp, fluorescence vertical illuminator, KG1 heat filter, BG23 blue filter and a Leitz I2 filter block. Twelve fields or a minimum of 400 cells per sample were counted.

PREPARATION OF CTX STANDARD

A 150g (wet weight) sample of clone MQ2 was disrupted in 100% methanol at 0°C with a Tekmar 500 watt sonic disrupter and filtered through a

Table 1. *Gambierdiscus toxicus* Culture Collection†, National Marine Fisheries Service, Charleston Laboratory

CLONE	ORIGIN	ISOLATOR
*CI03, *CI04, *CI05, CI08, CI09, CI10, CI12, CI13, CI14, CI15, *CI16, *CI18	Grand Cayman Is.	Babinchak
CZ2, CZ3, CZ4	Cozumel, Mexico	Babinchak
MQ1, *MQ2	Martinque	Babinchak
G03, *G05, #G06, *G15, *G16, *G17	Guam	Babinchak
#G02,	Palau	Babinchak
*G01, G20, *G23	Pohnpel, FSM	Babinchak
*MR-1	Moorea	Babinchak
*T01B, *T02B, #T03B, *T04B, *T11B, *T15B	Tahiti	Babinchak
*T01,	Tahiti	Bagnis
*AUS-1	Australia	Bomber
SB01, SB03, SB04	St. Barthelemy	Durand-Clement
*T04	Tahiti	Durand-Clement
*HIT-10, *HIT-25	Tahiti	Legrand
#TP125B	Dry Tortugas	Tomas
*T39	Hawaii	Withers

* Clones successfully mass cultured in micro-carrier system
\# Clones not successful in mass culture
†*G. toxicus* clones isolated by Babinchak are available from NMFS

0.22μm polycarbonate membrane. This crude extract was first fractionated on a G-10 Sephadex size exclusion column (0.5m x 3.8cm i.d.), using gravity flow with a 100% methanol mobile-phase. The Sephadex CTX fraction, as determined by PbTx competition assay, was further fractionated on a C18 (30μm particle size) reverse phase silica column (1.5m x 3.8cm i.d.). The sequence of elution solvents was 100% water, 50%, 25%, 12% and 6% water:methanol using gravity flow.

IATROBEAD FRACTIONATION

A crude extract of 5g (wet weight) from each of 6 (MQ2, T04, G15, Hit25, G17 and T11B) mass cultured *G. toxicus* clones was prepared as for the CTX standard. The methanol extract was evaporated to dryness and taken up to a final volume of 5-10ml chloroform. This solution was introduced to a Michel-Miller column (350mm x 40mm i.d.) equipped with a pre-column (130mm x 22mm i.d.). Both columns were packed with Iatrobeads, (porous, beaded silica; pH 6.8, 60μm particle size, 80Å pore size, Iatron Laboratories, Inc.). The solvent scheme used to fractionate the sample at pump flow rates of 5-8 ml/min was 100% chloroform, 2%, 5%, 10%,15%, 25% and 50% methanol:chloroform, 100% methanol and finally 15% water:methanol.

MOUSE BIOASSAY

Column fractions were evaporated to dryness and brought up in methanol and dilutions made up in 0.9% saline containing 1% Tween 20. Toxicities were determined by injecting 0.25-0.5ml of appropriate toxin concentrations intraperitoneally into female ICR mice weighing c.20g. Three mice were initially injected per dose which was increased to at least 5 mice for doses used to determine the LD_{50} toxicity. Total lethality is expressed in mouse units (MU), defined as the LD_{50} dose for a 20g mouse over 48hr.

CELL CULTURE

Rat pituitary tumor cells (GH_4C_1) were maintained at 37°C and 5% CO_2 in Hams F10 nutrient mixture supplemented with 2.5% fetal bovine serum and 15% horse serum in the absence of antibiotics (Hams F10+). The cells were passed at weekly intervals to maintain their exponential growth, for a maximum of 10 passages. These cells were used in the cytotoxicity assay and calcium uptake experiments.

CYTOTOXICITY ASSAY

Cytotoxicity was determined by a modification of the procedure of Mosmann (1983). GH_4C_1 cells were plated using 0.1ml Hams F10+ in 96-well tissue culture plates at a concentration of 5.0×10^5 cells/ml. Column fractions were evaporated to dryness and serially diluted in methanol. Duplicate wells of fraction-cell mixtures were then incubated for 18hr at 37°C. For determination of viability, 15μl of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/ml in PBS) were added to each well and the cells incubated 4hr at 37°C. Mitochondrial dehydrogenases in live cells convert the MTT to an insoluble formazan crystal. After incubation, the cells were solubilized by addition of 10% SDS

in 0.1N HCl and absorbance at 570nm was recorded using a Titre Tek 96-well plate reader. Non-specific absorbance due to media and non-converted MTT were subtracted to yield a corrected absorbance value. Cytotoxicity was considered positive if the reading was within 10% of the positive control, negative if within 20% of the negative control and partial if in between. In this study, a minimal lethal concentration (MLC) of toxin was defined as the last dilution to give only positive results.

Calcium Uptake Experiments

Calcium uptake experiments were performed by a modification of the method of Enyeart et al. (1986). GH_4C_1 cells were plated in 96-well plates in 0.1ml of Hams F10+ and allowed to attach overnight. For the assay, the medium was replaced with Hams F10+ containing 5μCi/ml $^{45}Ca^{2+}$ and test treatments. Cells were incubated with treatments for 10min at 37°C. To terminate the assay, the extracellular $^{45}Ca^{2+}$ was aspirated off and the cells rinsed 3 times with ice cold Hams F10+. Cells were solubilized by the addition of scintillation cocktail and the plates counted directly in a 96-well format microplate scintillation counter (Wallac). The calcium channel agonist, Bay K 8644, was used as a positive control. Samples were considered positive for values greater than 2 times the negative control.

Brevitoxin (PbTx) Displacement Assay

Assay of binding competition for the PbTx site on sodium channel receptors was carried out in 96-well plates by a modification of the method of Poli et al. (1986). All assays were carried out in the presence of binding buffer [50 mM Hepes, (pH 7.4), 130mM choline chloride, 5.5mM glucose, 0.8mM magnesium sulfate, 5.4mM potassium chloride, 1.0mg/ml BSA, and 0.01% Emulphor-EL 620]. To each sample well, a reaction mixture of 35μl of [^{3}H]PbTx3 (5nM) and 35μl of test treatment were added in binding buffer. To this reaction mixture, 135μl rat brain synaptosomes in binding buffer were added. Plates were incubated at 4°C for 1hr, then receptor bound [^{3}H]PbTx3 was trapped onto a glass fiber filter pad using a 96-well filtration apparatus (Milliblot, Millipore). The filter pad was dried and impregnated with solid scintillant and bound [^{3}H]PbTx3 detected by liquid scintillation spectroscopy in a 96-well format microplate scintillation counter (Wallac). Fractions which caused >50% decrease in [^{3}H]PbTx3 binding were considered positive. PbTx-3(1μM) was used as a positive control and caused complete inhibition of [^{3}H]PbTx3 binding at the receptors.

RESULTS

At an inoculum level of 500 cells/ml, the cell yield of Martinique *G. toxicus* clone, MQ2, in mass culture (4532±526 cells/ml, N=44) was equivalent to the yield obtained in 1l of culture using 2.8l Fernbach flasks (4735±1105 cells/ml, n=26). Before an aeration system was introduced into the mass culturing system, cell yields with MQ2 (3325±502 cells/liter, n=52) were 30% less than produced in Fernbach culture. With aeration, the media pH rose to 8.8 units, but without aeration the rise was to pH 9.9. When the inoculum size for MQ2 was doubled, the final biomass yield increased 60% (0.85–1.3 g/l, wet weight).

Four clones that produce copious amounts of mucoid material in culture caused a problem in mass culture when aeration was introduced (Table 1). Bubbling created by the aeration drove the mucoid secretions and entrapped cells onto the wall of the flasks above the medium, separating the cells from their nutrients. When this occurred, mass culturing was terminated. Final yields in mass culture of the remaining 25 clones ranged from 0.6 to 1.5 g/l (wet weight).

While preparing the CTX standard, 2 suites of maitotoxin eluted within 4hr during fractionation on G-10 Sephadex. The CTX family of toxins eluted from the column between 10-48hrs, but peaked at 15hr. When the CTX fraction was run on the C18 column in the next stage of purification, fractions collected during elution with 100% water and 50%-25% water:methanol were weakly cytotoxic. These represent fractions where any remaining MTX not separated from CTX on G-10 would be expected to elute off the C18 column. No toxicity was observed again until CTX containing fractions were eluted with 12%-6% water:methanol.

The CTX fractions used as the CTX standard, 9.0ml total volume, had a mouse LD_{50} toxicity of 0.007μl/MU for a total of 1.3 million MU produced from the 150g of MQ2 processed. The mice displayed intense lumbar contractions and progressive paralysis from hind to front legs. The toxin output for MQ2 was equivalent to one mouse unit per 2000 cells. The CTX standard had a cytotoxicity of 0.005μl/MLC and was negative or weakly positive in calcium flux and competitively inhibited labeled PbTx binding. The MTX fractions were calcium flux positive and did not inhibit PbTx receptor binding.

The toxin elution profiles for the 6 *G. toxicus* clones processed on Iatrobeads were similar and followed the solvent elution scheme. The first fraction isolated from the Iatrobead column with 100% chloroform was unique in that it displayed calcium flux activity, but was not toxic to mice or GH_4C_1 cells. CTX eluted in the next 1-3 fractions, 2%, 5% and occasionally 10% methanol: chloroform, inhibited PbTx binding, but did not affect mice or display cytotoxicity. After 1 or 2 fractions with no activity, 10% and 15% methanol:chloroform, MTX eluted with 25% and 50% methanol:chloroform and 15% water: methanol. The MTX fractions had high cytotoxicity, calcium flux activity, but did not inhibit PbTx binding on sodium channels receptors.

DISCUSSION

Dinoflagellates are generally considered sensitive to stirring in culture displaying cellular damage and reduced growth rates (White,1976; Galleron,1976; Tuttle & Loeblich,1975). However, the agitation of the micro-carrier flask system maintained *G. toxicus* in suspension without any detrimental effect on its growth rate, and with aeration, the pH of the medium stabilized during the final growth phase and provided final yields equivalent to Fernbach culture. Silicates which dissolve off the walls and crystallize while autoclaving seawater in borosilicate bottles (Brand et al.,1981) provided fine attachment sites for the epiphytic *G.toxicus* in the medium. Aeration supplied the carbon needed for growth and also stabilized the pH in seawater by keeping it from rising too high through the interaction of the carbonate system with pH (Guillard & Keller, 1984). A pH-stat system (Goldman et al.,1982), would provide finer control of the pH as well as inorganic carbon for photosynthetic uptake. This should produce higher biomass yields and the reduction in creation of bubbles would also allow culturing of dense mucoid producing clones.

The initial mass culturing of MQ2 was a balance between using available incubator space for growing inoculum and mass culturing vessels. Because of this, inoculum size was limited to 500 cells/ml. By doubling the inoculum size of MQ2 in later studies, a 60% increase in the final yield was achieved which would be expected to occur with other clones if optimal inoculum were used. Limited culturing (n=3) using 12 litre polycarbonate culture vessels (Nalgene Corp.), with 18 litre working capacity, provided a 50% increase in culturing capacity with the same biomass yield:medium volume ratio. The flasks occupied the same space as the borosilicate micro-carrier flasks, but were lighter and safer to handle.

It was critically important to filter and wash each inoculum and harvest cells in mid-Log growth phase while mass culturing. Neglect of these parameters increased the bacteria load and decreased growth vigor which resulted in reduced biomass harvest. Phenotypic changes that occurred during mass culturing that typified noncompliance to these parameters were increased mucoid production and adhesion of the cells to the vessel walls.

Wet weight yields at harvest were empirically related to the cellular volume of the clone. Generally, the larger the clone, the greater biomass produced per unit volume of medium. Acclimation to *in vitro* culture parameters was another determinate of biomass yield. Sixteen of the mass cultured clones were isolated less than 9 months previous to this study. *G. toxicus* can take up to one year to acclimate to culture conditions after isolation (Bomber et al.,1989). An increase in final yields would be expected for new isolates not yet acclimated to culture conditions.

The amount of CTX (purportedly) isolated from clone MQ2 using size exclusion G-10 Sephadex, followed by C18 reverse phase silica columns was 500-fold more on a per cell basis than previously isolated from this clone. However, previous separation of CTX from MTX was with a silicic acid column and a step-wise elution with chloroform and methanol (Tachibana,1980), a separation technique recommended for use in ciguatera research (Anderson & Lobel,1987). In the present study, the toxicity of CTX appeared to be unstable to contact with unprotected silica or the combination of silica and chloroform. This would explain the loss of toxicity, but retention of PbTx competitive binding activity when fractionated on Iatrobeads. To test this possibility, a sample of the CTX standard was fractionated on Iatrobeads. CTX was collected apart from the lipophilic, active calcium flux material using step-wise 100% ethyl acetate to 5% water/ methanol eluents. The CTX standard lost 50% of its mouse toxicity and greater than 50% of its cytotoxicity. A hypothesis for this observation could be that CTX toxins exist in nature as epoxides. Though possibly still toxic after such an epoxide is opened, much of the toxicity would be lost on silica which may facilitate epoxide ring opening. Though only a hypothesis, the CTX structures (and MTX) that are known, would provide excellent sites for epoxide formation.

Since CTX toxins were not stable on an Iatrobeads packed column, this was not an ideal one-column procedure useful for rapid screening of *G. toxicus* clones for CTX production. However, they have proved to be excellent column packing for MTX purifications providing excellent separation with no loss in toxicity. Unlike other silica media, Iatrobeads allow higher concentrations of water to be used as eluant without destroying or dissolving the silica.

When separating CTX from MTX by normal phase chromatography, a non-polar, non-toxic, calcium flux active fraction, not previously identified, was included with CTX. This led us to believe that MTX was still present in these fractions. If hexane:methanol partitioning was used before column purification, this lipophilic compound was extracted in the hexane phase, unlike CTX or MTX.

Additional CTX extractions from clone MQ2 are in progress to determine if the high yield of CTX in this study was a function of the separation procedure or a serendipitous production of CTX by clone MQ2 in culture. Pacific *G. toxicus* clone, G15, which produces several interesting fractions (isolated from the Iatrobead column) that competitively displaced PbTx binding to sodium channels, is now in mass culture. Although the identity of the CTX standard was confirmed with a negative calcium flux assay and positive competition for [^{3}H]PbTx3 binding, its yield from cultured *G. toxicus* is 500 times that previously reported (Balthrop & Herring,1990), which leaves lingering doubts that the CTX standard contained only CTX congeners. To eliminate this uncertainty, further production and purification of the CTX standard using HPLC and definitive confirmation of the compounds with mass spectroscopy and NMR are in progress.

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EFFECTS OF CIGUATOXIN-1 ON ELECTRICAL ACTIVITY RECORDED INTRACELLULARLY FROM RAT TAIL ARTERY IN VITRO. *Memoirs of the Queensland Museum 34(3): 454. 1994*:– Ciguatoxin-1 (CTX-1) is a lipid soluble toxin from the benthic dinoflagellate, *Gambierdiscus toxicus*; it is responsible for the disease ciguatera which exhibits a range of symptoms involving the peripheral nervous system. CTX-1 has been credited with a selective action on tetrodotoxin (TTX)- sensitive Na^+ channels and induces spontaneous nerve action potentials due to opening of sodium channels at normal resting potential. In this study the effects of CTX-1 on the rat tail artery have been investigated. Intracellular recordings were made from isolated sections of rat tail artery. Application of 0.002–0.2nM CTX-1 increased the occurrence of spontaneous excitatory junction potentials (SEJPs) and duration of the evoked excitatory junction potential (EJP), the decay phase no longer being fitted by a single exponential function. At 0.2nM CTX-1 also produced a large (25-30mV) maintained depolarization. The effects of CTX-1 were abolished by tetrodotoxin (0.3μM) and were calcium dependent. In addition EJPs and SEJPs were blocked by the purinoceptor antagonist suramin (1mM) and the maintained depolarization was blocked by the -adrenoceptor antagonist phentolamine (1μM). These data suggest the actions of CTX-1 are due solely to activation of the sympathetic nerves innervating the rat tail artery.

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RESPONSES OF VERTEBRATE NERVES TO CIGUATOXIN. *Memoirs of the Queensland Museum 34(3): 454. 1994*:– Electrophysiological studies were performed on a variety of nerve preparations from both mammals (rats and humans) and fish. Responses of human peripheral nerves to ingested ciguatoxin were assessed in the Sural nerves of 15 victims of ciguatera. In rats responses of the ventral coccygeal nerve in anaesthetised animals were studied after intoxication was induced by intraperitoneal injection of sub-lethal doses of ciguatoxin. In fish, isolated segments of the spinal nerves and the lateral line branch of the Vagus nerve of both 'carriers' and 'non carriers' of ciguatoxin were exposed to solutions of ciguatoxin in fish Ringer.

In all nerve preparations there were significant changes in a range of nerve conduction parameters including conduction velocity, amplitude, and the duration of refractory periods and the supernormal period. In all preparations there was a significant prolongation of an increase in the magnitude of the supernormal period. These changes conform with studies on isolated cells that suggest a fundamental action of ciguatoxin on Na^+ gating mechanisms.

Both rat and fish preparations have been used to assess the efficacy of a range of potential antagonists of the ciguatoxin response. In rats, ciguatoxin-induced changes in supernormality are unaffected by mannitol but significantly antagonised by lignocaine. In fish, lignocaine and tetrodotoxin antagonise the responses induced by ciguatoxin.

It has also been established that the nerves of fish respond to ciguatoxin in a similar manner to those of mammals and it is suggested that fish may have evolved some degree of protection against ciguatoxin by mechanisms that do not involve the Na^+ channel.

Michael F. Capra, John Cameron, Andrew E. Flowers & Christine E. Purcell, School of Life Science, Queensland University of Technology, Brisbane 4000, Australia; 12 April, 1993.

NATURAL VERSUS ANTHROPOGENIC DISTURBANCES TO CORAL REEFS: COMPARISON IN EPIDEMIOLOGICAL PATTERNS OF CIGUATERA.

RAYMOND BAGNIS

Bagnis, R. 1994 08 01: Natural versus anthropogenic disturbances to coral reefs: comparison in epidemiological patterns of ciguatera. *Memoirs of the Queensland Museum* 34(3): 455–460. Brisbane. ISSN 0079-8835.

Patterns of ciguatera fish poisoning vary from one location to another. In French Polynesia surveys have shown that outbreaks of the disease are associated with disturbances to live coral reefs. Anthropogenic damages such as undersea works, dumping of wastes, wreckage of ships, crashing of ship anchors may result in a flare up of poisonings in areas with no previous history of ciguatera; in this pattern, detritus-feeding herbivorous fish or invertebrates and carnivorous fish may become toxic in a confined area over variable periods of time. Natural catastrophes such as hurricanes, tsunamis, massive coral bleaching may be associated with a pattern of diffuse continuous risk of ciguatera poisoning from large predaceous fish, with periodic outbreaks involving fish from primary and secondary trophic levels. Seasonal disturbances such as storms, heavy swells, high freshwater drainage, red tides, seem to be consistent with a pattern of ciguatera poisoning in which the overall picture is stable, with the same fish species, most of the time large predators, toxic in well defined, extensive areas.

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In French Polynesia most islanders depend greatly on seafood; increases in toxicity of local fish does not go unnoticed. For decades, knowledge of ciguatera fish poisoning in most areas was based on information of variable reliability gathered from a diverse range of persons who had been in contact with the disease. These included medical staff treating it, persons afflicted with it, fishermen and administrators.

Fish poisoning was listed among the notifiable diseases to South Pacific Commission in 1974. Although the reporting is for all types of fish poisoning, it may be assumed that most cases deal with ciguatera (the pathognomonic cold-to-hot sensory reversal dysthesia makes it easier to distinguish from other types of fish poisoning). Reports from French Polynesia are completed by the Public Health Department staff (PHD) in Papeete and mailed, each month, to the South Pacific Epidemiological and Health Information Service (SPEHIS). Reporting is compulsory for each territorial Health Department Unit and voluntary for private medical practitioners. In both cases, it relies on the person in charge to provide accurate and timely monthly reports. Delays in reporting cases are beyond the control of both PHD and SPEHIS. Due to forwarding and transmission of mail, an outbreak occurring today may be notified one or several months later. So seasonal evaluation from rough official data is questionable. The number of cases reported depends on several factors. Severe cases that occur near a health unit are most accurately reported, whereas mild poisoning on the various islands without medical or paramedical staff is probably less accurate. Cases treated according to traditional medicine may also not be notified. Moreover, simplified official data do not provide information on the name or species of the accused fish, nor on the location of its capture.

Cases reported during 1974–1990 give an indication of the magnitude of the ciguatera problem in French Polynesia compared to other Pacific countries reporting in the same way. Data available at the PHD show that no island was completely immune from it, but morbidity is not evenly distributed and the overall data are not always significant for the region as a whole. For instance, data recorded in Tuamotu Islands are less accurate than those from the Gambier Islands. The 12,500 inhabitants of the Tuamotus live on 46 atolls separated by a few or by many miles of ocean and are spread over 400,000 square miles; they are served by two mobile medical practitioners, and have at their disposal one hospital in the main atoll of Rangiroa and five infirmaries located in Anaa, Fakarava, Hao, Makemo, Reao, notwithstanding the military medical staff in Hao and Moruroa. On the other hand, the Gambiers consist of 10 small volcanic

islands in one 600 square mile lagoon; the 600 inhabitants of the archipelago are grouped in one island, Mangareva, with an infirmary in the main village.

To assess accurately the qualitative, quantitative and time related aspects of evolution of ciguatera in the French Polynesian islands, a monitoring programme was developed from 1965 to 1990 by the author and the Malardé Institute staff.

MATERIAL AND METHODS

From 1965, a daily follow-up of cases in the whole territory was set up using the medical and paramedical staff of the Public Health Units, with the filling in of 15,000 standard questionnaires consisting of 26 medical and epidemiological parameters.

From 1967, nearly 20 tons of fish, caught in 30 different French Polynesian Islands exposed to ciguateric risk, and belonging to 30 families from various trophic levels, were bioassayed for ciguatoxicity. From 1976, 10,000 grams of macro-algae, among 40 species, were sampled for research and counting of *Gambierdiscus toxicus* cells.

Follow up of these various analyses, over 15–25 years, resulted in 3 indexes to monitor ciguateric risk (Bagnis et al.,1985a): the number of cases per 1,000 residents (CIR), percentage of toxic individuals in a group of fish from a given species, family or trophic level (PCI), density of the populations of the toxic dinoflagellate *G. toxicus* per gram of algae (GTD).

In addition to these research programs, the author had many discussions on fish poisoning problems with officials from fisheries and health departments, old natives and longtime residents, to assess what events, according to them, could be associated with ciguatera.

RESULTS AND DISCUSSION

Within the context of the monitoring programme, in each inhabited atoll or large valley, a person was officially appointed to fill in the questionnaires and, from 1965 to 1990, the ciguatera reporting system in French Polynesia has gradually become one of the most comprehensive in all of the Pacific area. It has been estimated that 70–80% of cases are reported (instead of 10–20% registered by the PHD in Papeete, from 1960 to 1965). During that time, about 30,000 cases of ciguatera were recorded (CIR: 8 ‰), caused by some 100 fish species from various trophic levels. Gambier Islands (11% of cases; CIR: 60 ‰), Marquesas Islands (19% of cases; CIR: 30 ‰), and Tuamotu Islands (17% of cases; CIR: 25 ‰) were the most involved, compared to Society Islands (52% of cases; CIR: 5 ‰) and Austral Islands(<1% of cases; CIR ‰). As patterns of ciguatoxicity may vary in time and space, from one island to another and from place to place in the same island, only data provided by the questionnaires give an account of the situation in each separate island. Thus, in the Tuamotu Islands, the average overall CIR was influenced by several flare ups that occurred in Hao (primarily) but also in Hikueru, Moruroa, Takaroa, Takapoto, Manihi, Reao, Fakarava, Mataiva, Anaa, Makemo from 1964–1975. In the Gambier Islands, from 1971 to 1980, the CIR never decreased below 300, reaching 560 at its highest point in 1975. In the Marquesas Islands, the CIR grew from about 10 in 1963 to 80 in 1971, and started decreasing gradually in 1976. In the Society Islands an outbreak occurred from 1964–1970 in Bora Bora, while at the same period a significant increase of the ciguatera poisonings by fishes caught in the area of Faaa, was reported in Tahiti, with a high surge in 1966–1967. In the Austral Islands, the least affected of the Polynesian archipelagoes, a few cases are episodically reported from each island but Rapa. In French Polynesia, there are also islands where for decades the ciguateric risk seems to be limited to some species of fish and to some well defined areas, with a level of ciguatoxicity apparently unchanged (Bagnis et al.,1985a).

The most significant data about the PCI concern Tahiti, Hao, Gambier and Marquesas Islands. In Tahiti, the study was carried out from 1967 (at the peak of the surge) to 1984, on the most frequently poisoned fish, the surgeon fish maito, *Ctenochaetus striatus*, and showed that the average PCI was cut by half during that period. In Hao, the PCI increased from c.0 (in July 1966) to c.70%(within 2 years) before decreasing from 1970 to reach 7.5% in 1982. In Gambier Islands, from 1969 to 1971, PCI increased five-fold to a maximum of 67% during 1975–1978, for detritus-feeding herbivorous fish and tenfold to a maximum of 80% during 1978–1979 for carnivorous fish, before decreasing gradually. The data related to Marquesas Islands indicate, from 1968–1984, a regular increase of PCI for herbivorous and carnivorous fish, from respectively 4.5 to 26% and 5.3 to 22% (Bagnis et al.,1985a).

A close relationship between PCI and GTD

could be observed in the Gambier Islands (Bagnis et al.,1985a) and on Hitiaa Reef in Tahiti (Bagnis et al., 1985b). In the Gambiers, where data were the most significant, the yearly average of GTD decreased gradually from 45,000 in 1977 (a year that fits roughly with the highest values of the PCI) to 40,000 in 1978, 10,000 in 1978–1980, 1,500 in 1982, <150 in 1984 to c.100 in 1989–1990. Elsewhere, the follow up was not accurate enough to allow comparative evaluation between the two indexes (PCI and GTD), within the same time span.

Previous data and several events associated with ciguatera, either from the monitoring of some flare-ups, or noted empirically by natives, such as various well identified anthropogenic and natural disturbances in the coral reef ecosystem (Bagnis,1987), support the hypothesis that the appearances of poisonous fish are directly linked to changes in some factors of their environment (Randall,1958). Helfrich & Banner (1968) suggested that the patterns of ciguatera in the Pacific fall into one of three main categories or occasionally a combination of these. These patterns are discussed below with reference to ciguatera in French Polynesia.

Epidemic Pattern

This pattern of poisoning concerns areas with no or a rare history of ciguatera. In such areas, after the initial outbreak, there was a general increase in toxicity reaching a peak of severity (based on the number of species affected and the level of the PCI) within 2–5 years. After some time, the toxicity began to decline. In the islands of French Polynesia where such flare-ups have been observed, this occurred c.5–10 years after the onset of toxicity. The decline may start sooner, but its exact timing is often obscured by i) the fact that the local population is reluctant to begin eating a fish after most of its members were stricken in the period following the initial outbreak (several months or years) and ii) by frequent clinical hypersensitization features which can be mistaken for true poisonings. The rate of decline in ciguatoxicity varies also according to areas. Grazing or browsing herbivores and detritus feeders, such as some acanthurids (*C. striatus* or *C. strigosus*), scarids (*Scarus gibbus* or *Scarops rubroviolaceus*) or mugilids (*Crenimugil crenilabis*), become toxic first, followed within a few months by the carnivores at higher trophic levels (serranids, lethrinids, lutjanids, carangids, labrids, muraenids). Thus the various links of the food web are progressively involved. At the peak of the flare up, an island that had not harboured any toxic fish in man's memory, may have all fish contaminated within a year, according to the natives. In the declining toxicity phase, herbivores become significantly less toxic first, followed by some species at higher trophic levels, until only a few of the large carnivores remain toxic.

Most flare-ups in the Tuamotu Islands, since investigations started, proceed with this pattern (Bagnis,1969,1982; Bagnis et al.,1973,1985a). Similar features have been observed in Society Islands, Austral Islands and Gambier Islands (Bagnis et al.,1988).

All these flare ups followed various anthropogenic damage to coral reefs, linked to public or military works (Bagnis,1987). The main disturbances were: tearing, blasting, disrupting, scouring of pieces of coral reef (to deepen or widen passes in the barrier reef, to open channels in lagoons, to drill shafts in the basaltic layer for nuclear tests), crashing of heavy ship anchors and ploughing caused by dragging anchor cables, dredging and shifting of sand, dumping of wastes or debris (chiefly metallic ones), wreckage of ship, building of piers, wharfs, roads, protective sea-walls on live coral reefs and any other damage less evident and more traditional, like massive diving for pearl-oysters, or usual surf-landing of whaleboats on the same outer reefs of atolls without a pass. Usually, human damages induce geographically limited flare ups (Bagnis, 1969,1981; Bagnis et al.,1973). In the Pacific area, outbreaks of fish poisoning during the Second World War were very likely related to man-made damages on coral reef (Halstead, 1967).

Endemo-Epidemic Pattern

This pattern of poisoning does concern areas with a history of continuous ciguateric risk for the local population, made up of periods of stable, relatively low fish toxicity, referred to as the 'quiescent stages' by Cooper (1964), alternating with periods of major outbreaks, usually only within the memory of the older inhabitants.

In this pattern, toxicity remains confined, during long periods, to large carnivorous fish: some snappers, groupers, jacks, emperor fish and moray eels. Periodically, such areas experience flare ups similar in form and duration to that described for the Epidemic Pattern. Thus, the detritus feeding herbivores (like some surgeonfish, parrotfish, mullets) become toxic, followed by some carnivores at higher trophic levels,

which were previously safe. Finally, all the food chain may be more or less affected by ciguatoxicity during a period of 5–20 years in extensive parts of the coast (Bagnis,1974). This pattern was first observed in Marquesas Islands where data obtained by the author (1965–1973) from inquiry with old natives, pointed out 4 flare ups since the beginning of the century. After multiple cross-checking, their dates and durations could be roughly established: 1905–1915, 1925–1935, 1953–1958, 1965–1985. The first broke out, according to the people interviewed, after the passage of some cyclones. Such an assumption prompted the author to look for a chronological relationship between the alleged flare ups and cataclysmic events, in these islands not protected by a barrier reef and occasionally exposed to tsunamis. Informations collected from the Geophysical Laboratory of Tahiti, the French Meteorological Service and the Hydrographic Department of the French Navy about tsunamis, strong storms, hurricanes which have affected Marquesas Islands since the beginning of the century, pointed out a close relationship between both sets of events (Bagnis,1980). Some data from Majuro in the Southern Marshall Islands (Bartsch & McFarren,1962), in certain Kiribati Islands (Cooper,1964), could also illustrate this pattern.

The episodic resurgence of toxicity involving detritus-feeding herbivorous fish, would indicate that *G. toxicus* (or another ciguatoxic microorganism) was recently (or is still) proliferating again and actively manufacturing the ciguatoxin or a precursor of it, very likely amongst the widespread macro-algal turf covering the many coral colonies damaged by the passage of a tsunami or a cyclone. This new toxin production would increase the overall ciguatoxicity of the food chain for years.

The long quiescent periods, during which only a few large carnivores are toxic, may be explained by the longevity of the fish themselves and their ability to retain toxin. The available data about longevity of tropical reef fish, indicate that some may live more than 30 years (moray eels). If the ciguatoxin were restricted to a 'pool' in the ecosystem bound in the organisms at trophic levels above the secondary one, and the reduction of toxin in the pool would be only by means of natural mortality and break-down by reducer organisms, assimilation by other consumer organisms would merely retain and recirculate it within the pool. As the overall level of the ciguatoxin in the pool slowly declines through loss of toxic individuals by natural mortality, reduction by microorganisms and possibly by slow natural excretion, only those animals with very great longevity would be expected to be toxic, and to contain a large quantity of ciguatoxin (Helfrich & Banner,1968).

Another example of potential recycling of the ciguatoxin in the pool of carnivores in a restricted area is frequently observed in Marquesas Islands (and elsewhere). Many lutjanids, serranids and lethrinids, considered as toxic in some areas, are thrown back into the water when caught. Such a practice would essentially act as a feedback mechanism in the ecosystem. In this condition, one can think that, rather than a gradual accumulation of the toxin at the higher trophic levels by the carnivores with the greatest longevity, as has been noted previously, the activity of the fishermen may continually recycle the toxin among the carnivores, favouring those with non-specialized food habits, such as the scavengers (some lethrinids for instance).

Another explanation of a resurgence in the ciguatoxicity (in both the level and the species involved) of reef fish during the 'quiescent stages' lies also in some anthropogenic disturbances. Thus, extensive blasting and dredging may release some of the ciguatoxin concentrated in the pool of sedentary eels, groupers,wrasses and distribute it to individual usually safe carnivorous fishes at various trophic levels in the community. Such events may result in a series of scattered cases of ciguatera incriminating fish other than detritus-feeding herbivorous fishes.

Endemic Pattern

This pattern occurs in areas in which the overall picture of toxicity seems to be quite stable, with the same species (most of the time predators) exhibiting the same level of toxicity within well-defined geographical areas. The condition is said to be unchanged as far as the local people can remember. Confirmation of the continued toxicity of the fish usually occurs when an outsider, such as a tourist or a member of a ship's crew, is poisoned or when a native who cannot resist the temptation offered by a meal of a fat, succulent grouper, snapper or eel, gamble on it being nontoxic and lose. According to old islanders, approximately the same species (most of the time carnivorous, but also detritus-feeding herbivorous fish) that were toxic in some reefs of Society, Marquesas, Tuamotu, Austral and Gambier Islands 50 years ago, may still be toxic today. The same stability is observed outside of French

Polynesia in some parts of New Caledonia, Vanuatu, Fiji, Samoa,Tonga, Guam, Marshall Islands. (for the Pacific), Reunion, Mauritius (in Indian Ocean) and in most Caribbean Islands.

Seasonal natural disturbances like heavy swells, abundant rains with freshwater drainage and soil runoffs, red tides, slight coral bleaching, crown of thorns proliferation, and any other kind of insidious disturbance, not taken into consideration by man, could be the most frequent causes of this pattern of ciguatera poisoning, with recycling of the toxin by the fishermen. Every disturbance destroying coral colonies and creating consequently new surfaces available for macro-algal colonization results either in a significant increase of genetically toxic strains of *G. toxicus* or in the development of toxicity by previously non toxic strains, because of some changes, very likely due to the microflora associated with *G. toxicus*.

From today's knowledge on the origin of ciguatera, natural disturbances would explain its antiquity and its occurence in many uninhabited islands and shoals distant from land. Through information provided by old islanders, one may safely say that no island of French Polynesia, save perhaps Rapa, is completely immune from it.

CONCLUSIONS

To understand the dynamics of the evolutive patterns of ciguatera, one should remember that the reef community is probably the most complex in the sea. A delicate balance must exist among competing organisms, so much so that a subtle change in environment can result in temporary proliferation of one at the expense of the others. Whatever the disturbances in lagoon, pass, fringing or barrier reef, the result is the same: occurrence of dead coral beds and new surfaces available for opportunist populations. We know that toxic *G. toxicus* is very poor in reef, in normal environmental conditions; but it may increase rapidly and significantly among the filamentous or calcareous algae growing on new or denuded surfaces, in normal ecological succession, fixing itself on the thalli of the algae, primarily the red ones. If we postulate *G. toxicus* as the single or main source of ciguatoxin, with herbivore ingestion as the chief means of transfer of the toxin to higher trophic levels, then the more widespread the macroalgal colonization, the more the toxic dinoflagellate bloom can be important and the higher the amount of toxin introduced and stored in the food-web. The detritus-feeding herbivorous fishes can be expected to remain poisonous, after the ciguatoxin producing dinoflagellates decrease in number, disappear or cease to be toxic, by a period of time equal to the maximum longevity of the fish species involved.

Discussion on patterns of ciguatera development is based on empirical data, from French Polynesia. It is admittedly tenuous and conjectural in some cases. Nevertheless, it supports Randall's (1958) hypothesis, resumed by Helfrich & Banner (1968), on the evolution of ciguatera.

The epidemic pattern seems to be most of the time associated with anthropogenic damage (Bagnis,1969,1982; Bagnis et al.,1973). The endemo-epidemic pattern may be related to natural catastrophes, as in the Marquesas Islands with their fringing patches of coral without the protection of barrier reefs and many shoals away from the shore, especially exposed to variations of oceanic hydrodynamism (Bagnis,1980). The endemic pattern may be associated with natural seasonal disturbances in most of volcanic islands with fringing or barrier reef where the detrimental action of man or cataclysms in the marine environment are negligible (as observed in many parts of French Polynesia). The combination of the three patterns is observed in Gambier, where all the islands are edged with a fringing reef and bounded with the same continuous half emerged 168 miles long barrier reef; the latter is bordering a relatively shallow lagoon, with a bottom rugged with many patch reefs, knoll reefs and coral ridges. In this archipelago, a close relationship between ciguateric fishes and anthropogenic and natural damage to reefs was pointed out (Bagnis et al.,1988).

ACKNOWLEDGEMENT

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GAMBIERTOXIN-INDUCED MODIFICATIONS OF THE MEMBRANE POTENTIAL OF MYELINATED NERVE FIBRES

EVELYNE BENOIT AND ANNE-MARIE LEGRAND

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The effects of external application of 1.2–24nM of gambiertoxin (CTX- 4B), extracted from the dinoflagellate *Gambierdiscus toxicus*, were studied on the membrane potential of myelinated nerve fibres isolated from the frog. At concentrations of 12 and 24nM, CTX-4B induced spontaneous action potential discharge at a frequency of 30–100Hz. In the presence of 24nM of CTX-4B, the amplitude and duration of these spontaneous action potentials were respectively decreased and increased compared to control action potentials. Toxin-induced spontaneous action potentials were suppressed by increasing the external calcium concentration or by lidocaine. It is concluded that the action of CTX-4B on membrane potential, in some respects, resembles that of moray-eel ciguatoxin previously reported (Benoit et al., 1986).

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Ciguatera toxins are responsible for the most common human food poisoning associated with the consumption of various tropical and subtropical fishes. The major source of these toxic compounds is the dinoflagellate, *Gambierdiscus toxicus* (Adachi & Fukuyo,1979).

Studies with ciguatoxin extracted from the moray-eel concluded that the toxin specifically interacts with Na channels of various preparations. In particular, in the frog node of Ranvier, the toxin induces spontaneous action potentials due to specific modifications of Na channels (Benoit et al.,1986).

In this work, we have investigated effects of the main toxic compound extracted from *G. toxicus*, gambiertoxin (CTX-4B), on the membrane potential of the frog myelinated nerve fibres.

MATERIALS AND METHODS

The experiments were carried out on single myelinated nerve fibres isolated from the sciatic nerve of the frog *Rana esculenta*. The membrane potential was recorded under current clamp conditions as previously described (Benoit et al.,1986). The Ringer's solution had the following composition (mM) : NaCl 111.5; KCl 2.5; $CaCl_2$ 1.8, HEPES 10; pH 7.4. The fibre ends were cut in a solution containing 120mM KCl. The temperature was 15–16°C.

Gambiertoxin (CTX-4B) was extracted from *Gambierdiscus toxicus* and purified. Samples were kept at –18°C and diluted immediately preceding experiments in Ringer's solution to give the final toxin concentrations indicated.

RESULTS

Effects of CTX-4B on Membrane Potential

CTX-4B at concentrations 1.2–6nM, applied for 12–22min, had no effect on the membrane potential, even when K currents were suppressed by replacing the solution bathing the cut fibre ends, i.e., the internal solution, with 110mM CsCl +10mM NaCl and by adding tetraethylammonium to make an external solution with a final concentration of 10mM. This was consistently observed on six different fibres, whether they were sensory or motor fibres.

In contrast, 2–8min after the addition of 12 or 24nM of CTX-4B to the Ringer's solution, spontaneous action potentials appeared at a frequency of 30–100Hz (Fig.1A). The toxin-induced spontaneous action potentials were often separated by silent periods and were less regular in amplitude and frequency than the spontaneous action potentials induced by moray-eel ciguatoxin (Benoit et al.,1986). Moreover, the resting membrane potential of fibres was apparently not modified by CTX-4B and maintained depolarizations to near –20mV were never observed, in contrast to the previously reported effects of moray-eel ciguatoxin (Benoit et al.,1986).

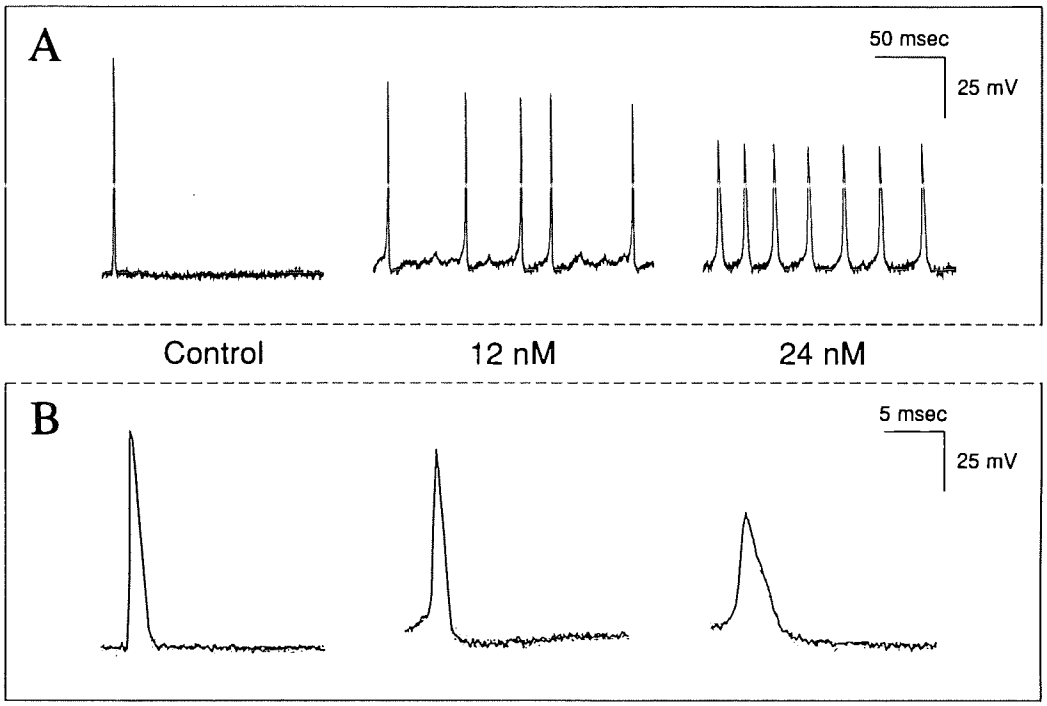

FIG.1. Control action potentials recorded in Ringer's solution evoked by 0.5msec depolarizing stimuli (left traces). Spontaneous action potentials recorded about 10min (middle traces) and about 12min (right traces) after bathing the nerve fibre in Ringer's solutions containing 12nM (middle traces) or 24nM (right traces) of CTX-4B. Note the difference in horizontal scales between (A) and (B).

In the presence of 12nM of CTX-4B, the amplitude of the spontaneous action potentials was only slightly reduced to 86±6% (n=3) of control on average and their duration, measured at the 50% level repolarization, was not significantly modified (mean 1.05±0.08%, n=3) compared to the control action potential elicited by a depolarizing stimulus (Fig.1B). This was not the case in the presence of 24nM of toxin, where the amplitude and duration of spontaneous action potentials were respectively reduced (mean 52±3%, n=4) and increased (mean 1.78±0.06%, n=4) compared to the control action potential (Fig.1B). Finally, it should be noted that the spontaneous action potentials induced by 12 or 24nM of CTX-4B (applied for 8–42min) were not suppressed after 18–36min wash out of toxin with control solution.

Effects of Tetraethylammonium on Spontaneous Action Potentials

Under control conditions, after the addition of tetraethylammonium to make the Ringer's solution with a final concentration of 10mM, the duration of elicited action potentials was 1.83±0.05 fold (n=3) greater than the duration of control action potential. Similar results were obtained when tetraethylammonium was added to a solution containing 12nM of CTX-4B, i.e., spontaneous action potentials were prolonged. In contrast, the subsequent addition of tetraethylammonium to the solution containing 24nM of CTX-4B or the addition of 24nM of toxin to the solution containing tetraethylammonium, did not noticeably further modify the duration of action potentials. As tetraethylammonium is well known to specifically suppress K current, these results strongly suggest that the nodal K current was not significantly affected by 12nM of CTX-4B, whereas it was reduced in the presence of 24nM of toxin.

Effects of Increasing External Calcium Concentration on Spontaneous Action Potentials

The effects of increasing the external con-

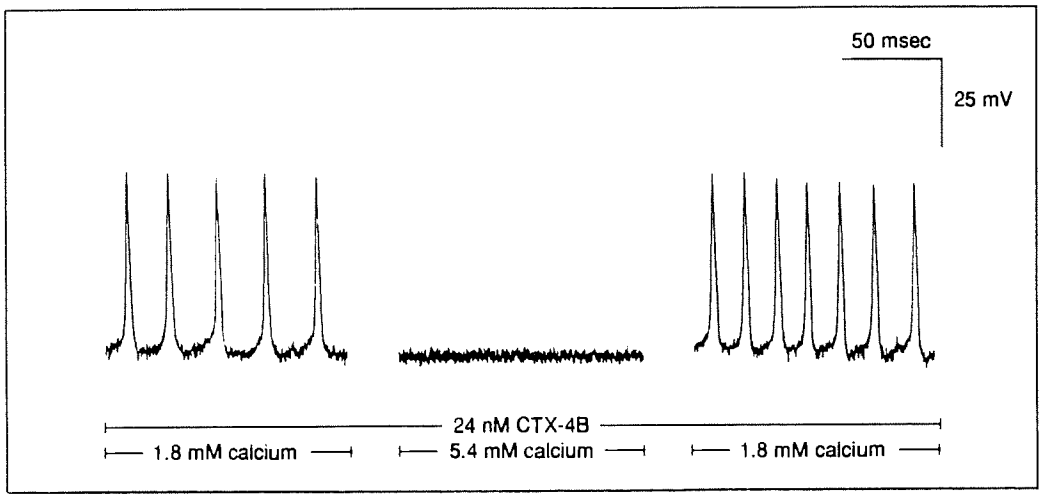

FIG. 2. Spontaneous action potentials recorded in a 24nM solution of CTX-4B made up in Ringer's solution containing successively 1.8mM (left trace), 5.4mM (middle trace) and 1.8mM of calcium (right trace).

centration of calcium were studied on spontaneous action potentials recorded in the presence of 12nM or 24nM of CTX-4B, or during wash out of CTX-4B with a Ringer's solution. Increasing the external calcium concentration from 1.8 to 5.4mM suppressed spontaneous action potentials in <30sec (Fig.2). In the presence of 5.4mM of calcium, an action potential could be elicited by a depolarizing stimulus. The effects of increasing external calcium concentration were reversed within 1–2min by superfusing the fibre with solutions containing 1.8mM of calcium (Fig.2).

Effects of Lidocaine on Spontaneous Action Potentials

Addition of 50μM of lidocaine to the Ringer's solution containing 12nM or 24nM of CTX-4B, inhibited spontaneous action potentials in less than 30sec (Fig.3). However, under such conditions, as previously described in the presence of 5.4mM of calcium, an action potential could be observed when the myelinated nerve fibre was stimulated by a depolarizing current. It should be noted that in the presence of CTX-4B, the frequency of appearance of spontaneous action potentials was 30–100Hz (Fig.1A) whereas the depolarization-induced action potentials were

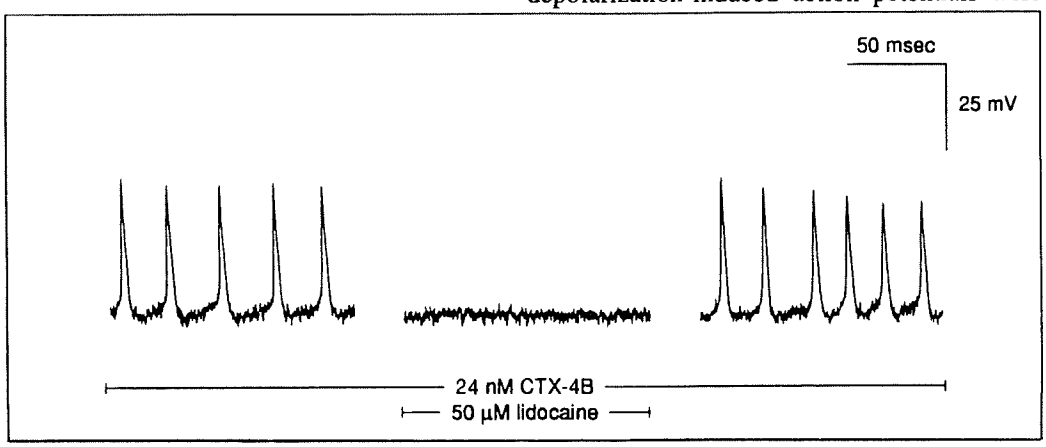

FIG. 3. Spontaneous action potentials recorded in a 24nM solution of CTX-4B made up in a Ringer's solution, in the absence (left trace), presence (middle trace) and after wash out (right trace) of 50μM of lidocaine.

usually elicited at a frequency of 1Hz. The inhibitary action of lidocaine has been shown to be more effective when the frequency of events is increased (Hille,1977). This may explain in part why lidocaine was more effective at blocking spontaneous action potentials than the elicited action potentials. The effects of lidocaine were reversed by a 2–3min wash with a Ringer's solution containing CTX-4B (Fig.3).

DISCUSSION

These results show that CTX-4B induces spontaneous action potentials in frog sciatic nerves which are suppressed by increasing the external concentration of calcium or by lidocaine.

Appearance of spontaneous action potentials was also observed in the presence of moray-eel ciguatoxin (Benoit et al.,1986). However, ciguatoxin was active at concentrations as low as 0.22nM, whereas in the present experiments concentration of at least 12nM of CTX-4B was needed to induce spontaneous action potentials. Thus, CTX-4B appears to be about 50 fold less effective than ciguatoxin on the membrane potential of myelinated nerve fibres. Finally, the effects of ciguatoxin were completely reversed upon washing out of toxin with a Ringer's solution, in contrast to those induced by CTX-4B.

It is concluded that, in some respects, the action of CTX-4B on membrane potential of myelinated nerve fibre resembles that of moray-eel ciguatoxin.

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MANNITOL THERAPY FOR ACUTE AND CHRONIC CIGUATERA FISH POISONING

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Blythe, D.G., Fleming, L.E., Ayyar, D.R., deSylva, D., Baden, D. & Schrank, K. 1994 08 01: Mannitol therapy for acute and chronic ciguatera fish poisoning. *Memoirs of the Queensland Museum* **34**(3): 465–470. Brisbane. ISSN 0079-8835.

An evaluation of Intravenous (IV) mannitol therapy for treatment of the marine toxin disease, Ciguatera Poisoning, in 107 persons from the South Florida/Caribbean area. 70 patients with ciguatera poisoning received IV mannitol treatment (1g/kg) within hours to 1000 days from exposure, and 37 patients with ciguatera poisoning received only supportive therapy, if any. The treated and non-treated groups were comparable, except for prolonged time until presentation of the untreated group. 29 out of 32 (91%) patients treated with mannitol within the first 48 hours from exposure had complete reversal of symptoms. Although not a formal randomized clinical trial, this case series does provide valuable information and support for the use of intravenous mannitol in the treatment of acute and chronic ciguatera poisoning.

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Dinoflagellates in the marine genus *Gambierdiscus* elaborate a number of toxins which are bioconcentrated in the food chain through reef-feeding herbivores to larger predatory fish. When these larger species are eaten by humans, they lead to Ciguatera Poisoning. Ciguatoxin (CTX) is responsible for the majority of human illness associated with ciguatera poisoning (Carmichael et al.,1986; ILO,1984).

Ciguatoxin is a lipid soluble, heat stable and acid resistant neurotoxin (Carmichael et al.,1986, Sakamoto et al.,1987). It causes no adverse effects to the fish, and cannot be detected by differences in smell or taste, nor is it eliminated by cooking, freezing or other preparation procedures (Lewis,1986). The mechanism of action of ciguatoxin is as a sodium channel toxin (Lewis, 1986; Baden et al.,1990).

In the past, a variety of bioassays (including feeding and injections into cats, mongoose and mice) have been used to test for CTX in fish. Intraperitoneal injection in mice has been one of the most widely accepted bioassays, and more recently, rat brain synaptosome (Lange,1987; ILO,1984). In addition, to being impractical for routine use in the fish industry, there has been no test available for the evaluation of human ciguatera in clinical practice. Several new tests have been developed. One of these is a radioimmunoassay for ciguatoxin, a so-called 'stick test', which can be used to test for ciguatoxin in fish and has been widely used in Hawaii (Hokama, 1985). A highly sensitive ELISA test for assay in human biologic fluids is currently being trialled (Trainer & Baden,1990). Until these assays are established in human populations, diagnosis of ciguatera can only be arrived at clinically.

In the United States, nearly half of the reported foodborn disease outbreaks of chemical origin are due to marine toxins, with CTX causing at least one third of these outbreaks (Lange,1987). Ninety percent (90%) of the reported cases of ciguatera poisoning come from Florida and Hawaii (Lange,1987). In Miami, an average annual incidence of at least 5 cases/10,000 persons was estimated by reports to the Public Health Department and based on clinical diagnosis (Lawrence et al.,1980). In certain islands of the South Pacific up to 43% of the population has experienced at least one episode of Ciguatera (Rodgers & Muench,1986) and in Puerto Rico, up to 7% of the residents (Holt et al.,1984).

The human disease entity of 'Ciguatera Poisoning' is a direct result of the stimulation of adrenergic and cholinergic nervous system due to the opening of the sodium-dependent channels by ciguatoxin (ILO,1984; Lange,1987). It presents as an acute syndrome characterized by a variety of gastrointestinal, neurologic and cardiovascular symptoms within a few hours of contaminated fish ingestion. Most commonly, patients experience acute nausea, vomiting, diarrhea, gastrointestinal cramping, paresthesias, and bradycardia. Fatality, usually due to respiratory

TABLE 1. Subject characteristics in mannitol treatment study

Group	Number	Age (mean)	Sex (%Female)	Time Present	Sx	Fish
All	107	39y	48%	46d (0.3-1000)	12%GI 76% neuro	27%grouper 25%king
No Rx	37	41y	43%	111d (1-1000)	5%GI 92% ne uro	40%king 27 %grouper
Rx	70	39y	50%	11.5d (0.3-365)	16%GI 67% neuro	27%grouper 17%king
Rapid Rx	19	36y	56%	9d (0.4-70)	16%GI 42% neuro	63%grouper 11%barr acuda
Slow Rx	51	38y	47%	12.5d (0.3-365)	16%GI 76% neuro	23%king 14 %grouper

failure, cardiac arrhythmias and possibly cerebral edema, is reported to range from 0.1–12% of cases (Lange,1987; Morris et al.,1982; Bagnis et al., 1979). In addition to the acute illness, the chronic symptoms of ciguatera poisoning, especially the paresthesia, can persist in varying severity for months to years after the acute illness, with significant long term disability as a result. Chronic effects of ciguatera poisoning have been largely ignored in the literature, probably due to inaccurate diagnosis by inexperienced healthcare workers and lack of available human diagnostic testing (Lange,1987; Blythe & deSylva,1992).

A variety of treatment modalities have been tried for intervention in ciguatera poisoning. These include antihistamines, corticosteroids, calcium supplements, amitriptyline, fluoxetine, and lidocaine derivatives (Lange,1987; Berlin et al.,1992; Pearn et al.,1989; Gillespie et al.,1986). None of these therapies have withstood the test of time. 23 cases of clinically diagnosed acute ciguatera poisoning in the Marshal Islands were treated with an intravenous infusion of 20% mannitol (1g/kg at a rate of 500cc/hr) over 30 mins 'piggy backed' on an intravenous infusion at 30 cc/hr of either 5% dextrose in Ringer's or saline solution; there was complete resolution of symptoms within 48 hours in 17/23 patients (Palafox et al.1988). Pearn et al. (1989) published a case series of 12 patients treated with IV mannitol (0.5–1g/kg over 30mins); there were dramatic results in the 5 acutely ill patients. They postulated that mannitol might reduce axonal edema and/or act as a scavenger of hydroxyl radicals located on the ciguatoxin molecule.

We present a case series of 107 subjects with clinically diagnosed ciguatera poisoning from south Florida and the Caribbean collected since 1985. Seventy of these were treated with IV mannitol and 37 were not treated because they either presented prior to the Palafox publication, or mannitol was not offered or was declined.

METHODS

Patients of all ages and both sexes were diagnosed clinically with ciguatera poisoning if they gave a history of a) consuming reef fish from South Florida or the Caribbean and b) the onset of gastrointestinal symptoms within 6–24 hours of consumption and c) when relevant, subsequent onset of neurologic symptoms, usually after 24–48 hours from consumption. The gastrointestinal symptoms include nausea, vomiting, abdominal pain and cramps, and diarrhea; these gastrointestinal symptoms rarely last more than 48 hours from exposure with or without treatment. The neurologic symptoms reported include paresthesia (in the extremities and around the mouth) and weakness; these symptoms can persist upto 3 years in the case of one patient without treatment.

The patients were collected by two clinicians (DB, DRA) with known interest in ciguatera poisoning in the Miami (Florida) area from their clinical practice since 1985. A Ciguatera Network with referral telephone number had been set up by the authors to give advice and recommend treatment. Due to an ongoing research study, the following information was collected for each patient at presentation: age, sex, time from exposure until time of presentation, symptoms at presentation, and type of fish implicated.

Patients with clinical features consistent with either acute (ie. within 48 hours of exposure) or chronic ciguatera poisoning seen after the publication by Palafox et al. (1988) were offered mannitol treatment. Both clinicians gave mannitol in a dose of 1g/Kg; one clinician administered it over 3–4 hours (Slow) and the other over 30 minutes (Rapid).

Patients were asked to rate their response im-

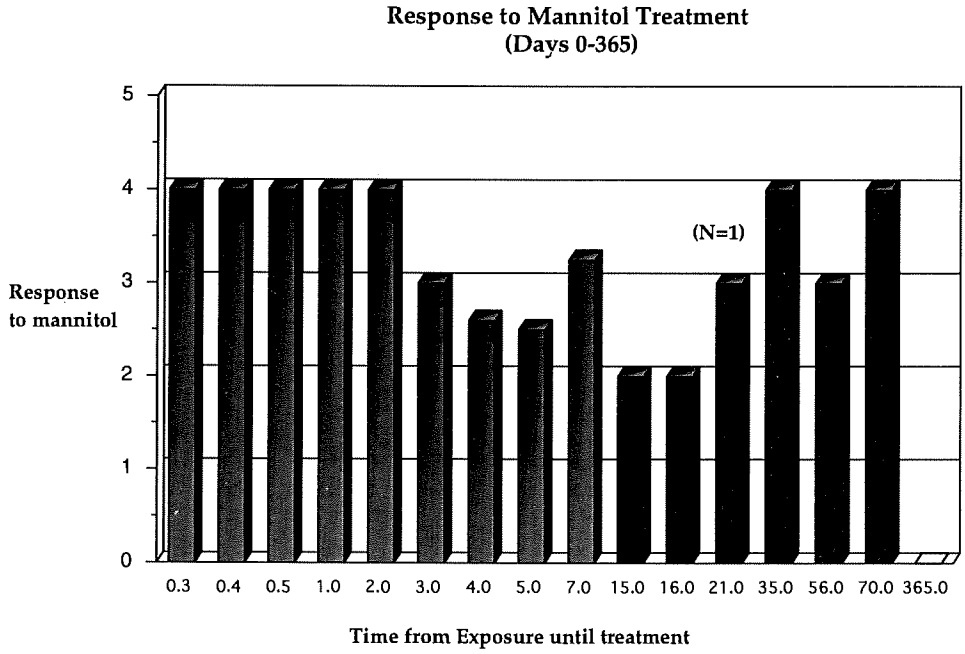

FIG.1. Response to Intravenous Mannitol Treatment (Days 0–356)

mediately after receiving the mannitol intravenous treatment (i.e. after 3–4 hours with the 'Slow' treatment and after 30 minutes with the 'Rapid' treatment). The responses were rated on a scale of 0 to 4+, in which 4+ meant recovery to normal without further treatment needed and 0 meant no change in their symptoms. In any case in which the reportedly positive effects of mannitol treatment did not last and neurologic symptoms returned, repeat treatments were offered under the same protocol until a 3+ to 4+ response was obtained and maintained.

Statistical analysis was performed on SE 30 MacIntosh using STATVIEW (Brainpower) statistical program.

RESULTS

Of 107 subjects, 70 (65%) were treated with mannitol (Table 1). 48% were women and 52% men with a mean age of 39 (± 15.9) and range of 1–79 years. The mean time from exposure to presentation was 46 days (± 149.5) with a range to 0.3–1000 days. Symptoms consisted of purely gastrointestinal in 12%, neurologic in 76% and combination of the two types in 12%. The pathognomonic symptom of cold to hot reversal was reported by the majority, but not all patients. The fish types reported were 27% grouper, 25% kingfish, 9% amberjack, 8% barracuda, 8% snapper, 8% other, and 15% unknown.

The symptoms at presentation had the following relationship to time from exposure: gastrointestinal symptoms alone were reported by those presenting within the first 24 hours, then both neurologic and gastrointestinal symptoms were reported by those presenting 24 hours from exposure until day 22, while neurologic symptoms alone were reported by those presenting after day 1 and up to day 1000 from initial exposure.

Of the 37 patients who did not receive any mannitol, there were 57% men and 43% women with a mean age of 41 (± 15.24), range 1–67 years. Their mean time to presentation from exposure was 111 days (±235.3) with a range of 1–1000 days. The symptoms reported at the time of presentation were 5% gastrointestinal, 92% neurologic and 3% both; mannitol was not offered and no other intervention (ie. supportive) relieved these symptoms. Fish types identified

were 40% kingfish, 27% grouper, 11% snapper, 3% amberjack, 8% other, and 11% unknown.

70 patients were treated with IV mannitol. 50% were males and 50% females with a mean age of 39 years (±16.4), range 2–79 years. The mean time to presentation from exposure was 11.5 days (±44.5), range 3–365 days. The symptoms reported at the time of presentation were 16% gastrointestinal, 67% neurologic, and 17% both. Fish types reported were 27% grouper, 17% kingfish, 13% amberjack, 11% barracuda, 7% snapper, 10% other, and 14% unknown.

Of the 70 treated patients, 51 (73%) were treated with the 'slow' mannitol treatment and 19 (27%) by 'rapid' treatment. The mean overall response to mannitol treatment was a score of 3.3+ (±0.94) with a range of 0–4+. There were no adverse side effects reported to receiving mannitol treatment, either Rapid or Slow treatment.

32 (46%) of the 70 individuals treated within the first 2 days from exposure, 91% had +4 response, and 100% had 3+ or 4+ (Fig.1). There were 31 (44%) of the 70 individuals treated after day 2 through day from exposure; 23% had a 4+ response, 31% had a 3+ response and 35% had a 2+ response to mannitol treatment. Finally, 7 (10%) of the 70 treated individuals were 15 to 365 days from exposure; 33% had a 4+ response, 33% had a 3+ response and 33% had a 2+ response while the individual who was 1 year from exposure had no response to mannitol treatment.

As mentioned by Pearn et al. (1989), multiple treatments (mean 2 treatments, but up to 4 treatments) were required in five cases to maintain the initial positive response to treatment. All the multiple treatment persons presented within two days from exposure. There were no further reports of symptom recurrence or necessity for further medication from successfully treated patients after completing the mannitol treatments.

32 persons were treated within the first two days from exposure; 50% were male and 50% were female with a mean age of 31 years (range 2–60 years). Of these 32 persons, 28% reported purely gastrointestinal symptoms, 38% reported a mixture of gastrointestinal and neurologic symptoms, and 34% reported purely neurologic symptoms at the time of presentation. The majority (89%) of those persons reporting purely gastrointestinal symptoms at the time of presentation were 24 hours or less from exposure, while neurologic symptoms were reported after 24 hours from exposure.

Of the 51 patients treated by 'slow' mannitol treatment, there were 53% men and 47% women, with a mean age of 38 years (±17.7), range 2–79 years. The mean time to presentation (and treatment) was 12.46 days (±50.7), range 0.3–365 days. The presenting symptoms reported were 16% gastrointestinal, 76% neurologic and, 8% both. The fish types identified were 23% kingfish, 14% grouper, 14% amberjack, 12% barracuda, 10% other, and 19% unknown. The mean overall response to "slow" mannitol treatment was rated 3.1+ (±0.995), range 0–4+.

Of the 19 patients treated with 'rapid' mannitol treatment, 42% were men and 56% were women with a mean age of 36 years (±12.1), range 12–68 years. The mean time to presentation (and treatment) was 9.04 days (±19.3), range 0.4–70 days. The symptoms reported were 16% gastrointestinal, 42% neurologic, and 42% both. The fish types identified were 63% grouper, 11% snapper, 11% barracuda, 11% amberjack, and 5% other. The mean overall response to "rapid" mannitol treatment was rated +3.7 (0.56), range 2–4+.

By ANOVA analysis (Table 2), there were no differences between those with and without treatment with respect to sex (F=0.437, p=0.51), age (F=0.37, p=0.54), type of fish (F=1.67, p=0.19), and type of symptom (F=0.167, p=0.68). There was a statistically significant difference between those with (11.5 ±16.4 days) and without treatment (111 ±235.3 days) with regards to time to presentation from exposure (F=11.8, p=0.0008).

There was no difference by ANOVA analysis (Table 2) between the two treatment groups with respect to age (F=0.57, p=0.45), sex (F=0.638, p=0.43), type fish (F=1.37, p=0.246), time to presentation (F=0.081, and p=0.776). There was a statistically significant difference between Slow and Rapid treatment groups with regards to response to treatment (F=6.9, p=0.01) and type of symptoms at presentation (F=5.14, p=0.03), with Rapid treatment resulting in a better response.

By correlation analysis, positive response to mannitol treatment (ie. by the rating scale 0 to 4+) was correlated with the type of treatment (ie. Rapid vs Slow mannitol) (r=0.31) and time until presentation from exposure to contaminated fish (r=-0.442). By regression analysis in a model with the variables of type of treatment and time until treatment, both variables were statistically significant and predictive of successful response to treatment (F=12.6, p=.001).

DISCUSSION

Although not a random controlled clinical trial, treated and untreated patients with ciguatera

TABLE 2: Results of ANOVA Analyses of Treatment Group Outcomes

Group Treatment	Response	Age (mean)	Sex (%Female)	Time Present	Sx	Fish
Rx vs No Rx	N/A	F=0.37 p =0.54	F=0.44 p=0.51	F=11.8 p=0 .008*	F=0.17 p=0 .68	F=1.67 p=0 .19
Rapid Rx vs Slow Rx	F=6.9 p= .01*	F=0.57 p =0.45	F=0.64 p =0.43	F=0.08 p=0 .77	F=5.14 p=0 .03*	F=1.37 p=0 .25

poisoning from south Florida were identified which are comparable with respect to age, sex, type of symptoms, and type of fish consumed.

The only significant difference between the treated and untreated groups was that the treated group were more likely to present to the authors earlier in the course of their disease than untreated patients, even though the symptoms and disease entity were the same. We believe that the treated patients as a group presented earlier in the course of their illness due to new expectations for successful treatment and improved early diagnosis thanks to the community work in south Florida on ciguatera by these investigators. The majority of the persons in the untreated group had presented early in the course of their illness to other healthcare facilities and were treated unsuccessfully with a variety of treatments prior to being seen by the authors.

With regards to IV mannitol treatment, it appears to be effective in all ages and both sexes. There were no reported side effects to mannitol treatment. It is most effective if given within the first 48 hours from exposure. Mannitol treatment was moderately effective if given from 3–14 days from exposure (responses 2+ to 4+). In addition, based on data derived only from single individuals, moderate success was seen with treatment of individuals upto 70 days from exposure; the one individual treated 1 year from exposure had no response to mannitol treatment.

Multiple treatments (upto 4 additional treatments) were necessary in 5 individuals; there was complete resolution of symptoms with repeat treatments. The absence of interviewer blinding and of an objective measurement of response are weaknesses in this study. However, persons who reported a maintained successful response to mannitol treatment did not return for further treatment of any kind, while those who were not treated by mannitol continued to report symptoms even 3 years after exposure.

The prolonged symptoms with accompanying significantly increased time from exposure to presentation (mean 111 $\pm$235.3 days) among the untreated control group support the need to consider mannitol treatment for ciguatera poisoning, especially acutely. Although only a few individuals were treated after 14 days from exposure, in our experience it is worthwhile attempting mannitol treatment because it may relieve or even eliminate the debilitating neurologic symptoms of chronic ciguatera.

It appears that the more rapid administration (30 minutes) may be slightly more effective, although there were only 19 patients who received this treatment and as a group, they presented earlier in the course of the illness (9.04 vs 12.46 days) which correlates with a better response to treatment. However, the slow intravenous administration (over 3–4 hours) may be more appropriate with small children and others who cannot tolerate a heavy fluid load.

As opposed to the symptom course described in Pacific ciguatera poisoning (Bagnis et al., 1979), in our Atlantic experience the gastrointestinal symptoms universally preceded the neurologic symptoms. As such, it would be important to consider treatment with IV mannitol of any person presenting with the acute onset of gastrointestinal symptoms within 6–24 hours of consuming a large reef fish from a tropical area, even though the more classic neurologic symptoms have not yet presented.

Multiple fish types were reported in these ciguatera cases, although all were large reef fish species from tropical areas. Ciguatera poisoning has been associated with over 400 species worldwide. Also of interest to clinicians and epidemiologists, the ciguatera cases often presented in clusters due to sharing of fish among family and friends.

The social and economic impact of ciguatera poisoning, due both to the threat and the actual disease, is enormous. For example, in several highly endemic areas, local fish are avoided as a food source, as in south Florida where the sale of barracuda (a major source of ciguatera poisoning in the past) has been banned (Lawrence et al., 1980). Fear of ciguatera poisoning has lead to depression of local and exporting fishing industries and of tourism, and indirectly on human health due to avoidance of fresh fish consumption (despite its nutritional value) (Lewis,1986). The

impact of ciguatera and other marine toxins on the fishing industry is evidenced by the FDA Recommendations before Congress to require mandatory testing for marine toxins in all marine fish imported and sold in the United States.

Further work is needed on diagnosis, treatment and epidemiology of ciguatera poisoning. First, biomarkers in human fluids, as well as fish, are needed for the diagnosis and management of acute and chronic ciguatera poisoning. Animal studies of IV mannitol treatment are necessary to understand the mechanism of action. Random controlled double blind trials of IV mannitol in humans with biomarker-diagnosed ciguatera poisoning are needed. Biomarkers would be useful in determining the extent of acute and chronic ciguatera poisoning in humans worldwide. Finally, education of healthcare workers in endemic areas is crucial for the correct recognition and early intervention in ciguatera poisoning.

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MANAGEMENT OF CIGUATERA FISH POISONING IN THE SOUTH PACIFIC

PAUL DALZELL

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Catches of near-shore or coastal fish are a major source of animal protein in the South Pacific region. Nominal landings amount to about 90,000 tonnes/yr, about half of which is reef fish. Ciguatoxic fishes are ubiquitous but in some areas, such as Niutao in Tuvalu, there is a higher risk of intoxication associated with eating reef fish. Commonly ciguateric fishes are normally avoided, but where alterative food sources are not available, they are readily consumed. The risk of poisoning is considered acceptable by the community and ciguatera is not regarded as a significant health problem in most island countries. This risk of ciguatera poisoning is reflected in the attitude of the medical community which is reluctant to see ciguatera given priority over other public health problems. Although ciguatera is of relative low priority as a medical problem in the region, fisheries development initiatives in high risk areas should aim to reduce the incidence of ciguatera by improving supplies of non-toxic deep reef slope and pelagic species. Elsewhere, ciguatera management is mainly required to limit the impact of fish poisonings on tourism and reef fish exports. Initiatives to improve the management of ciguatera such as the South Pacific Commission's seafood poisoning database are discussed.

Paul Dalzell, Inshore Fisheries Research Project, South Pacific Commission, BP D5 Noumea Cedex, New Caledonia; 2 February, 1994.

Dalzell (1992) presented an overview of ciguatera in the South Pacific from the perspective of fisheries development. In common with statistics on other illnesses, ciguatera cases are incompletely documented in the region. The South Pacific Epidemiological and Health Information Service (SPEHIS) records numbers of cases of fish poisoning reported by national health departments, which it is assumed represent mostly ciguatera intoxications. The annual number of reported cases fluctuates (Dalzell,1992, fig.1). Lewis (1986) suggested that reported cases account for c.20% of incidence of ciguatera in the region. The increased incidence of fish poisoning from 1985 on (Dalzell,1992, fig.1) may be due to improvements in diagnosing ciguatera and in data collection rather than a rise in the occurrence of ciguatera.

Landings of coastal fishes in most of the islands of the South Pacific come from coral reefs and lagoons (Dalzell,1992, table 1). In many locations fish is still a principle source of animal protein and forms a major component of subsistence diets. In the smaller countries of the region, fish stocks represent one of the few viable economic resources that have potential for development. Outbreaks of ciguatera, besides being a health hazard, may have detrimental effects on fish production and marketing through adverse publicity and litigation.

Key issues that face health and fisheries workers in the South Pacific are:1, How big a problem is ciguatera to community health and the economy? 2, If ciguatera is a serious problem, how and where should resources be focused to manage it?

Dalzell (1992) summarized Pacific Island fisheries and discussed possible effects of ciguatera on development fisheries. A similar summary is given here as a preliminary to assessing steps that may limit ciguatera outbreaks.

SOUTH PACIFIC REEF FISHERIES

Statistics on the composition and distribution of landings from reef and other coastal fisheries in the South Pacific, are poorly developed. Coastal fishing is characterised by small scale methods catching a wide variety of species and landing fish at many locations. This, plus the limited resources of most fisheries departments in the region, results in poor coverage of fisheries production.

The nominal coastal fin-fish landings for the South Pacific region (Dalzell,1992, table 1) should not be confused with absolute values, rather this is the best estimate that can be made given data sources available. Estimated fisheries production for the region is about 90,000 tonnes/yr (Dalzell,1992, table 1), although this is almost certainly an underestimate. Assuming a regional average value of US$2.00/kg for these

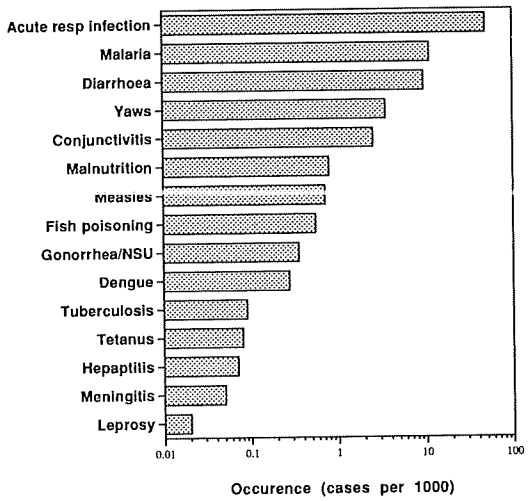

FIG.1. The 15 most commonly reported illnesses from the SPEHIS database in ranked order of occurrence (averaged between 1988 and 1992).

landings, then the potential nominal value is c.US$174,000,000/yr.

Given the quality of the data on fisheries landings and on the incidence of ciguatera, it is difficult to draw any firm conclusions (Dalzell, 1992, table 1). The volume of fish production per capita tends to be highest in the smaller, less-developed islands and atolls with limited land area and long traditions of fishing. These locations also record the highest incidence of ciguatera. Fresh fish production (and consumption) is lowest in the large islands of Melanesia, where some of the population live in the interior (especially PNG) and people traditionally look to the land as a principal source of food.

The percentage of the total fish landings that comprise reef fish ranges from 7–88% with a mean of 51% (Dalzell,1992, table 1). About half the families of reef fish landed in the Pacific contain species known to have been ciguateric (Dalzell,1992, table 2). Certain species such as the small surgeon fish *Ctenochaetus striatus*, the snapper *Lutjanus bohar* and barracudas (*Sphyraena* spp.) are known health hazards but are still occasionally consumed. In some areas, these species are known not to be ciguatoxic and are safely consumed.

Fishing for reef fish in the South Pacific is accomplished mainly with hand-lines, traps, nets and spears, deployed from dinghies or canoes. Lock (1986) presented observations of the composition of the catch taken by different fishing gears deployed on the South Papuan Barrier Reef off Port Moresby. Hand-lines select mostly for carnivorous species, which may be ciguatoxic through the ingestion of already contaminated prey species. Spearing and net fishing take a wider range of species, including reef herbivores that become ciguatoxic through ingestion of the dinoflagellate, *Gambierdiscus toxicus*. Catch rates of such small scale artisanal gears are modest at best – on average 1–4kg/man-hour, depending on the gear (Dalzell & Wright,1986) and there may be a disincentive for fishermen to return a portion of the catch to the sea simply because of suspected ciguatoxicity.

Food accounts for c.20% of imports into Pacific Island countries as opposed to <10% of imports into the metropolitan countries (SPC,1992). Domestic food production, and hence fisheries production, is important to diminish the reliance of the Pacific nations on imported foods. However, as island populations increase, fish harvests will also increase, both in subsistence and commercial sectors. In some countries of the region, harvest levels from coastal fisheries are already very high and fishing pressure is unlikely to diminish. The main objectives for fisheries managers and administrators are developing fish production and maintaining it at a sustainable level.

CIGUATERA AS A REGIONAL HEALTH ISSUE

A range of different illnesses and afflictions are recorded in the SPEHIS database (Fig.1). The level of under-reporting is unknown and some countries may report few medical statistics. Fish poisoning (which it is assumed represents mainly ciguatera) is ranked eighth. The information is misleading as the high incidence of yaws is based on cases reported mainly from the Solomon Islands. Similarly, the incidence of malaria is confined to Papua New Guinea, the Solomon Islands and Vanuatu. However, acute respiratory infections and gastro-intestinal infections are more evenly spread throughout the Pacific and are the major health issue in the region. Dalzell & Gawell (1989) reported that respiratory tract infections and gastro-intestinal diseases are the main concern of the Health Department in the Federated States of Micronesia, particularly amongst the young who comprise the majority of the population.

The non-communicable nature and relatively

low mortality rates from ciguatera mean that while the risk of fish poisoning is acknowledged, health departments place higher priorities on other health problems. The most effort health departments are likely to devote to ciguatera is publicising those species which are dangerous to consume and sometimes restricting marketing of those species.

Despite the possible impact of ciguatera on the fisheries in the South Pacific, there appears to be little concern about this form of intoxication from fisheries departments in the region. Fisheries officers may express interest in ciguatera, particularly if there is an outbreak in their country, but in general it does not appear to be given a very high priority.

Ciguatera was discussed at the first SPC fisheries conference (SPC,1952); was the subject of an SPC technical paper (Banner et al.,1963); and has been the topic of several workshops and conferences (SPC,1968,1978,1981,1988). The Commission has also produced a handbook on ciguatera (Bagnis,1973) and the Inshore Fisheries Research Project formed a Ciguatera Special Interest Group in 1991 which has published two bulletins.

Regular international meetings, symposia and workshops have addressed the subject of ciguatera. Despite these meetings, reports and publications there has been little in the way of requests for assistance from the various member countries of the Commission to deal with ciguatera.

THE SPC SEAFOOD POISONING DATABASE

In the absence of any reliable information on ciguatera from most of the South Pacific (French Polynesia, Hawaii and Australia are the exceptions), the South Pacific Commission established a regional seafood poisoning database in late 1990.

Both fisheries and health workers have been contacted to report (Dalzell,1992, fig.3) cases of fish poisoning. The database has been publicised repeatedly in SPC publications and in national newspapers and by radio. Despite repeated attempts to encourage the reporting of cases, the response has been variable.

Over 400 case histories have been collected from eight countries in the South Pacific (Table 1). Case histories from elsewhere are limited or non-existent, even from countries known from the SPEHIS database to have a high incidence of ciguatera such as Kiribati, Tokelau and the Marshall Islands (Dalzell,1992: table 1). This may be due to the priority accorded to ciguatera by national medical departments and the lack of training for medical and fisheries personnel in the collection of case history data.

Not all intoxications (Table 1) are likely to be due to ciguatera and case histories involving skipjack tuna (*Katsuwonus pelamis*), herring (*Herklotsichthys quadrimaculatus*) and snake mackerel (*Promethichthys prometheus*) are probably the result of some other form of poisoning. Several invertebrates such as crabs, lobsters, clams and sea cucumber, have also been involved in a number of poisonings, although again, another form of toxin other than ciguatera probably was responsible for causing illness.

Given the complexities of coral reef fish taxonomy and the majority of case histories recorded by medical personnel, it is not surprising that many reports do not identify precisely the fish responsible for intoxications. Thus it is common, for example, for the person poisoned to give a local generic term for surgeonfishes, groupers and parrotfish. Occasionally some local words for particular fish are for individual species. Good examples are the blue-line surgeon fish, *Acanthurus lineatus*, and the convict tang, *Acanthurus triostegus*, which in Tuvalu are known as 'ponelolo' and 'manini' respectively. Indeed, manini is a common name for *A. triostegus* in much of Polynesia, where it is a common food fish. However, in many of the poisonings reported from Tuvalu involving surgeonfish, the term 'pone' is used as a generic term for all acanthurids.

Intoxications involving surgeonfish (20.7%) and parrotfish (12%) together account for c.1/3 of case histories in the database. Other families responsible for 10% of intoxications include the groupers (15%) and the snappers (10%). Besides *A. lineatus* and *A. triostegus*, other species commonly implicated in poisonings are the snappers *Lutjanus bohar* and *L. monostigmus*, the groupers *Plectropoma* spp. and *Cephalopholis argus*, and the soapfish, *Grammistes sexlineatus*.

Surgeonfish and parrotfish are two of the common families of fishes on coral reefs and often form major fractions of the landings in a reef fishery. At two locations in Papua New Guinea, they formed c.12% of landings from reef fisheries in the north and south of the country (Dalzell & Wright,1986). Parrotfish and surgeonfish accounted for 1/3 of commercial landings of reef fish in Palau during 1976–1990 (Kitalong & Dal-

Species	Fiji	Federated States of Micronesia	Kiribati	Marshall Is	Nauru	New Caledonia	Niue	Tuvalu
FINFISH								
Abudefduf spp.								1
Acanthuridae								41
Acanthurus leucopareius								1
Acanthurus lineatus		1						18
Acanthurus triostegus								22
Balistidae								3
Carangoides ferdau								4
Caranx lgnobilis			1					2
Carcharinus longimanus								7
Cephalopholis argus								9
Chaetodontidae						1		1
Chelinus undulatus		4						
Ctenochaetus striatus		2						
Decapterus macarellus		1						
Epinephelus cyanopodus						1		
Epinephelus fuscoguttatus								3
Epinephelus melanostigma								1
Epinephelus microdon						1		1
Epinephelus spp.				2				
Grammistes sexlineatus								16
Gymnothorax javanicus		1						5
Herklotsichthys quadrimaculatus		1						
Holocentridae								12
Katsuwonus pelamis		1						1
Kyphosidae								2
Lethrinidae				1		4		
Lethrinus elongatus								3
Lutjanidae						2		4
Lutjanus argentimaculatus		1				1		
Lutjanus bohar		7		1	1			12
Lutjanus gibbus								1
Lutjanus monostigmus								12
Lutjanus sebae						2		
Monocanthidae								12
Monotaxis grandoculus		1						4
Mugilidae						1		
Mullidae								1
Naso brevirostris								1
Naso unicornis						1		
Plectropoma spp.						10		
Promethichthys prometheus		1						
Sargocentrum spiniferum								2
Scaridae		1				5		45
Serranidae						9		25
Shark		1						
Siganidae								1
Soleidae						1		
Sphyraena jello						2		
Sphyraenidae		9					2	5
Symphorus nematophorus						1		
Unknown fish		12				4		21

Species	Fiji	Federated States of Micronesia	Kiribati	Marshall Is	Nauru	New Caledonia	Niue	Tuvalu
CRUSTACEA								
Carpilius maculatus								1
Panulirus spp.								4
Scylla serata		1						
MOLLUSCS								
Chama pacifica								2
ECHINODERMS								
Holothuroidea		8						

TABLE 1. Species implicated in fish poisoning by country in the South Pacific Commission seafood poisoning database. Fish that could not be identified to species are grouped under family headings.

zell,1994). In the Philippines, parrotfish and surgeonfish formed 16 and 27% of landings from two coral reef fisheries (Bellwood,1988; Alcala & Russ,1990). Sims (1988) estimated that imports of fish into Rarotonga from Palmerston Atoll were composed mainly of parrotfish (70–80%) and that annual landings of parrotfish ranged from 15–20t. Smith & Dalzell (1993) found that surgeonfish and parrotfish comprised about 74% of landings from community spear and net fishing on Woleai Atoll in Micronesia and that the Scaridae and Acanthuridae accounted for 60–90% of the fishable biomass on lagoon back reefs.

Grazing herbivores such as the parrotfish and surgeonfish are likely to be toxic if a ciguatera outbreak occurs on a reef and thus a sizeable fraction of the fishable stocks on reefs may become a health hazard. The same is true of the snapper *Lutjanus bohar*, which is one of the most common predatory species on coral reefs and is widely distributed throughout much of the tropical Indian and Pacific Oceans. This species may account for up to half the fish caught by handlines in some areas (Dalzell & Preston,1992) but may be rejected due to its reputation for toxicity. Sale of *L. bohar* is prohibited in Mauritius due to its toxic reputation, although this species is a dominant feature of handline catches at the banks and islands of the western Indian Ocean (Wheeler & Ommanney,1953).

The five most common maladies from ciguatera are joint aches, headache, temperature reversal, diarrhoea and muscle cramps (Fig.2). The five most common symptoms from eating herbivorous fishes were joint aches, headache, diarrhoea, temperature reversal and vomiting (Fig.2). Eating carnivorous fishes most commonly caused joint aches, headache, temperature reversal, muscle cramps and tingling & numbness (Fig.2). Gastro-intestinal maladies such as vomiting and diarrhoea occur but with less frequency.

Collection of case history data will continue and further efforts will be made to increase the rate of reporting, particularly from those countries where ciguatera is relatively common but case histories are not forthcoming.

MANAGEMENT OF CIGUATERA

If ciguatera is not perceived as a major health hazard in most places, then this should guide the approaches taken to manage this problem. In locations such as Papua New Guinea (PNG), the incidence of ciguatera is very low although cases do occur intermittently. The PNG Medical Journal (1950s–1993) did not uncover a single case of ciguatera poisoning, although reports were given of turtle meat poisoning (Dewdney,1967), paralytic shell fish poisoning (Rhodes et al.,1975) and scombroid fish poisoning (Barrs,1985). According to the Department of Fisheries and Marine Resources (DFMR) 5 outbreaks of ciguatera occurred between 1971 and 1981 at Port Moresby, Finschhafen, Milne Bay and New Hanover (Anonymous 1988b).

The species involved in the poisonings were coral trout (*Plectropoma* sp.), red bass (*Lutjanus bohar*), parrotfish (*Scarus strongylocephalus*) and barracuda (*Sphyraena* sp.). In all cases no fatalities were recorded and most people recovered in 3–5 days. The only instance of a commercial ban on the purchase of species on the basis of suspected ciguatera is that of the Milne Bay Fishing Authority in regard to the red bass, *L. bohar*. The general consensus from the DFMR summary is that ciguatera is a minor problem and that the importance of fish to the economy and

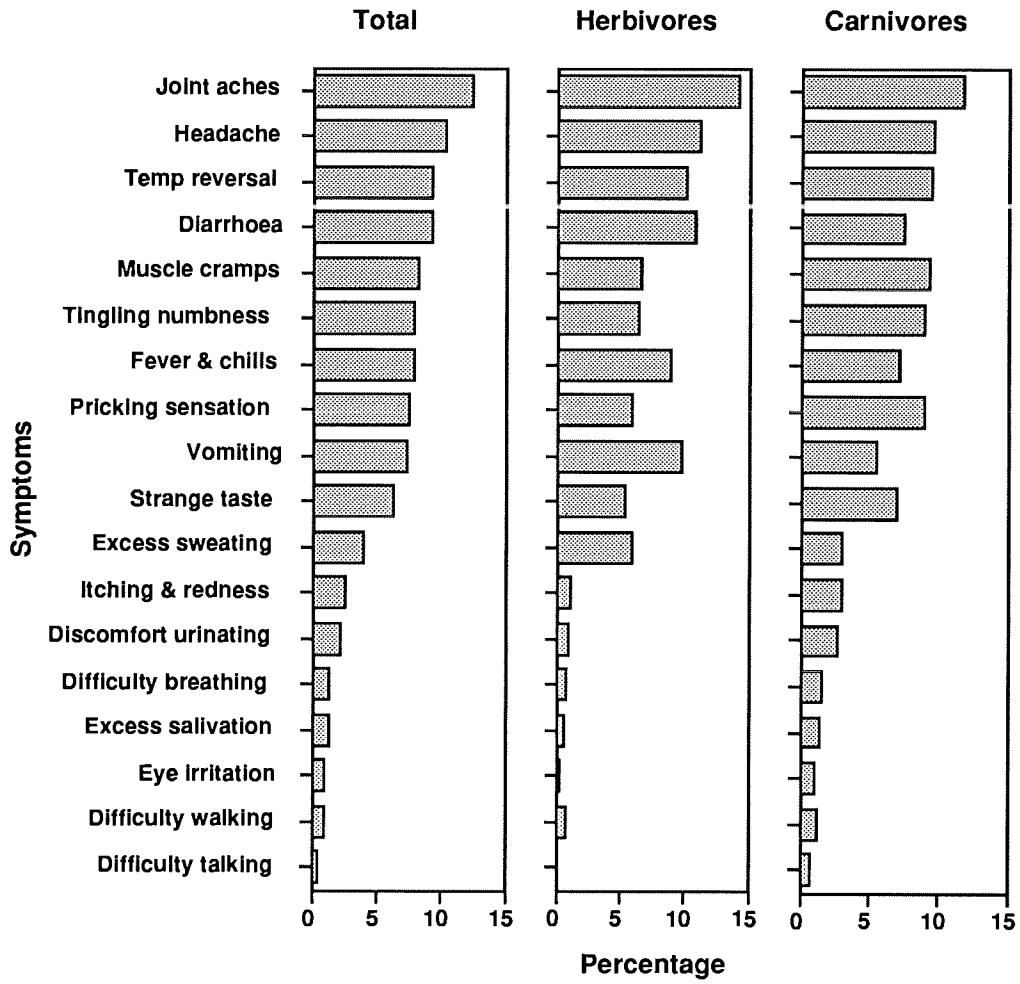

FIG. 2. Ranking of ciguatera symptoms from the consumption of all fishes, and separately the herbivores and carnivores in the South Pacific reported to the SPC seafoods poisoning database (N=380).

diet of the country is such that it would be imprudent to ban all or any species of which a few individuals may be dangerous to eat.

There are, however, places where ciguatera is a serious problem. These are usually small atolls or coral islands where per-capita fish and seafood consumption is very high (Dalzell,1992: table 1). A good example is Niutao Island in the Tuvalu archipelago. Since the establishment of the SPC database in 1990, over 200 cases of ciguatera have been reported from Niutao Island. For a population of almost 1000 people this is an incidence equal to about 10% of the population per year. Niutao is a coral island of 226ha, that encloses a small landlocked brackish lagoon and is surrounded by a fringing reef of c.108ha. Kaly et al. (1991) reported 80 case histories from the 1988 Niutao ciguatera outbreak, some months prior to a programme of subtidal reef blasting to create boat passages. Some blasting was conducted on the reef at Niutao in 1981 to create a boat channel but this was not followed by a serious outbreak of ciguatera. According to Kaly et al., there were on average fewer than three cases of ciguatera per year prior to 1988 resulting from the consumption of susceptible fish such as *L. bohar*. The outbreak, which begun in 1988, shows no signs of abating. Monthly occurence of

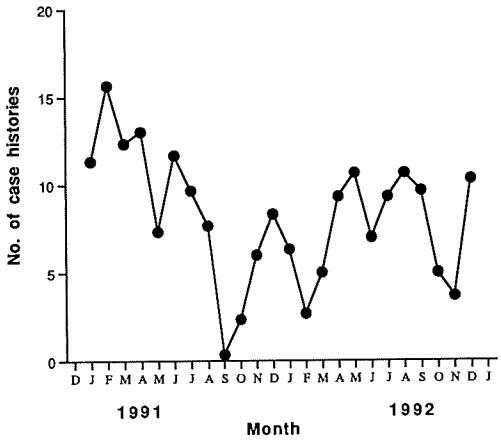

FIG.3. Number of case histories of ciguatera fish poisoning recorded in Niutao. Data smoothed by a running average of 3.

ciguatera cases (Fig.3), shows that intoxications occur throughout the year but with much variation between months.

Given the isolation and size of Niutao animal protein necessarily comes from the sea. However, reef fish are a risk on this island and persons are likely to be exposed to ciguatoxins on a regular basis through the consumption of reef fish. The most effective approach to managing the problem is to increase the supply of non-toxic fish. This means targeting pelagic species that live beyond the reef, and demersal snapper and grouper of the deep reef slope. Most pelagic and deep slope species are rarely ciguatoxic.

Deep reef slope fishes include mainly large snappers, groupers, emperors and a mix of other species including oilfish, jacks and barracuda. The deep slope dwelling snappers (Etelinae) have not been implicated in fish poisoning, as opposed to the shallow reef snappers (Lutjaninae). Although carnivorous, the diet of the Etelinae is mainly restricted to fish and benthos from the immediate environment (Parrish,1987) and not shallow reef dwelling herbivores. The same is probably true for groupers on the deep reef slope.

Stocks of deep slope species are more limited then pelagic fishes, particularly around small islands where the steep submarine gradient off the shelf limits the deep slope habitat. However, subsistence catches of deep slope species could play their part in displacing reef fish from the diet where ciguatera is a severe problem. The South Pacific Commission has been instrumental in successfully introducing the simple technology required to catch deep slope fishes in a number of countries.

Deep reef slope species may range between the shallow reef and deeper waters away from the reef. In Hawaii the amberjack (*Seriola dumerili*) has been implicated in a number of ciguatera outbreaks, despite being caught predominantly from the deep reef slope. Outbreaks of ciguatera led to the demise of the fishery for this species which amounted to about 30 tonnes annually (Humphreys,1986). Similarly, catches of deep slope fishes in the South Pacific include the snapper *Lutjanus bohar*. Unlike the eteline snappers, this species is found throughout the water column and is an opportunistic predator (Wright et al., 1986) with a diet that includes reef fish. *L. bohar* comprised 8% by weight of the catches of deep slope species in the South Pacific (Dalzell & Preston,1992). For the atolls and coral islands only, catches of *L. bohar* amounted to about 15% of total landings. Thus, although deep slope species can be an important supplement to the diet, potentially toxic fish may still be involved.

Similar strategies can be adapted for other countries where small islands are severely affected by ciguatera. In larger coral atolls where there is a substantial lagoon and reef area, sites that are known to produce ciguatoxic fish can be avoided in favour of safe fishing grounds. Development of pelagic and deep slope fisheries should be pursued, however, so that the resource base is widened and reef fish is not the sole source of fish protein. However, people may persist in fishing in known ciguateric sites and eating fishes which are recognised as being potentially dangerous. It will be difficult to control consumption of fish in most countries and there is an accepted risk in eating reef fishes even in those islands where they may be ciguatoxic. Management under these circumstances should be restricted to publicising those species which are known to be implicated in causing ciguatera.

A number of countries and territories of the South Pacific are heavily dependant on aid to maintain their economies. The typical island state is small and it has few natural resources. Trade between other islands is also limited because what one island can offer, the products of subsistence farming and fishing, can be found on most other islands. Islands that produce cocoa, palm oil and copra are competing against much larger producers elsewhere in the tropics. Revenues are accrued from permitting foreign fishing vessels to catch tuna within the 200 n.mi exclusive

economic zones (EEZ) of some of the Pacific Islands.

Some manufacturing enterprises do exist but are small and generally serve limited domestic markets. Further, imports of raw material tend to be costly because relatively small quantities have to be shipped over long distances. Probably the greatest economic potential for many of the South Pacific Islands, particularly those with limited natural resources, is tourism (Anon 1991). In some countries such as Fiji, Vanuatu, and parts of Micronesia, tourism is a major source of revenue and employment. Other countries in the region are trying to develop a better tourist industry infrastructure and increase earnings from this sector of the economy.

Ciguatera can be a threat to the hotel and restaurant business and to the tourist trade in general. The results of intoxication may include loss of business for the individual establishment and potentially for a particular location if the problem is severe enough. Further, there is the added risk of litigation brought by persons who have been poisoned. Hotels and restaurants in countries where tourism is important should be clearly informed about which fishes are in the high risk category. This is particularly important in institutions that have chefs and kitchen staff from overseas and who may not be familiar with potentially toxic fish, and where fishermen may tend to sell suspect fish that might be rejected by local consumers.

Most nations in the South Pacific have expanding commercial fishing industries. As described earlier, increasing volumes of reef, pelagic and deep slope fishes are being sent to markets in Japan, Hawaii, New Zealand and Australia from Pacific Islands to take advantage of high market prices. Outbreaks of ciguatera poisoning can have adverse effects on all coastal fisheries since, in the mind of retailers and consumers, even fish which are perfectly safe may be feared. As with tourism, litigation may be brought against fishermen and exporters with adverse consequences for an expanding fishing industry.

If countries manage to establish exports of reef fish, then high risk species such as *Lutjanus bohar*, moray eels, barracuda and some surgeonfish (eg. *Acanthurus lineatus*, *Acanthurus triostegus*, *Ctenochaetus striatus*) might be prohibited from export. Unlike subsistence production, the scale of fish exports is likely to be such that control and management can be effectively implemented. Fishes that are sent for export should be scrutinised and checked as to the location of the catch, particularly if reef areas with ciguatoxic fish are known to exist. Affordable tests for ciguatera (Park,1992) should be routine for export fishes.

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EVALUATION OF A SOLID-PHASE IMMUNOBEAD ASSAY FOR DETECTION OF CIGUATERA-RELATED BIOTOXINS IN CARIBBEAN FINFISH

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Dickey, R.W., Granade, H.R. & McClure, F.D. 1994 08 01: Evaluation of a solid-phase immunobead assay for detection of ciguatera-related biotoxins in caribbean finfish. *Memoirs of the Queensland Museum* **34**(3): 481–488. Brisbane. ISSN 0079-8835.

The Ciguatect™ solid-phase immunobead assay for detection of ciguatera-related polyether biotoxins in finfish was evaluated for consistency with the mouse bioassay. Fifty finfish from ciguatera-endemic waters of St. Thomas, U.S. Virgin Islands, and one fish remnant from a confirmed case of ciguatera poisoning were mouse bioassayed. The 51 specimens were then assayed using the Ciguatect™ assay with 3 different methods of tissue sampling: single exposure, triple exposure and single exposure to solvent extract from flesh (REM™: rapid extract method). Qualitative statistical analyses ascertained false positive and false negative rates. Positive matches for the single, triple and REM™ methods of tissue sampling were 58, 85 and 94%, respectively, and negative matches were 17, 22 and 12%, respectively. Corresponding false negative rates were 82, 55 and 50%, and false positive rates were 44, 33 and 33%. Predictive indices for Ciguatect ™ performance under ciguatoxin contamination rates ranging from 5 to 75% project that high false negative and false positive values might be expected in market situations.

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The major impediment to management strategies for the ciguatera public health hazard is the difficulty in detecting highly potent ciguatoxins (and maitotoxins) in seafood matrices. Ciguatoxin and maitotoxin structures have been elucidated from Pacific sources (Murata et al., 1989, 1990,1992,1993; Lewis et al.,1991; Yokoyama et al.,1988; Holmes et al., 1990), but Caribbean forms have not been resolved and analytical methods to detect and quantify them are not yet available. Several potential assessory toxins have also been characterized (Murakami et al.,1982; Torigoe et al.,1988; Nagai et al.,1992; Fukui et al., 1987). Difficulties with developing ciguatera detection methods are due to lack of analytical standards. An immunochemical assay for detection of ciguatera-related polyethers was developed (Hokama,1985,1990; Hokama et al.,1990, 1992) and modified into a 'kit' format, 'Ciguatect™' (Park et al.,1992). We compare this assay with the mouse bioassay, the most widely recognized method for identification of ciguatoxic finfish.

EXPERIMENTAL

APPARATUS

Eberbach 8017 explosion-proof blender (Eberbach Corp., Ann Arbor, MI); Brinkmann/Buchi rotary evaporator Model RE-111 (Brinkmann Instruments, Inc., Westbury, NY); Mettler Model PM2000 top-loading balance (Mettler Instrument Corp., Hightstown, NJ); Sartorius Model R180D analytical balance (Sartorius Corp., Bohemia, NY); Savant Model SS-2 centrifugal evaporator (Savant Instruments, Inc., Farmingdale, NY).

REAGENTS AND MATERIALS

Solid-phase immunobead assay (SPIA) materials were purchased from Hawaii Chemtect International, Inc. (San Diego, CA) on 24 May 1992 (immunobead lot number 051492) and again on 6 October 1992 (immunobead lot number 100592). All solvents were of reagent grade or better (J.T. Baker Chemical Co., Phillipsburg, NJ). Silica gel solid-phase extraction columns were obtained from Varian Associates, Inc. (Sunnyvale, CA). Swiss white mice (Crl:CFW (SW)BR) were obtained from Charles River Laboratories (Wilmington, MA).

SPECIMEN COLLECTION

Potentially ciguatoxic finfish were collected 4–13 June,1992 from ciguatera-endemic waters S of St. Thomas, U.S. Virgin Islands (McMillan et al.,1983). Fish were solicited from local sports fishermen. Fish were collected by spearfishing. Participants in an annual fishing tournament were

notified of the need for specimens, and donations of fish were accepted at the weigh-in station. Larger fish of suspect species from areas noted for ciguatera were procured as they have the highest probability of being ciguatoxic. This approach was taken knowing that a significant percentage of finfish are non-ciguatoxic even from high incidence areas. Specimens were identified, weighed, tagged, frozen and air-freighted to the FDA Gulf Coast Seafood Laboratory (GCSL), Dauphin Island, AL. All specimens arrived frozen with no evidence of thawing or decomposition. Fish specimens were stored at -20°C.

TEST SAMPLE PREPARATION

Fifty Caribbean fish were selected, including *Sphyraena barracuda* (70%), *Caranx latus* (20 %), *C. hippos* (2%), *Seriola dumerili* (4%), *Seriola rivoliana* (2%) and *Scomberomorus cavalla* (2%). From each specimen two muscle tissue samples were taken from the anterolateral part of the body immediately behind the head: 10g for use in the SPIA procedure and 450g for extraction and mouse bioassay. Samples also were taken from a fillet portion of barracuda (specimen VI-108: courtesy of Dr. Norbert Mantor, St. Thomas Hospital) that was responsible for ciguatera poisoning in St. Thomas and from a *Cynoscion arenarius* (white trout: specimen 92-07-1; not included in the tables) obtained from the retail seafood market of Dauphin Island, AL, as a negative control. A portion of the latter specimen was consumed without toxic effect. The 10g samples were frozen at -20°C. The 450g samples were weighed to the nearest 0.1g, and extracted and partitioned (McMillan et al., 1983; Yasumoto et al.,1984). The dried products from the extraction and partitioning procedures were dissolved in chloroform and applied to 60mL (10g) silica gel solid-phase extraction (SPE) columns that had been preconditioned by washing with 60mL of the same solvent. The SPE columns were washed with an additional 120mL chloroform and then eluted with 120mL 10% methanol in chloroform. The eluting solvent mixtures were removed by rotary evaporation, and residues transferred in methanol (c.2–3mL) to tared 13x100mm Teflon-capped test tubes. Solvent was removed again by vacuum centrifugation and residue weights were recorded to the nearest 0.1mg for each final product. Final SPE products were dissolved in methanol (c.1–2mL) and stored at -20°C.

MOUSE BIOASSAYS

Methanol was removed from the muscle tissue SPE products by vacuum centrifugation. The residues were redissolved in known total volumes of saline (0.15M NaCl) containing 1% Tween-60. Appropriate aliquots of the saline solutions were taken such that the dosing of mice was equal to muscle tissue fresh weight equivalents (MTE) of 45, 90 or 180g per mouse. Residue weights administered to mice did not exceed 20mg, and ranged as low as 318μg when adjusted to the chosen MTE. All bioassay solutions were adjusted to a total injection volume of 0.5mL prior to bioassay. The 0.5mL solutions were administered by intraperitoneal injection into Swiss white mice (Crl:CFW(SW)BR) weighing 18-21g. Control mice were administered saline-Tween solution only. The mouse bioassays were performed in duplicate for controls and for each MTE of the final SPE products. Mice were observed post-injection for a period of 48hr. Signs of toxicity were noted and when death occurred, times from injection were recorded. Mouse bioassays were conducted according to the principles provided in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animals Resources, National Research Council, NIH Pub. No. 85-24.

CIGUATECT ™ SPIA

A 9x10mm white membrane, affixed to a 90x9mm plastic strip is exposed to muscle tissue taken from behind the head of the fish. The membrane is permitted to air-dry and is then immersed in methanol for no more than 1sec. After the membrane is completely dry, it is immersed in an anti-ciguatoxin-antibody-latex bead suspension (gently mixed prior to use) for 5 or 10min. The latter solution is blue. The membrane is then dipped in and out of a phosphate-buffered saline solution 3 times, placed flat on an absorbent towel and gently blotted to remove excess saline solution. Adsorption of ciguatera biotoxins to the membrane and subsequent conjugation with the anti-ciguatoxin antibody bound to the surface of the blue latex beads should produce a blue color (cast) in the membrane affixed to the plastic strip if the fish contains ciguatera biotoxins. Kit positive-control membrane strips are processed from the point of exposure to antibody-latex bead solution. Kit negative-control membrane strips are processed from the point of exposure to methanol. All Ciguatect ™ SPIA materials are stored at 4°C.

Instructions for the first set of materials (received 24 May 1992) directed the user to expose the membranes to fish tissue only one time

TABLE 1. Mouse bioassay dosing, fish muscle tissue equivalents (MTE) and survival times. * indicates dose of 180g MTE administered to mice.

Specimen	45g MTE dose (mg/kg)	45g MTE survival time (hr)	90g MTE dose (mg/kg)	90g MTE survival time (hr)
VI-13	46.0 42.6	>48 >48	83.9 77.9	>48 >48
VI-17			1018.9 856.9	0.20* 0.36*
VI-26			369.0 337.8	25.00 >48
VI-27			258.7 236.8	3.60 1.80
VI-32			301.0 275.4	>48 >48
VI-33			183.8 219.3	0.33 0.32
VI-34			255.4 230.6	1.9 13.00
VI-35			265.2 250.5	1.00 1.56
VI-37			295.7 365.3	0.55 0.63
VI-38	122.5 119.3	0.33 0.63		
VI-48	73.3 65.3	>48 >48	121.3 130.7	>48 2.21
VI-49			80.3 68.6	0.15 0.15
VI-52	17.4 15.8	0.66 0.45	34.4 35.6	>1.00 >1.00
VI-53			82.1 79.8	2.50 6.40
VI-54	7.9 8.7	>48 >48	15.2 14.9	>48 >48
VI-55	135.8 124.4	>48 >48	218.0 229.2	>48 >48
VI-57	56.8 53.0	0.50 0.45		
VI-58	63.4 75.3	>48 >48	126.0 149.4	0.61 0.30
VI-59			123.6 121.8	>48 >48
VI-60	73.2 73.6	>48 >48	133.3 141.8	>48 >48
VI-61	131.5 133.0	3.0 >48		
VI-62	74.9 76.1	6.10 >48		
VI-63	87.4 90.5	>48 >48	157.7 154.5	>48 >48
VI-64	175.6 170.4	15.50 >48		
VI-65	81.7 83.4	>48 >48	162.2 146.9	16.00 >48
VI-67	51.5 51.8	1.51 >48	107.3 113.8	0.92 0.91
VI-70			195.3 210.8	>48 >48
VI-71	33.3 34.1	>48 >48	62.9 70.2	2.50 14.25
VI-72	35.9 31.6	>48 >48	62.1 63.7	>48 >48
VI-73	41.1 40.2	>48 >48	73.1 72.1	1.10 4.66
VI-75	110.6 120.3	>48 >48	202.3 218.4	>48 >48
VI-76	44.1 39.7	>48 >48	81.8 79.0	>48 1.68
VI-77	52.7 45.4	0.66 0.63		
VI-79	84.2 83.3	2.13 >48		
VI-81	69.7 61.4	>48 >48	131.9 128.5	>48 >48
VI-85	44.1 41.3	2.92 5.50	93.8 92.8	1.21 1.61
VI-86	54.9 56.4	28.00 4.50		
VI-88			236.3 238.7	>48 >48
VI-90	44.1 40.1	15.50 >48		
VI-91	67.0 66.7	1.53 >48		
VI-92	27.4 26.2	>48 >48	50.8 45.5	>48 >48
VI-93	70.7 71.5	>48 >48	127.2 127.2	>48 >48
VI-97	74.5 74.1	>48 >48	128.3 130.1	>48 >48
VI-98	78.0 71.1	>48 >48	123.0 129.1	>48 >48
VI-99	61.3 61.3	0.86 0.88	138.8 123.1	0.10 0.23
VI-100			54.1 54.4	>48 >48
VI-102			46.2 44.9	>48 >48
VI-105			288.1 278.6	0.13* 0.55*
VI-106	64.0 62.3	>48 >48	120.2 124.6	0.15 0.15
VI-107	54.3 48.7	>48 >48	90.9 90.0	1.10 0.73
VI-108	55.6 55.6	>48 >48	146.0 153.7 400.0 412.6	0.83 1.83 0.35* 0.22*

Specimen	single exposure		triple exposure		REM™ duplicates*	Mouse bioassay
	Mean	SD	Mean	SD		
VI-13	**1.5**	0.5	0.2	0.4	**3,3**	N
VI-17	1.8	0.4	1.8	0.4	**0,1**	T
VI-26	2.3	0.5	2.3	0.5	5,5	T
VI-27	1.8	0.4	1.0	0.0	5,6†	T
VI-32	**1.8**	0.4	**3.8**	1.2	**3,3**	N
VI-33	3.2	0.7	3.2	0.4	4,4	T
VI-34	2.5	0.5	3.8	0.4	3,3	T
VI-35	2.0	0.0	3.0	0.6	5,5	T
VI-37	3.7	0.5	2.0	0.0	4,5	T
VI-38	**0.5**	0.5	3.7	0.5	5,5	T
VI-48	**0.7**	0.5	1.8	0.4	3,3	T
VI-49	1.5	0.5	**0.7**	0.5	2,2	T
VI-52	**0.5**	0.5	3.7	0.9	2,2	T
VI-53	1.8	0.4	2.7	1.1	3,3	T
VI-54	**1.8**	0.4	**2.5**	0.5	**3,3**	N
VI-55	**2.3**	0.5	**1.2**	0.4		N
VI-57	**1.0**	0.0	2.7	1.2	5,5	T
VI-58	**0.8**	0.4	3.3	1.1	5,6†	T
VI-59	**2.2**	0.4	**2.7**	1.1	**5,6†**	N
VI-60	**2.0**	0.0	**3.8**	1.3	5,5	N
VI-61	1.5	0.5	1.8	0.4	2,2	T
VI-62	**1.0**	0.0	2.3	0.5	**1,1**	T
VI-63	**2.5**	0.5	**2.7**	0.7	1,1	N
VI-64	1.8	0.4	3.0	0.8	4,4	T
VI-65	**0.2**	0.4	**0.3**	0.5	4,5	T
VI-67	1.7	0.7	3.0	0.6	4,5	T
VI-70	**3.0**	0.6	**2.3**	0.5	**3,4**	N
VI-71	**0.7**	0.5	1.3	0.5	5,5	T
VI-72	**1.8**	0.4	**1.3**	0.7	**4,5**	N
VI-73	**0.8**	0.4	**0.0**	0.0	5,5	T
VI-75	**1.3**	0.5	**1.2**	0.4	**4,4**	N
VI-76	**1.0**	0.0	**1.0**	0.0	2,3	T
VI-77	1.7	0.7	1.2	0.4	3,3	T
VI-79	2.0	0.0	1.3	0.5	5,5	T
VI-81	0.8	0.4	1.0	0.0	**4,5**	N
VI-85	1.7	0.7	2.0	0.6	5,5	T
VI-86	3.5	1.0	2.5	0.8	5,5	T
VI-88	**3.3**	0.5	**2.0**	0.0	**4,4**	N
VI-90	**0.3**	0.5	1.8	0.4	3,3	T
VI-91	**0.3**	0.5	1.5	1.6	4,4	T
VI-92	0.7	0.5	0.7	0.5	**4,5**	N
VI-93	**2.2**	0.4	**1.5**	0.5	**3,3**	N
VI-97	**2.0**	0.0	0.0	0.0	**5,5**	N
VI-98	**1.3**	0.7	**1.3**	0.7	**2,2**	N
VI-99	1.8	0.4	**0.8**	0.4	2,3	T
VI-100	**1.2**	0.7	**2.5**	1.0	0,1	N
VI-102	0.5	0.5	**2.2**	0.4	**3,3**	N
VI-105	**0.8**	0.4	2.7	1.7	4,4	T
VI-106	1.2	0.7	1.8	0.4	2,2	T
VI-107	2.0	0.0	1.5	0.5	2,2	T
VI-108	**0.5**	0.5	2.2	0.4	5,5	T

TABLE 2. Ciguatect™ SPIA mean scores (n=5) and standard deviations (SD) for three variations of procedure. Boldface values indicate false positive or false negative scores relative to mouse bioassay results (T = ciguatoxic; N = survival beyond 48hr). * indicates data set provided by Dr D.L. Park, Univ. of Arizona. †indicates apparent inconsistency with regard to immunoassay scoring scale.

(single exposure) and to allow the membrane to dry before proceeding. Weak color development prompted the manufacturer to modify the procedure for the second set of materials (received 6 October 1992). The latter procedure instructed the user to expose the membranes by contact with fish tissue three times (triple exposure) with drying periods following each exposure. Consequently, Ciguatect™ immunoassays were performed twice on each 10g test sample. The instructions for immunoassay setup, fish assay and use of positive and negative kit controls were followed precisely. Positive and negative control test strips (provided with SPIA materials) were developed prior to fish tissue assays. The SPIA color developments for controls and fish tissues were read and interpreted independently by 6 analysts. Participants were asked to assign a value of 0 (no color) to 5 (strong color) to color development of each test strip by comparison with a standard series of positive and negative control test strips supplied by the kit manufacturer.

The 10g test samples that were used for Ciguatect™ SPIA evaluation at the FDA GCSL were frozen and delivered 'blind' (i.e., each test portion was identified only by code number) to the laboratory of the kit manufacturer for a repetition of the SPIA procedures.

Statistical Analysis

The Ciguatect™ SPIA method was assessed using procedures for evaluating screening tests (Fleiss,1981). Method performance is described through four interrelated rates: sensitivity, specificity, false positive, and false negative. The sensitivity rate (P_+) is the proportion of correctly

TABLE 3.Ciguatect™ SPIA performance rates relative to mouse bioassay results. * indicates data set provided by Dr D.L. Park, University of Arizona.

Performance criterion	Performance rate		
	Single-exposure immunoassay	Triple-exposure immunoassay	REM™ immuno-assay*
SENSITIVITY	19/33 (58%)	28/33 (85%)	31/33 (94%)
SPECIFICITY	3/18 (17%)	4/18 (22%)	2/17 (12%)
FALSE NEGATIVE	14/17 (82%)	5/9 (55%)	2/4 (50%)
FALSE POSITIVE	15/34 (44%)	14/42 (33%)	15/46 (33%)

classified 'known' positive test samples. Specificity rate (P_-) is the proportion of correctly classified 'known' negative test samples. False positive rate (PF_+) is the proportion of positive classified test samples that are misclassified 'known' negatives. False negative rate (PF_-) is the proportion of negative classified test samples that are misclassified 'known' positives. A 'known' positive or 'known' negative test sample is a test sample that has been classified as positive or negative by the reference method (i.e., mouse bioassay). False positive and false negative rates, as defined by Fleiss, are predictive rates. These were used to assess the Ciguatect™ SPIA method when used on populations of fish with different proportions of ciguatoxic specimens.

RESULTS AND DISCUSSION

Mouse Bioassay

Characteristic signs of ciguatoxicity (Hoffman et al.,1983; Kimura et al.,1982) and death within 48hr were chosen as the determinants for the presence of ciguatera-related biotoxins. The first three fish SPE products bioassayed were administered at doses of 180g MTE. Survival times were short for the three products ($\leq$0.36hr). All subsequent bioassays were conducted at an MTE of either 90 or 45g in order to record the presence or absence of characteristic signs of ciguatoxicity. The mouse bioassays resulted in 33/51 (65%) mortalities (Table 1). A total of 79 pairs of mice were injected (duplicate bioassay for each MTE). In 10 of the 79 pairs 1 mouse expired within 48hr, and the other survived beyond 48hr. Signs of ciguatoxicity in mice included inactivity; piloerection; vasodilation in ears; cyanosis of tail, feet and muzzle; lacrimation; salivation; diarrhea; dyspnea; unsteady gait; tremor/convulsive jumping; straub tail; convulsions; and death. Not all mice displayed each of these signs of toxicity. Some characteristic signs of toxicity were not observed in mice that expired within 1hr (15/33: 45%). In 50% of short survivals the dosage was reduced to 45g MTE (lowest dose level administered) and longer survival times or survivals through 48hr were noted with characteristic signs of toxicity. The mouse bioassay of specimen VI-108 resulted in survival beyond 48hr at 45g MTE, survival times of 0.83 and 1.83 at 90g MTE, and survival times of 0.35 and 0.22 at 180g MTE. Specimen 92-07-1 resulted in survival beyond 48hr at 180g MTE without any sign of toxicity. Fish specimens from which corresponding SPE products produced mouse death with characteristic signs of toxicity at 90g MTE were considered ciguatoxic.

Ciguatect™ SPIA

SPIA ratings of the fish specimens were scaled from 0 to 5 by comparison of color development with a standard series of pre-developed membranes supplied by the manufacturer. The manufacturer specified that ratings of 0 or 1 were to be considered non-ciguatoxic and ratings above 1 through 5 ciguatoxic at progressively greater levels. The means and standard deviations of ratings for SPIA responses were recorded (Table 2). However, for comparison with mouse bioassays the specimens were scored as either positive (ciguatoxic) or negative (non-ciguatoxic) by SPIA. Results from single exposure of test-strip membranes to muscle tissues indicated that 34/51 (67%) of fish specimens were ciguatoxic. Based upon the precedent (McMillan et al.,1983; Yasumoto et al.,1984; Hoffman et al., 1983; Kimura et al.,1982; Vernoux et al., 1985) that the reference mouse bioassay accurately reflects ciguatoxicity, results of the 3 sample treatment methods with the immunoassay technique are shown in Table 3. Single-exposure SPIA scored the human illness case sample (specimen VI-108) negative (mean 0.5, SD=0.5) and the non-toxic white trout specimen positive (mean 2.7, SD=0.7). The SPIA results following triple exposure of test-strip membranes to muscle tissues indicated that 42/51 (82%) of the fish specimens were ciguatoxic. Triple-exposure SPIA scored VI-108 (mean 2.2, SD=0.4) and the white trout specimen (mean 4.0, SD=0.5) both positive. Mean scores for the kit internal controls for single exposure of test strips included 2/2 positive matches and 2/2 negative matches. Scores for kit internal controls for triple exposure

TABLE 4.Predictive indices of Ciguatect™ performance, given ciguatoxins contamination rates from 5 to 75%. PF_+ = false positive; PF_- = false negative.

Contamination (%)	Predictive index					
	Single exposure		Triple exposure		REM™	
	PF_+	PF_-	PF_+	PF_-	PF_+	PF_-
5	0.9649	0.1181	0.9457	0.0346	0.9486	0.0264
15	0.8913	0.3100	0.8386	0.1074	0.8462	0.8333
25	0.8128	0.4590	0.7333	0.1852	0.7444	0.1466
35	0.7288	0.5782	0.6300	0.2685	0.6432	0.2172
45	0.6389	0.6756	0.5284	0.3581	0.5426	0.2965
55	0.5422	0.7566	0.4286	0.4545	0.4426	0.3864
65	0.4380	0.8254	0.3305	0.5587	0.3432	0.4889
75	0.3254	0.8842	0.2340	0.6716	0.2444	0.6071

of test strips included 2/2 positive matches, 1/2 negative matches and 1 false positive (mean of 1.2 on 5-point scale). False negatives were not observed among the kit control test strips.

The Ciguatect ™ SPIA results obtained by the manufacturer's laboratory for the fish test samples evaluated by FDA GCSL do not correspond to results obtained by GCSL. The manufacturer laboratory first performed extraction and partitioning procedures (REM™ :rapid extraction method) on each test sample. The SPIA was then performed on the REM™ products (Tables 2, 3). SPIA results obtained by following the REM™ modification of the procedure indicated that 46/50 (92%) of the fish specimens were ciguatoxic. VI-108 scored positive (duplicate scores of 5 and 5).

The sensitivity and specificity test performance rates for each variation of the SPIA procedure (Table 3) were used to evaluate how the Ciguatect™ SPIA would be expected to perform when used on fish populations with different proportions of ciguatoxic specimens. Table 4 presents a summary of the predictive values for each SPIA method by population ciguatoxicity rate. Assuming that a true ciguatoxins contamination rate of 55% is encountered in a hypothetical lot of tropical fish, the predictive indices generated from the present study indicate that single-exposure Ciguatect™ SPIA would produce a false negative rate of 76% and a false positive rate of 54%. Triple-exposure Ciguatect™ SPIA would produce a false negative rate of 45% and a false positive rate of 43%, and the REM Ciguatect™ SPIA would produce a false negative rate of 39% and a false positive rate of 44%.

The Ciguatect™ SPIA was examined for interference from potential fish decomposition products and for possible non-specific toxicity unrelated to ciguatera in the mouse bioassay. A repeat sampling of 13 muscle tissues (25%) from the original 51 fish provided a cross-section of tissue that were SPIA positive and negative, mouse bioassay positive and negative, and SPIA false positive and false negative. The tissues were analyzed for putrescine and cadaverine by the method of Staruszkiewicz & Bond (1981) and for histamine by the fluorometric method (AOAC, 1990). Determination of putrescine was not possible because of matrix interference. Cadaverine levels were below the lowest calibration standard of 0.5μg/g in 10 of the 13 tissues. Three of the tissues contained 3.7, 7.2 and 8.4μg/g cadaverine (Table 5). Cadaverine levels above 6μg/g in tunafish indicate decomposition. Tolerance levels for other species of fish (including those used in the present study) are not established. Histamine levels did not exceed 0.1mg% in 11 of the 13 tissues, and did not exceed 0.2mg% in the two remaining tissues. The defect action level for histamine in the United States is 20mg%, and 50mg% poses a human health hazard. There was no correlation between cadaverine or histamine and SPIA or mouse bioassay findings.

Thirteen tissue extract products (corresponding to the specimens analyzed for decomposition products) were evaluated for sodium channel activity by using a tetrazolium-based neuroblastoma cell bioassay for neurotoxins active on sodium channels (Manger et al.,1993). Sodium channel potentiating activity is indicative of the ciguatoxins. The bioassay indicated that sodium channel activity was present in all 13 tissue products and provided a general ranking of sodium channel activity for those tested (Table 5). The activity ranking correlated well with mouse bioassay survival times. Specimen extract products shown to be highly toxic by the mouse bioassay also produced significant reductions in cell viability through sodium channel potentiating effects. Inhibitory doses which reduced cell viability by 90% after 22 hr exposure (ID_{90}) ranged from 2 to 6mg MTE per 200microliter cell culture (96μL well format). Specimen extract products classified non-toxic by the mouse bioassay were found to possess sodium channel effects at ID_{90} dosing that ranged from 7 to 40mg MTE. The latter finding, if considered in conjunction with mouse bioassay results from the ciguatera case specimen VI-108 (i.e., mouse survival at 45g MTE and expiration at 90g MTE), suggests that utilization of 90g MTE in the mouse bioassay may approximate a correlate for human toxicity.

TABLE 5. Sodium channel activity of specimen solid-phase extraction products (ID_{90} =inhibitory dose which reduces cell viability by 90% at 22hr exposure). Values expressed in milligram muscle tissue equivalents per well; mouse bioassay survival times; Ciguatect™ SPIA scores and decomposition indicators. * indicates data provided by Dr D.L. Park, University of Arizona. † indicates apparent inconsistency with regard to scoring scales. # indicates dose of 45g MTE administered to mice.

Specimen	Sodium channel ID_{90} (mg MTE/well)	90g MTE mouse bioassay TD (hr)	Ciguatect™ score			Decomposition products	
			Single-exposure (SD)	Triple-exposure (SD)	REM*	Cadaverine µg/g	Histamine mg%
VI-13	>40	>48 >48	1.5(0.5)	0.2(0.4)	3,3	<0.5	0.1
VI-59	32	>48 >48	2.2(0.4)	2.7(1.1)	5,6†	<0.5	0.1
VI-63	22	>48 >48	2.5(0.5)	2.7(0.7)	1,1	<0.5	0.1
VI-32	20	>48 >48	1.8(0.4)	3.8(1.2)	3,3	<0.5	0.1
VI-81	9	>48 >48	0.8(0.4)	1.0(0.0)	4,5	7.2	0.2
VI-54	7	>48 >48	1.8(0.4)	2.5(0.5)	3,3	<0.5	0.1
VI-73	6	1.10 4.66	0.8(0.4)	0.0(0.0)	5,5	<0.5	0.1
VI-65	4	16.00 >48	0.2(0.4)	0.3(0.5)	4,5	<0.5	0.1
VI-53	2	2.50 6.40	1.8(0.4)	2.7(1.1)	3,3	8.4	0.2
VI-85	2	1.21 1.61	1.7(0.7)	2.0(0.6)	5,5	<0.5	0.1
VI-77	2	0.66 0.63	1.7(0.7)	1.2(0.4)	3,3	<0.5	0.1
VI-38	2	0.33# 0.63#	0.5(0.5)	3.7(0.5)	5,5	<0.5	0.1
VI-57	2	0.50# 0.45#	1.0(0.0)	2.7(1.2)	5,5	3.7	0.1

CONCLUSION

Ciguatect™ SPIA performance with ciguatoxic Caribbean finfish may be characterized by low specificity rates and high false positive and false negative values. Extrapolating these performance characteristics to a market situation implies that a proportion of wholesome fish might falsely be identified as ciguatoxic and an equally significant proportion of ciguatoxic fish might reach the marketplace undetected. Conclusions of the present study cannot be extended to Ciguatect™ SPIA performance with Pacific Ocean finfish (where the immunoassay originated) without separate evaluation of the method using fish from the Pacific region.

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ASSESSMENT OF CIGUATERIC FISH IN HAWAII BY IMMUNOLOGICAL, MOUSE TOXICITY AND GUINEA PIG ATRIAL ASSAYS

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Hokama, Y., Asahina, A.Y., Titus, E., Ichinotsubo, D., Chun, S., Hong, T.-L.W.P., Shirai, J.L., Asuncion, D.A. & Miyahara, J.T. 1994 08 01. Assessment of ciguateric fish in Hawaii by immunological, mouse toxicity and guinea pig atrial assays. *Memoirs of the Queensland Museum* **34**(3): 489–496. Brisbane. ISSN 0079-8835.

Ciguatera studies were determined at the Waianae Boat Harbor, Oahu when 12 or more individuals became ill after eating freshly caught mullet (*Mugil cephalus*, amaama) in January–March,1991. Typical clinical manifestations of ciguatera were shown by the patients. Immunological assay for ciguatoxin and polyethers with monoclonal anti-ciguatoxin (MAb-CTX) showed 80% of the mullet to be borderline to positive. The herbivores, *Ctenochaetus strigosus* (kole), *Acanthurus sandvicensis* (manini) and other *Acanthurus* sp. (palani), showed high levels of toxins. The mackerels showed little or no toxic levels, while the carnivores (jack, amberjack) showed borderline to positive toxicity. Abundant green algae (*Bryopsis*), 30–60cm below the seawater surface, found at all five sites examined, contained *Gambierdiscus toxicus* in moderate numbers. At 2 sites, when *Bryopsis* disappeared (summer - early winter), no *Gambierdiscus toxicus* was found. Fish extracts of mullet and other herbivores (palani, manini, kole-surgeonfishes) were highly toxic to mice. Guinea pig atrium analysis of the wild *Gambierdiscus toxicus* and fish extracts showed typical ciguatoxin-like inotropic response strongly inhibited by tetrodotoxin.

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In January–March,1991, an outbreak of ciguatera poisoning occurred among individuals eating mullet (*Mugil cephalus,* amaama) from Waianae Boat Harbor. Approximately 12 people exhibited classical symptoms of ciguatera poisoning (Hokama,1988) with an older couple hospitalized for several days. Based on knowledge of the ciguatera food chain (Randall, 1958; Hokama et al.,1986; Hokama et al.,1993), a systematic study of Waianae Boat Harbor was carried out and included:

1.Examination of algae species;

2.Examination of ciguatoxin producing *Gambierdiscus toxicus*; 3.Immunological testing of as many species of fish from the boat harbor;

4.Analysis of alga and *G. toxicus* chemical extracts for ciguatoxin by mouse toxicity bioassay and by the guinea pig atrium assay;

5.Analysis of fish extracts using the mouse toxicity and guinea pig atrium assays; and

6.Water quality data (from the Department of Health).

Basis for this systematic assessment is that of Yasumoto et al., (1979,1980,1984) and Hokama et al. (1993).

METHODS

SURVEY AREA

The Waianae Boat Harbor (117057m^2) includes 4 ramps and 3 docking areas for boats. Two water inflow outlets are the entrance channel to the ocean, and a tunnel located between the boat docks (fresh water and seawater runoff). Five areas are surveyed within the boat harbor (Fig.1).

ANALYSIS FOR *G. TOXICUS*

Algae specimens (0.5kg) were collected in a 5 litre ziplock bag containing 1 litre of seawater. The contents were shaken for 2 minutes to loosen epiphytic dinoflagellates from the alga. The salt water–algal suspension was passed through a 125μm sieve to remove larger algal fragments and then through a 37μm sieve. This residue was backwashed with a filtered seawater media, transferred into a 100ml sterile glass bottle and capped loosely to provide aeration. After gently shaking the algal sample bottle, 1.0ml was removed and transferred onto a Sedgewick Rafter Cell Counting Slide. Cell counts were carried out in tripli-

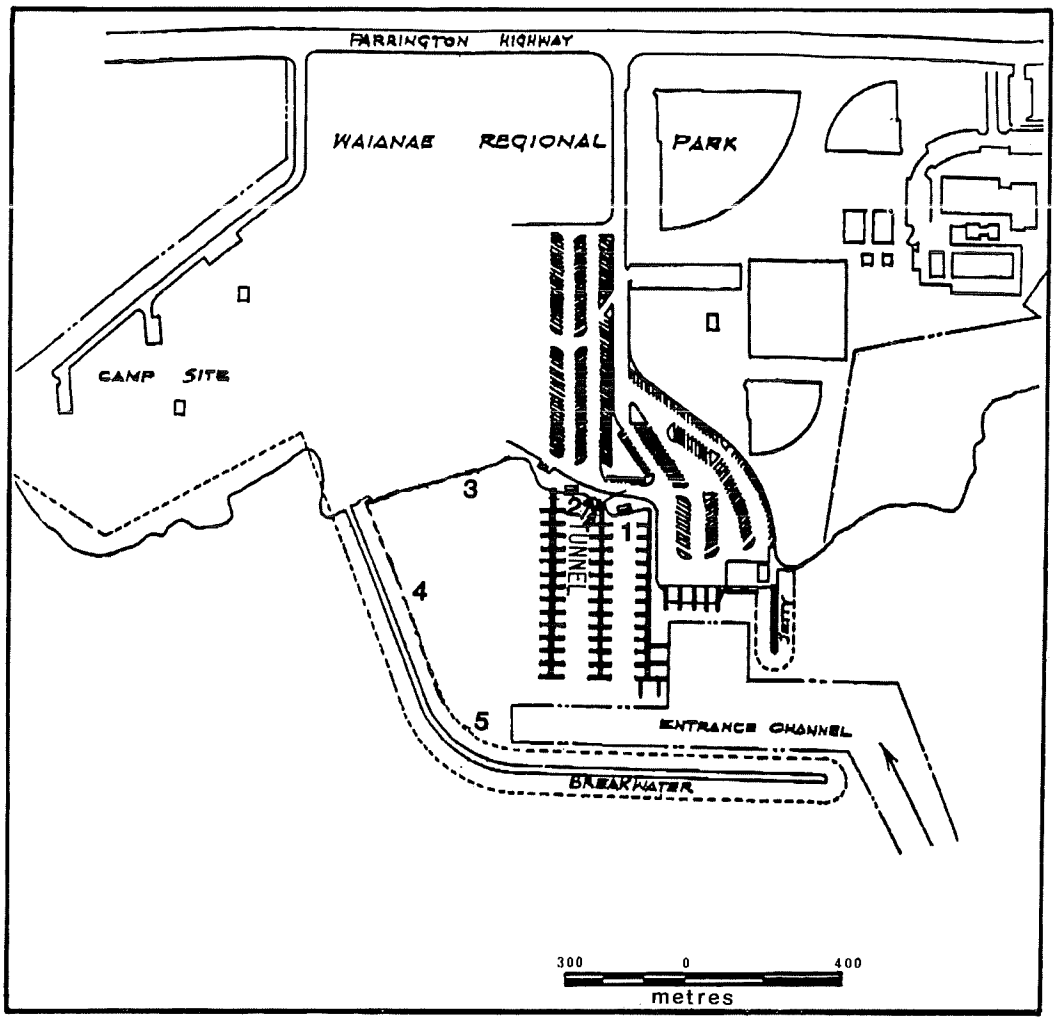

FIG. 1. Map of the survey area of Waianae Boat harbor (WBH). Tunnel is in the area of Section 2.

cate and the average number of cells determined per millilitre.

COLLECTION OF FISH

Fish samples (Table 4) were taken by divers mainly using spear-guns but also nets and lines and were identified from Tinker (1982).

FISH GUT SMEARS

An incision was made in the belly of the fish, in particular mullet (amaama), surgeonfish (kole) and Sandwich Island surgeon fish (manini) to extract gut contents which were mixed with 0.1ml of 0.85% NaCl and one drop was smeared onto a slide. The mixture was air dried and analyzed for *G. toxicus* with a microscope (Zeitz) at x400. Presence of other algal fragments were noted.

STICK ENZYME IMMUNOASSAY (S-EIA)

The S-EIA procedure comprised: a, make incision in fish tissue; b, insert skewered liquid paper-coated end of bamboo stick into flesh; c, air dry coated end of stick; d, immerse coated end of stick into absolute methyl alcohol for 0.5 sec; e, air dry; f, immerse in 1ml monoclonal anti-ciguatoxin-horse radish peroxidase (MAb-CTX-

TABLE 1. Mouse toxicity assay scoring

Toxicity	Description of Visible Clinical Symptoms in Mouse After Extract Injection
0	No ill effect
1	15-60 min: muscle contraction in lower back area (flexion), increased respiration, immobile (inactive), recovery.
2	Same as 1, but recover in 2-3 hours, pilo-erection
3	Recover in 12-24 hours: same as 2, muscle contraction, paralysis in extremities (usually hind legs), rapid and irregular breathing, immobile, closed eyes, pilo-erection, slight cyanosis (tail).
4	Symptoms as in 3, but death within 24*-48 hrs.
5	Symptoms of 3 and 4, death in less than 6 hrs.

*1 mouse unit, death within 24hrs of 20gm mouse; contains 7-9ng ciguatoxin in the sample of 100mg of crude extract/mouse injected (IP) (Hokama, personal estimation from Department of Health confirmed ciguateric fish extracts).

HRP); g, wash end of coated stick twice in buffered saline; h, immerse stick in horse-radish peroxidase (HRP) substrate; i, score color intensity of substrate as previously reported (Hokama,1988; Hokama et al.,1989). Final color: 0−1.2 is scored as negative, 1.3−1.9 borderline and 2.0 as positive. All fish scoring 1.3 are reported as rejections (not edible). S-EIA values of 1.3 generally represent 0.4ng ciguatoxin or related polyether per gram of fish tissue.

MOUSE BIOASSAY (Kimura et al.,1982)

Swiss Webster mice weighing 20−25g were utilized in this study to assess the toxicity of fish extracts. 100mg of crude fish extract was resuspended in 1ml of 1% Tween 60 in saline and injected intraperitoneally (IP) into mice. Symptoms displayed by the mouse were observed at 30min, 1, 2, 4, 6, 8, 24 and 48hours after injection and rated on a scale of 0−5 according to toxicity (Table 1). One mouse unit equals 7−9ng of ciguatoxin per 100mg of crude extract which kills a mouse within 24hours. This is based on our estimation of ciguatoxin from known cases of ciguateric fishes obtained from the Department of Health.

GUINEA PIG ATRIAL ASSAY (Miyahara et al., 1989)

100mg of each fish extract was resuspended in 1ml of Krebs-carbonate solution. 100μl of the suspension was tested on the guinea pig atria. Subsequent inotropic and chronotropic actions were noted in addition to its pharmacological response to TTX (tetrodotoxin), verapamil and the adrenergic blockers (propranolol and phentolamine). The inhibitors, TTX, verapamil and adrenergic blockers, were given after the in-

TABLE 2.WBH Water Quality Physical Analysis and *G. toxicus* in 1992 (Bloom Year's Analysis).

		Sections*				
		1	2	3	4	5
Temp (°C)	mean ± S.D.	26.3 ± 0.85	26.2 ± 0.8	26.3 ± 0.8	26.2 ± 0.7	26.1 ± 0.7
	range	25.1-27.2	25.1-27.2	24.9-27.2	25.1-27.0	25.1-26.9
pH	mean ± S.D.	8.1 ± 0.2	8.1 ± 0.2	8.1± 0.3	8.1 ± 0.2	8.1 ± 0.2
	range	8.0-8.6	8.0-8.6	7.9-8.6	7.9-8.6	7.9-8.4
DO (mg/l)	mean ± S.D.	6.2±1.3	6.1±1.7	6.3±1.6	5.9±1.3	5.9æ0.8
	range	5.2-8.6	4.8-7.5	4.1-9.4	4.3-8.6	5.0-7.1
Salinity (%)	mean ± S.D.	3.5±0.4	3.5æ0.3	3.5±0.4	3.5±0.3	3.5æ0.3
	range	3.5-3.6	3.4-3.5	3.4-3.5	3.5	3.4-3.5
Conductance	mean ± S.D.	53.5±0.5	53.5±0.4	53.4±0.5	53.6æ0.3	53.6±0.4
	range	52.7-54.1	52.5-54.0	52.4-54.0	53.1-54.0	52.9-54.2
G. toxicus (cells/gm weight of alga)	mean ± S.D.	23.4±26.4	10±10.9	18.3æ37.0	15.5±27.7	45.4±87.7
	range	0.9-65.6	0-26.5	0-120.3	1.3-90.7	0-268.9

*1.No relationship of physical properties of seawater to G. toxicus growth.
2.All values are means± S.D. for 9 months evaluation of each section (1 to 5).
3.The salinity in % of WBH generally below 4.0% maximum of seawater is due to the underground fresh water along the Leeward coast of Oahu.

otropic responses at 12.5µl of a 10^{-3}M concentration.

WATER QUALITY

Throughout Sections 1-5 (Fig.1), the salinity, temperature, pH, dissolved oxygen and conductance were measured utilizing a Surveyor 2 (Hydrolab Corp., Austin, Texas) (Table 2).

RESULTS

G. TOXICUS – DATA FOR 1992

Bryopsis growth was most prominent in Sections 3, 4 and 5 throughout the years. *Bryopsis* was generally sparse at Sections 1 and 2, between which the rainfall and wash water outlet is present. If non seawater enters, it is expected to lower the salinity and may interfere with algal growth. However, the lowering of salinity has not been observed in Sections 1 or 2 (Table 2). *G. toxicus* cell counts were best in all Sections on June 25,1992, especially Sections 3 and 5. October 29,1992 also showed moderate to high counts of *G. toxicus* in Sections 4 and 5.

It is suggested that constant presence of *Bryopsis* and *G. toxicus* helps to maintain the high toxicity level of herbivore fishes in the WBH (Table 4).

GUT SMEAR ANALYSIS

Gut smear analysis by microscope at x400 magnification revealed on several occasions, *G. toxicus* in smears of *Mugil cephalus* (mullet). This involved 3 samples of fish gut.

TABLE 3.Number of *Gambierdiscus toxicus*/gm of Alga in Waianae Boat Harbor at Various Time Periods and Stations

	Station Number*				
Dates (1992)	1	2	3	4	5
Mar. 27	1.4	2.1	0.0	3.2	4.0
Apr. 28	6.6	27.0	9.8	4.0	27.0
May 29	0.9	0.0	0.0	1.3	0.8
Jun. 25	15.0	20.0**	120.0	10.0	269.0
Jul. 24	NA	NA	3.2	2.5	8.0
Aug. 28	9.0	NA	2.7	7.0	16.0
Sep. 18	56.0	NA	17.0	30.0	0.0
Oct. 29	NA	NA	17.0	91.0	129.0
Nov. 25	NA	8.5	3.0	2.2	1.1

*NA, no *Bryopsis* available
**20 cells/gm *Ulva*, not *Bryopsis*

TABLE 4.Stick Enzyme Immunoassay (S-EIA) Evaluation of the Fish in Waianae Boat Harbor for Ciguatera (1992)

	No. of Fish	>2 Positive	1.3–1.9 Border line	<1.2 Negative
Ctenochaetus hawaiiensis (Hawaiian kole)	18	4	12	2
		(22.2)	(66.7)	(11.0)
Acanthurus sandvicensis (Manini)	110	53	33	24
		(48.1)	(30.0)	(21.8)
Mugil cephalus (amaama, mullet)	100	27	42	31
		(27.0)	(42.0)	(31.0)
Kuhlia sandvicensis (aholehole)	51	18	18	15
		(35.3)	(35.3)	(29.4)
Savotherodon sp. (Tilapia)	10	1	8	1
		(10.0)	(80.0)	(10.0)
Acanthurus dussumieri (palani)	8	0	5	3
		(0.0)	(62.5)	(37.5)
Abudefduf abdominalis (mamo)	7	0	7	0
		(0)	(100)	(0)
Trachiurops crumenophthalmus (akule, halalu)	6	0	1	5
		(0)	(16.7)	(83.3)
Caranx sp. (papio)	5	5	0	0
		(100)	(0)	(0)
Mulloidichthys auriflamma (weke)	4	1	1	2
		(25)	(50)	(25)
TOTALS	319	109*	127*	83
		(34.2)	(39.8)	(26.0)

*positive plus borderline represent 75% of fish rejected (non-edible)

STICK ENZYME IMMUNOASSAY OF FISH

There is a high rejection rate of *Mugil cephalus* (Table 4) which is the species that caused the outbreak of ciguatera poisoning in the early months of 1992. Halalu (*Trachiurops crumenophthalmus*) was mostly negative (Table 4), and has never been implicated in ciguatera poisoning. This species has been continuously caught and eaten throughout 1991 and 1992 with WBH with no known incidence of ciguatera poisoning. Aholehole has shown a large number in the rejection category (Table 4), but no known toxicity has been reported. All the other species are not evaluated individually because of the numbers. Evaluation of all fish (67 in 1991) suggests a high prevalence of fish in the rejection category (borderline plus positive = 77.6%). This may be due to the continuous presence of *G. toxicus* in the

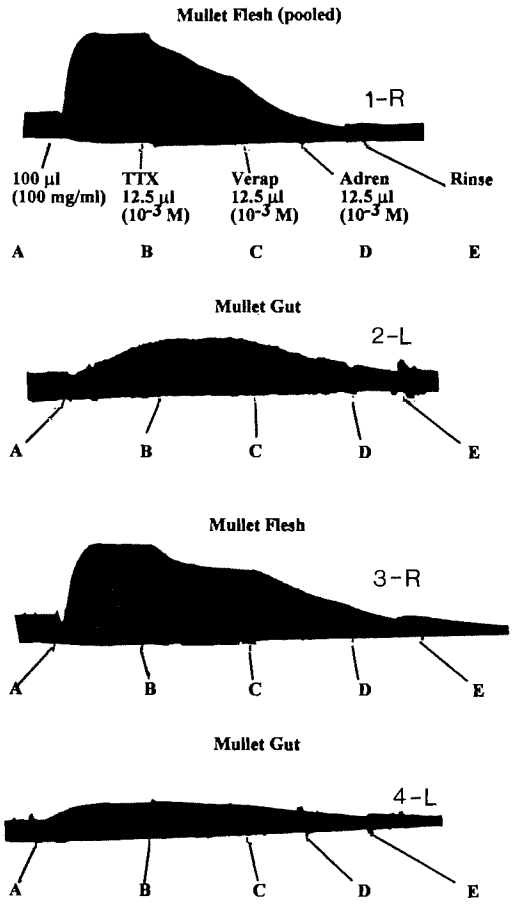

FIG. 2. Guinea pig atrium assay with two mullet flesh and gut samples from WBH, 1992. 1-R; A, S-EIA positive pooled mullet flesh extract; B, addition of TTX; C, addition of verapamil; D, addition of adrenergic inhibitors; and E, rinse of physiobath with medium. 2-L; A, S-EIA positive pooled mullet gut extract; B, TTX; C, verapamil; D, adrenergic inhibitors; and E, rinse. 3-R; A, S-EIA positive pooled mullet flesh extract; B, TTX; C, verapamil; D, adrenergic inhibitors; and E, rinse. 4-L; A, S-EIA positive pooled mullet gut extract; B, TTX; C, verapamil; D, adrenergic inhibitors; and E, rinse. R=right atrium; L=left atrium.

enclosed WBH, which has only one outlet to the ocean. The non-toxicity of halalu suggest the significance of the food consumption habit and source of this species is different from the herbivores feeding on algae (*Bryopsis*).

S-EIA data for 1992 (Table 4) show rejection rates in descending order: mamo (100%), *Caranx* sp. (100%), tilapia (90%), Hawaiian kole (87.9%), manini (78.2%), aholehole (70.6%), mullet (69%) and palani (62%). The samples of mamo, weke and *Caranx* sp. are too small to evaluate, though the high value of toxicity in *Caranx* sp. may be significant.

Mullet flesh and gut were all of high toxicity in all samples collected, except mullet flesh from 4/92. Surprisingly, extracts from negative mullets from 6/92 and 9/92 were highly toxic to mice. Similarly, Hawaiian koles were all highly toxic with mouse assay values of 5+. Manini flesh and gut extract showed variable results, although the majority of samples were highly toxic in mouse (5+). The 5/92 samples of *Caranx* gave a high toxicity value in the gut (5+) and a low toxicity value (2+) in the flesh. This is generally the pattern when examining flesh and gut separately in carnivores. Palani appeared to be of high toxicity in both flesh and gut extracts for samples on 3/92 and 6/92. Mamo and aholehole extracts gave comparable results with the flesh extract showing low toxicity (2+) and the gut extracts high toxicity (5+). Of interest is the high toxicity level of whole tilapia extracts obtained from mostly borderline tested fish in the S-EIA. Whether this is truly ciguatoxin (congeners) or other polyethers remains to be determined for the tilapia. The highly toxic gut extracts were essentially non-ciguatoxin-like in the mouse assay.

GUINEA PIG ATRIAL ASSAY

The results of the mullet tissue analysis (Table 5, Figs 2,3) suggest ciguatoxins as noted by the consistent inhibition of the inotropic effect by tetrodotoxin (TTX). See also the inotropic patterns presented for the mullet tissue extracts (Fig.2). In some cases, verapamil (verap.) also shows the inhibition of the Ca^{++} ion suggesting a maitotoxin-like toxin. The Hawaiian kole extracts showed variable inotropic response unlike the mullet, but with slight inhibition by TTX and verap. The manini flesh and gut extracts gave moderate inotropic responses with inhibition by both TTX and verap. This suggests possible ciguatoxin and maitotoxin-like toxins in these extracts. Though not shown here, a new toxin with sodium channel inhibition was observed in the guinea pig atrium assay. This toxin(s) was unlike TTX or PSP in solubility.

DISCUSSION

The Waianae Boat Harbor appears to be an excellent model for the solution of possible en-

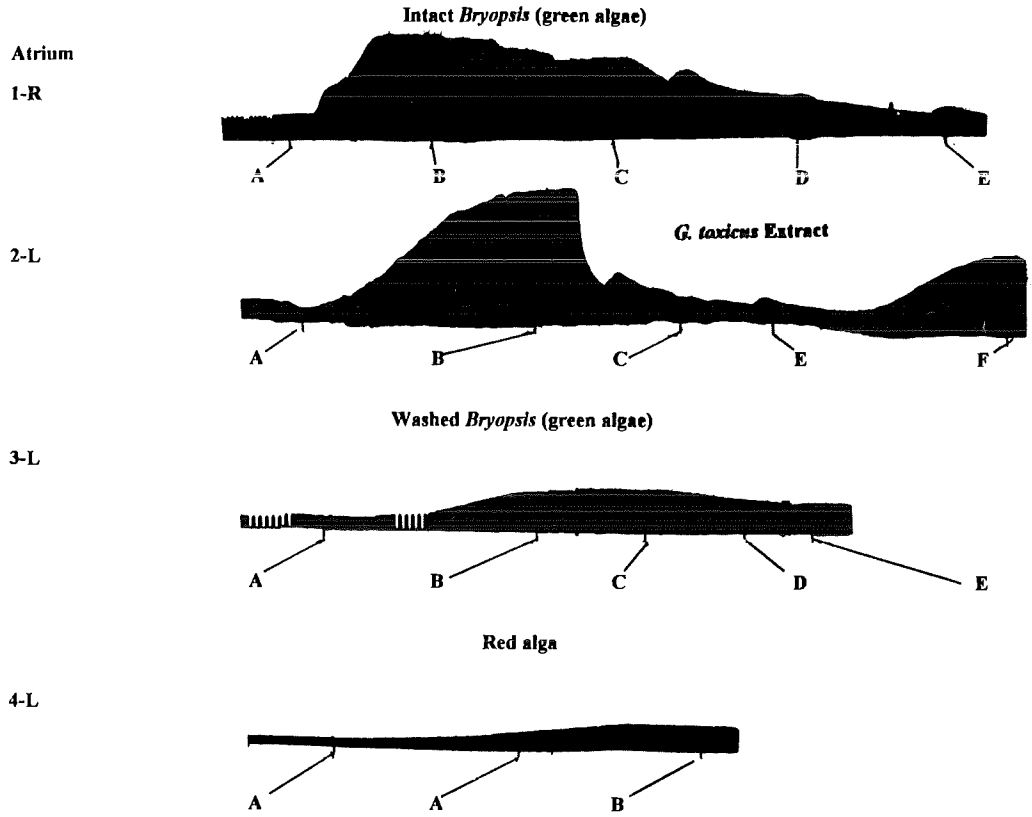

FIG. 3. Ciguatoxin in *Gambierdiscus toxicus* ciguatoxin-like toxins or no ciguatoxin in green and red algae. 1-R; A, 100µl of a 100mg/ml solution of intact *Bryopsis* acetone extract; B, addition of TTX (12.0µl of a 10^{-3} M solution); C, addition of verapamil (12.0µl of a 10^{-3} M solution); D, addition of a 12.0µl of a 10^{-3} M solution each of phentolamine and propranolol; and E, rinse with medium. 2-L; A, 100µl of a 100mg/ml solution of *G. toxicus* extract of a 22mg/ml solution; B, TTX added; C, verapamil added; E, rinse; F, return of inotropic response initiated by A. This indicates the CTX in *G. toxicus* extract remains tightly bound to atrium and the inhibitor (TTX) is removed by rinse. 3-L; A, 100µl of washed *Bryopsis* added (concentration 79 mg/ml, B, TTX added; C, verapamil; D, adrenergic inhibitors; and E, rinse. Concentrations added same as in 2-L. 4-L; A, 100µl added (50mg/ml solution); A, second addition of A; TTX added, same concentration as 1, 2, and 3. R=right atrium. L=left atrium.

vironmental control of *G. toxicus* and hence possible control of the fish poisoning problem associated with ciguatoxin and other polyethers: for example, understanding the growth stimulant, displacement of algae favourable to *G. toxicus* growth with algae inhibitory to *G. toxicus*, and control of the growth factors coming from the inlet tunnel into the harbor. In Waianae Boat Harbor, this means 1, diverting the tunnel outside the harbor to prevent rainfall bringing soil extract; 2, determination of the life cycle of *G. toxicus*; 3, inhibiting by appropriate compounds the weak link of the *G. toxicus* life cycle and; 4, assessing the toxic fish level and variability in toxicity. But thus far, the data reveal no biological cyclic effect. This probably results in the continuous presence of *G. toxicus* throughout the year. The food chain concept has been verified in this study: *G. toxicus* growth near *Bryopsis* is eaten or taken in by mullet, kole, manini, etc., then travels up to the carnivores such as ulua and kahala and finally to man. In the case of WBH, the major ciguatera causative fish was mullet. A few mullet revealed *G. toxicus* in the gut smear

Table 5.Results of Crude Fish Extracts in the Guinea Pig Atrium and Mouse Toxicity Assays

Date	Fish	S-EIA pre-extract results	Guinea Pig Assay**					Mouse Toxicity
			Atria	Inotropic	TTX	Verap	Adren	
4/92	Mullet flesh	B & P	L	+	Sl	Sl	-	5
6/92	Mullet flesh	ND	R	+	+	+	-	5
6/92	Mullet gut	B&P	R	Sl	Vsl	Vsl	-	5
4/92	Mullet flesh	Neg	L	-	-	-	-	0
6/92	Mullet flesh	Neg	R	-	+	-	ND	5
6/92	Mullet gut	Neg	R	+	+	Sl	-	5
3/92	Mullet flesh	Pooled all fish	R	+	+	+	-	5
5/92	Mullet flesh	Pooled all fish	L	+	Sl	-	-	
3/92	Mullet gut	Pooled	L	+	+		ND	5
4/92	Mullet gut	Pooled	L	+	-	Sl	-	5
5/92	Mullet gut	Pooled	R	+	+		-	5
4/92	Hawn kole flesh	B	L	Vsl	-	Vsl	-	5
4/92	Hawn kole gut	B	R	Sl	-	Sl	-	5
5/92	Hawn kole flesh	Pooled	R	+	-	Sl	-	5
5/92	Hawn kole gut	Pooled	L	Sl	-	Sl	-	
4/92	Manini flesh	B&P	R	+	-		-	0
5/92	Manini flesh	B&P	L	+	Sl	Sl	-	5
6/92	Manini flesh	B&P	L	Sl	Vsl	Vsl	-	4
6/92	Manini gut	B&P	L	Vsl	Vsl		-	1
4/92	Manini flesh	Neg	L	-	-		-	2
5/92	Manini flesh	Neg	L	+	Sl	Sl	-	5
6/92	Manini flesh	Neg	L	+	Sl	Sl	-	5
6/92	Manini gut	Neg	L	+	Sl		-	5
	Manini gut	Pooled					-	
4/92	Manini gut	Pooled	L	+	Sl		-	5
5/92	Manini gut	Pooled	R	-	-		-	5
5/92	Papio flesh	Pooled	R	-	-		-	2
5/92	Papio gut	Pooled	L	Vsl	-		-	5

*S-EIA Results: B, borderline; P, positives; ND, no data; Neg, negative; Pooled, pooled extracts of the same species (negative, borderline and positive S-EIA results)

**Atria: L, left; R, right; TTX, tetrodotoxin; Verap, verapamil, Adren, adrenergic; Sl, slight; Vsl, very slight; +, positive; -, negative (no response)

analysis. It is also suggested that until the *G. toxicus* is diminished, herbivores and carnivores in WBH should not be consumed. The *only* safe fish have been the akule and halalu and they are being caught and eaten by recreational fishermen without ciguatera outbreaks. These findings are essentially similar to those reported for Puako, Hawaii (Hokama et al.,1993), except for differences in the presences of algae species associated with *G. toxicus*. Since the major outbreak in 1991, no other reports of toxicity due to WBH fish have been observed to date. This is to be expected, since the species of potentially toxic fish in WBH have been continuously monitored and reported to the public by the Department of Health. These species include the mullet and all herbivores and carnivores within WBH. These reports of warning also include the presences of *G. toxicus* among *Bryopsis*.

The guinea pig atrial analysis revealed at least 2 toxins in the fish obtained from WBH. These included a ciguatoxin-like (inotropic response inhibited by TTX) and a maitotoxin-like (inotropic response inhibited by verapamil). The *G. toxicus* extract showed that it contained mostly ciguatoxin-like toxin. In addition, a new sodium channel inhibitory toxin(s) has been noted and is being studied.

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THE ORIGIN OF CIGUATERA

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Holmes, M.J. & Lewis, R.J. 1994 08 01: The origin of Ciguatera. *Memoirs of the Queensland Museum* **34**(3), 497–504. Brisbane. ISSN 0079-8835.

Ciguatera is caused by eating fish contaminated with ciguatoxins. Ciguatoxins-1, -2 and -3 are the major ciguatoxins found in the flesh and liver of ciguateric fishes with ciguatoxin-1 the most toxic and most abundant. Gambiertoxin-4b is the likely precursor of ciguatoxin-3 which is in turn oxidatively metabolised in fishes to ciguatoxin-1. Consequently, gambiertoxin-4b accounts for more than 90% of the toxicity of ciguateric fishes. Gambiertoxin-4b has been extracted from biodetritus containing large numbers of the benthic dinoflagellate *Gambierdiscus toxicus*, indicating that *G. toxicus* is the primary source of toxins involved in ciguatera. Putative gambiertoxins have also been detected in certain strains of cultured *G. toxicus*. However, the link between *G. toxicus* and ciguatera remains circumstantial since gambiertoxin-4b has not yet been unambiguously identified from cultures of this dinoflagellate.

Michael J. Holmes & Richard J. Lewis, Southern Fisheries Centre, Queensland Department of Primary Industries, PO Box 76 Deception Bay, Queensland 4508: 22 November, 1993.

Ciguateric fishes are poisonous because their flesh and viscera contain elevated concentrations of lipid-soluble polyether ciguatoxins (Murata et al.,1990; Lewis et al.,1991; Lewis & Sellin, 1992). Ciguatoxins-1, -2 and -3 (Fig.1) have been isolated fromthe flesh of toxic carnivorous fish with ciguatoxin-1 being most abundant and most toxic (Lewis & Sellin,1992). Some minor toxins (presumably ciguatoxins) remain to be characterised from carnivorous and herbivorous fishes (Murata et al.,1990; Lewis et al.,1991; Lewis & Sellin,1992; Legrand et al., 1992). Ciguatoxins-2 and -3 do not have the secondary hydroxyl on carbon 54 and are therefore less-polar than ciguatoxin-1 (Lewis et al., 1991). Ciguatoxin-3 is thought to be an intermediate in the oxidative metabolism of a less-polar precursor, gambiertoxin-4b, to cigua- toxin-1 (Lewis et al., 1991) whereas ciguatoxin-2 is a diastereomer of ciguatoxin-3 (Lewis et al., 1993) which may originate from a different precursor. The stereochemistry at carbon 52 indicates that ciguatoxin-2 has a different structural backbone to ciguatoxins-1 and -3 (Lewis et al., 1993). Ciguatoxins-2 and -3 induce similar bioassay signs in mice, including hind limb paralysis, the only bioassay sign that differentiates these less-polar ciguatoxins from ciguatoxin-1 (Lewis et al.,1991). Precursors of ciguatoxins-2 and -3 are thought to enter the marine food web incidentally upon ingestion by lower trophic level fishes (e.g. herbivores/ detritivores like surgeonfishes (Randall,1958; Lewis et al.,1991)) or invertebrates (Kelly et al.,1992; Lewis et al.,1994). These species are in turn preyed on by carnivorous fishes.

Randall's (1958) hypothesis that the ciguatera toxins originate from a small benthic organism received strong but circumstantial support when Yasumoto et al. (1977a,b) extracted ciguatoxin-like and maitotoxin-like toxins from a benthic detrital sample containing large numbers of the dinoflagellate *Gambierdiscus toxicus* Adachi & Fukuyo. Yasumoto et al. (1979a) and Bagnis et al. (1980) were able to repeat the extraction of such toxins from biodetritus from the Gambier Islands and to extract maitotoxin from cultures of *G. toxicus*. However, the column and thin-layer chromatography of the ciguatoxin-like toxin found by Yasumoto's group was not consistent with the major ciguatoxin (ciguatoxin-1) found in fishes but instead was indicative of a less-polar ciguatoxin-like toxin (Lewis,1985). The role of *G. toxicus* in ciguatera remained in doubt since meagre amounts of this ciguatoxin-like toxin, if any, were produced by cultured *G. toxicus* (Yasumoto et al.,1979a; Bagnis et al.,1985a; Holmes et al.,1990; Murata et al.,1990; Yasumoto, 1990) and this toxin could not be extracted from wild *G. toxicus* outside of French Polynesia (Gillespie et al.,1985a). It was not until gambiertoxin-4b (precursor of ciguatoxins-1 and -3) was extracted from biodetritus samples containing large numbers of wild *G. toxicus* (collected in 1979 from the Gambier Islands and kept at -20° for many years) and its structure compared with that of ciguatoxin-1, that *G. toxicus* was once again considered the likely origin of ciguatera (Murata et al.,1990; Legrand et al.,1990,1992). Nine gambiertoxins have since been extracted from wild *G. toxicus*, including gambiertoxin-4c

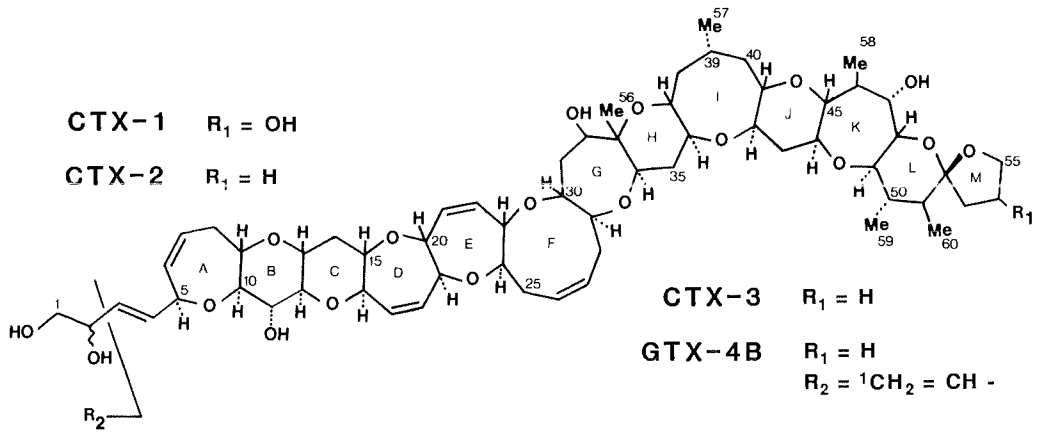

FIG. 1. Structures of ciguatoxins (CTX)-1, -2 and -3 and gambiertoxin-4b (GT-4b) (Murata et al.,1990; Lewis et al.,1991). Ciguatoxin-2 is a diastereomer of ciguatoxin-3 (Lewis et al.,1993).

(the major toxin in terms of mouse lethality) and the isomers, gambiertoxin-4a and -4b (Legrand et al.,1992). Of these, only the structure of gambiertoxin-4b is known (Murata et al.,1990). However, gambiertoxin-4b appears to be the most important gambiertoxin contributing to ciguatera as its oxidative products, ciguatoxin-1 and -3, account for more than 90% of the toxicity of ciguateric fish (Lewis et al.,1991; Lewis & Sellin,1992). The site of biotransformation of gambiertoxin-4b to ciguatoxin-3 and of ciguatoxin-3 to ciguatoxin-1 remains to be established, but likely occurs in the liver of fishes (Lewis et al.,1991).

Extraction of gambiertoxins from reef biodetritus containing wild *G. toxicus* is circumstantial evidence that *G. toxicus* is the origin of the toxins that cause ciguatera. Further support for this hypothesis was obtained by Holmes et al. (1991) and Holmes & Lewis (1992) who found two putative gambiertoxins (called major and minor based upon their relative contribution to total lethality) in cultures of certain strains of *G. toxicus*. These toxins were less-polar than ciguatoxins-1, -2 or -3 but were considered closely related to the ciguatoxins since: (i) both gambiertoxins produced bioassay signs in mice similar to those produced by ciguatoxins-2 and -3, (ii) the major (more-polar) toxin was shown to be a Na^+ channel activator toxin (as are the three ciguatoxins) that produced pharmacological responses in isolated tissues similar to those produced by the ciguatoxins (especially ciguatoxin-3; Lewis & Wong Hoy,1993) and (iii) the major toxin competitively inhibited the binding of [^{3}H]brevetoxin-3 to rat brain synaptosomes. The ciguatoxins (and brevetoxins) are the only toxins known to competitively inhibit brevetoxin binding to the Na^+ channel (Lombet et al.,1987; Lewis et al.,1991). However, gambiertoxin-4b has not been unambiguously identified from cultured *G. toxicus* and therefore the origin of the precursor of ciguatoxins-1 and -3 remains to be established. Many reports have claimed to extract ciguatoxin or ciguatoxin-like toxins from cultures of *G. toxicus* (Bergmann & Alam,1981; Withers,1982; Shimizu et al.,1982; Miller et al.,1984; Lechat et al.,1985; Durand et al., 1985; Durand-Clement,1987) but these investigations relied upon a liquid-liquid partition (eg. diethyl ether-water) to completely separate ciguatoxin-like toxins from the considerable amounts of maitotoxin present in crude extracts. Since maitotoxin can partition into both the lipid- and water-soluble phases (Yasumoto et al., 1979a; Holmes et al.,1990) these former studies are unlikely to have completely separated any ciguatoxin-like material from the maitotoxin.

Production of gambiertoxins in cultured *G. toxicus* appears to be strain-dependent, with most clones only producing maitotoxins (Holmes et al.,1991). These authors found that only two of 13 cultured strains produced putative precursors of the ciguatoxins, indicating that the aetiology of ciguatera is likely restricted to genetic strains of *G. toxicus* which can produce gambiertoxins. The most striking evidence for this genetic variability was that putative gambiertoxins were produced by only one of four *G. toxicus* clones

isolated from the same site, with only one of two clones isolated from the same site and at the same time producing gambiertoxins (Holmes et al.,1991). This result has important implications for ecological studies of *G. toxicus*, as it indicates that the size of *G. toxicus* populations does not necessarily reflect the potential for these populations to cause ciguatera. Wild populations of *G. toxicus* from the Gambier Islands, Kiribati and Platypus Bay, Queensland have been found to produce gambiertoxins (Legrand et al.,1990, 1992; Holmes et al.,1991; Holmes et al.,1994) whereas a large population of wild cells from Flinders Reef, south Queensland did not produce detectable levels of these toxins (Gillespie et al., 1985a; Lewis et al.,1988a).

Only relatively low concentrations of gambiertoxins have so far been detected from cultured compared with wild *G. toxicus* cells (Holmes et al.,1991,1994; Holmes and Lewis,1992). Considerable variation can also occur in the concentration of gambiertoxins produced by wild *G. toxicus* (Holmes et al.,1994). Holmes et al. (1994) have proposed the existence of "super-producing strains" of *G. toxicus* to explain some of this variation in gambiertoxin production between cultured and wild *G. toxicus*. However, environmental factors are also likely to affect toxin production since the concentration (or type) of gambiertoxin produced can change in culture (Holmes & Lewis,1992). Environmental conditions obviously effect the growth of *G. toxicus* as they do for any other algae, but it is not known if environmental parameters can selectively affect the growth and toxicity of super-producing strains over non-producers. It is quite likely that the conditions which enhance growth will not necessarily be the conditions which enhance toxin production. Future research could focus on the effect of different combinations of genetic and environmental parameters on the rate of gambiertoxin production.

Ecological studies need to also consider the effect of different rates of turnover of *G. toxicus* populations. A large standing crop of *G. toxicus* does not necessarily indicate a greater potential to cause ciguatera compared with a smaller population, if the lower biomass is simply a reflection of higher productivity *and* higher rates of consumption by herbivores. Recent evidence that fishes can excrete/metabolise the ciguatoxins (Tosteson et al.,1988; Lewis et al.,1992) suggests that considerably greater quantities of gambiertoxins are entering the marine food web than would otherwise be expected by the frequency of ciguatera. Seasonal patterns of ciguatera in some Pacific Island countries (Sorokin, 1975; Dawson,1977; Bagnis,1979; Bagnis et al.,1992; Lewis,1992) may also reflect seasonal patterns in the abundance and/or gambiertoxin production by wild *G. toxicus*. There are numerous reports of seasonal variation of populations of *G. toxicus* and other benthic dinoflagellates (Yasumoto et al.,1979b; Carlson & Tindall,1985; Gillespie et al.,1985b; Bagnis et al., 1985b; Ballantine et al., 1985,1988; Bomber et al., 1988; McCaffrey et al., 1992; Holmes et al.,1994). However, seasonal patterns of *G. toxicus* abundance have been correlated with fish toxicity only in French Polynesia (Bagnis et al.,1985b).

Most strains of *G. toxicus* appear not to produce gambiertoxins, while most produce a maitotoxin (Holmes et al.,1991). Maitotoxins are generally referred to as water-soluble toxins although they are soluble in a range of organic solvents and a butanol-water liquid-liquid partition will recover nearly 100% of maitotoxin in the butanol phase (unpublished result). The maitotoxins have a cyclic polyether structure as do the gambiertoxins and ciguatoxins (Yokoyama et al.,1988; Murata et al.,1991, 1992, 1993). Interestingly, the type of maitotoxin produced by *G. toxicus* is dependent upon the strain being cultured, with each strain apparently producing only the one type of maitotoxin (Holmes et al.,1990). Holmes & Lewis (in press) have recently found that large maitotoxins (maito-toxins-1 and -2, with molecular weights >3,000) were produced by strains of *G. toxicus* which do not produce gambiertoxins, whereas the small maitotoxin-3 (molecular weight 1,060 for the disodium salt) was produced by a clone which also produces gambiertoxins. The molecular weight of maitotoxin-3 is the same as gambiertoxins-4a and -4b (Murata et al.,1990; Legrand et al.,1992). Holmes & Lewis (in press) have suggested that the biosynthesis of gambiertoxins and maitotoxins may be linked in strains of *G. toxicus* which produce both of these types of toxins.

Maitotoxin has been found in the gut contents of surgeonfishes (Yasumoto et al.,1976) but there is little evidence that maitotoxin is accumulated in the flesh of these or other fishes. Water-soluble, maitotoxin-like toxins have been extracted from the flesh of fishes in Hawaii and Queensland (Iwaoka et al.,1993; Endean et al., 1993). However, these studies based the detection of these toxins, at least in part, on intraperitoneal (i.p.) injections into mice of at least 100 mg of crude extracts (≥5 g extract/kg mouse

body weight). Doses of crude fish extracts >1 g/kg i.p. can produce non-specific toxic effects (Banner et al.,1961; Lewis et al.,1988a). Unsaturated fatty acids extracted from shellfish have also been shown to produce toxic effects when injected i.p. into mice (Takagi et al.,1984). Calculations of total toxicity based upon lethal doses of such large amounts of extract would likely result in the overestimation of the quantity of toxin present. Additionally, the water-soluble extracts of fish flesh killed mice quickly (5 and 13 min, Iwaoka et al.,1993; 3–30min, Endean et al., 1993). However, based upon these rapid deaths, we conclude that any water-soluble toxins isolated were not maitotoxins, since very large doses of native maitotoxin (e.g. >100 lethal units) would be required to produce such short death times. The three maitotoxins characterised so far are potent but slow acting toxins with the shortest survival times (calculated according to Molinengo, (1979)) being greater than 41 min (Holmes et al.,1990; Holmes & Lewis in press).

Maitotoxins are the most toxic toxins produced by *G. toxicus*, often comprising more than 99% of total toxicity (Holmes & Lewis,1994). Fishes fed cultured *G. toxicus* cells display abnormal swimming behaviour (Davin et al.,1986,1988; Kelly et al.,1992) probably as the result of maitotoxin intoxication. Maitotoxin poisoning of fishes in the wild may result in these fishes being preferentially preyed upon. This could be a mechanism for concentrating gambiertoxins through the food chain of fishes when herbivorous fishes ingest strains of *G. toxicus* which produce both gambiertoxins and maitotoxins.

Toxins other than ciguatoxins-1, -2 and -3 have been suggested as causes of ciguatera. Scaritoxin, extracted from parrotfish (*Scarus gibbus*) from the Gambier Islands (Chungue et al.,1977), may be a less-polar form of ciguatoxin (Lewis et al., 1991; Legrand et al., 1992). Vernoux & Talha (1989) detected fast-acting ciguatoxins in fish flesh; instability and quick death-times induced by these toxins distinguish them from ciguatoxins-1, -2 and -3, which are stable and slow acting (Lewis et al.,1991). Other toxins suggested as causal agents of ciguatera include palytoxin, okadaic acid and other toxins (predominately water-soluble toxins) produced by the benthic dinoflagellate species *Ostreopsis* spp. and *Prorocentrum* spp., and toxins produced by the planktonic cyanophyte (cyanobacterium) *Oscillatoria* (*Tricodesmium*) *erythraea* (Yasumoto et al.,1980; Nakajima et al.,1981; Murakami et al., 1982; Tindall et al.,1984,1990; Norris et al.,1985; Holmes et al.,1988; Kodama et al., 1989; Dickey et al.,1990; Juranovic & Park,1991; Hahn & Capra,1992).

Palytoxin is a potent water-soluble toxin isolated from various *Palythoa* coral species (Moore & Scheuer,1971; Habermann,1989). Palytoxin (or one of its congeners) has been found in the flesh and viscera of triggerfish *Melichthys vidua* (Fukui et al.,1987a,b) and smoked mackerel *Decapterus macrosoma* (Kodama et al.,1989). Palytoxin is also thought to be responsible for intoxications caused by eating parrotfish liver (*Ypsiscarus ovifrons*) from western Japan (Noguchi et al.,1987). The extent of human poisoning from palytoxin is not known but we believe it is a separate poisoning distinct from ciguatera. Hospitalised cases present with signs distinguishable from ciguatera including elevated serum enzyme levels (Noguchi et al.,1987; Kodama et al., 1989). However, mild palytoxin poisoning may be mistaken for ciguatera.

Okadaic acid is a lipid-soluble polyether toxin with similar chromatography to ciguatoxins (Yasumoto et al.,1980; Murakami et al.,1982). Okadaic acid was originally isolated from the black sponge *Halichondria okadai* (Tachibana et al.,1981) but has been isolated from the benthic dinoflagellates *Prorocentrum lima* (Murakami et al.,1982) and *P. concavum* (Dickey et al.,1990) and from the temperate dinoflagellates *Dinophysis* (Lee et al.,1989). Okadaic acid is one of the toxins that can accumulate in shellfish to cause a disease known as diarrhetic shellfish poisoning (Lee et al.,1988). However, the only fishes from which okadaic acid has been extracted are barracuda from the Caribbean (Gamboa et al.,1992). This result requires confirmation, including an estimate of whether the levels detected were sufficient to cause human poisoning. The primary basis for linking okadaic acid (and the other toxins produced by *Ostreopsis* spp. and *Prorocentrum* spp.) with ciguatera is (i) the dinoflagellates that produce these toxins would likely be ingested by the same or similar herbivores that ingest *G. toxicus*, and (ii) the diverse range of symptoms of ciguatera could result from a combination of several toxins. However, there is little evidence to indicate that the toxins produced by these dinoflagellates accumulate in fish flesh to cause human poisoning. Symptoms of ciguatera could result from ingestion of different relative amounts of different ciguatoxins (Lewis & Sellin,1992) and/or different doses of

ciguatoxin (Yasumoto et al.,1984; Lewis et al., 1988b).

Evidence linking *O. erythraea* to ciguatera is similarly unconvincing. *O. erythraea* is a tropical and sub-tropical planktonic, filamentous cyanophyte common off the east and west coasts of Australia (Hallegraeff,1991). However, this species is also common in areas where ciguatera has not been reported (Lewis,1988; unpublished observations). Toxins have been extracted from Caribbean and Australian samples of *O. erythraea* (Hawser et al.,1991; Hahn & Capra, 1992) but plankton-feeding fishes, which would be the most likely to ingest this algae, apparently do not cause ciguatera (Randall,1958). Hahn & Capra (1992) showed that a toxic fraction could be briefly accumulated by filter-feeding bivalves and that this toxin may be subsequently accumulated in the viscera of the molluscivorous fish *Trachinotus blochi*. However, only one case of human poisoning by *Trachinotus* sp. has been recorded in Australia since 1965 (unpubl. data).

Ciguatera is caused by eating fish which have accumulated toxins from their diet. Nearly all benthic dinoflagellates produce toxins but not all of these toxins are accumulated to harmful levels in the flesh of fishes. Evidence to-date suggests that, of the benthic dinoflagellates, only *G. toxicus* produces toxins, especially gambiertoxin-4b, responsible for the human poisoning syndrome known as ciguatera. The ciguatoxins are the major toxins found in ciguateric fishes and must be considered the primary cause of ciguatera. Other toxins and other sources of these toxins have been suggested as being involved in ciguatera but their involvement, if any, remains to be established.

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THE ORIGIN OF CIGUATERA IN PLATYPUS BAY, AUSTRALIA

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Holmes, M.J., Lewis, R.J., Sellin, M. & Street, R. 1994 08 01: The origin of ciguatera in Platypus Bay, Australia. *Memoirs of the Queensland Museum* **34**(3), 505–512. Brisbane. ISSN 0079-8835.

Platypus Bay on the northwestern side of Fraser Island is the only site in Queensland known to frequently harbour ciguateric fishes. Platypus Bay is not typical of areas normally associated with ciguateric fishes as it contains no corals but has a sandy bottom covered with an unattached green macroalgae (*Cladophora* sp.). Benthic biodetritus samples sieved from the *Cladophora* during seven sampling trips between May 1988 and February 1990 contained *Gambierdiscus toxicus* with mean population densities of 4–556 cells per gram of *Cladophora*. Biodetritus samples from six of the trips were extracted for toxins. Putative major and minor gambiertoxins (precursors of the ciguatoxins) were detected, suggesting that these *G. toxicus* populations are the origin of the toxins in ciguateric fishes caught in Platypus Bay. However, gambiertoxins were detected from only one of the six samples. This indicates that not all strains of *G. toxicus* produce these toxins in the wild. The concentrations of major and minor gambiertoxins produced by these wild *G. toxicus* can be considerably greater than the highest levels found from cultured *G. toxicus* clones isolated from Platypus Bay. 'Super-producing' strains of *G. toxicus* are hypothesised to explain the high concentrations of these toxins.

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Ciguatera is a disease caused by eating fish contaminated with ciguatoxins which are thought to originate from less-oxidised precursors called gambiertoxins that are produced by the benthic dinoflagellate *Gambierdiscus toxicus*. Gambiertoxins are produced by only a minority of cultured *G. toxicus* strains (Holmes et al., 1991). In Australia, *G. toxicus* has been found along the east coast from Flat Rock, E of Brisbane, to Alexandra Reef N of Cairns, co-occurring with corals (Gillespie et al., 1985a; Gillespie, 1987). *G. toxicus* is the most common benthic dinoflagellate on Queensland coral reefs, except on some reef flats where *Ostreopsis* spp. dominates (Gillespie et al., 1985a). Population densities of *G. toxicus* on Queensland reefs are generally <5 cells/g of substrate. Flinders Reef in south Queensland is an exception which seasonally produces >1,800 *G. toxicus*/g of macroalgal substrate (Gillespie et al., 1985a). However, gambiertoxins and ciguatoxins have not been extracted from *G. toxicus* or fish from Flinders Reef (Gillespie et al.,1985b; Lewis et al.,1988a).

Platypus Bay, on the northwestern side of Fraser Island (Fig.1), is the only site in Queensland known to frequently harbour ciguateric fishes (Lewis & Endean,1983,1984; Gillespie et al., 1986; Lewis,1987). Mackerels (*Scomberomorus* spp.), especially narrow-barred Spanish mackerel (*S. commersoni*) caught near the mouth of Wathumba Creek, have caused most of these poisonings (Lewis & Endean,1983; Gillespie et al.,1986; Lewis,1987; Lewis et al., 1988b). Since prohibition, in 1987, on the capture of *S. commersoni* and barracuda (*Sphyraena jello* and *Agrioposphyraena* spp.) from Platypus Bay, the majority of cases of ciguatera in Queensland have been caused by demersal fish from the Great Barrier Reef (unpubl. data). However, small demersal fishes from Platypus Bay have caused ciguatera, including the blotched javelin (*Pomadasys maculatus*) (Lewis et al.,1988b) and less often, the rabbitfish *Siganus spinus* (unpubl. data). Ciguatera is typically associated with fishes of coral reefs (Randall,1958; Bagnis et al., 1989) so its occurrence in Platypus Bay where corals are absent seems unusual. The benthic biodetritus of Platypus Bay was examined for dinoflagellates and extracted for toxins. Putative gambiertoxins from a biodetrital fraction with large numbers of *G. toxicus* indicates that the origin of ciguatoxins in fishes caught in Platypus Bay is likely to be *G. toxicus* in Platypus Bay.

MATERIALS AND METHODS

BIODETRITUS FROM PLATYPUS BAY

Large areas of the bottom of Platypus Bay

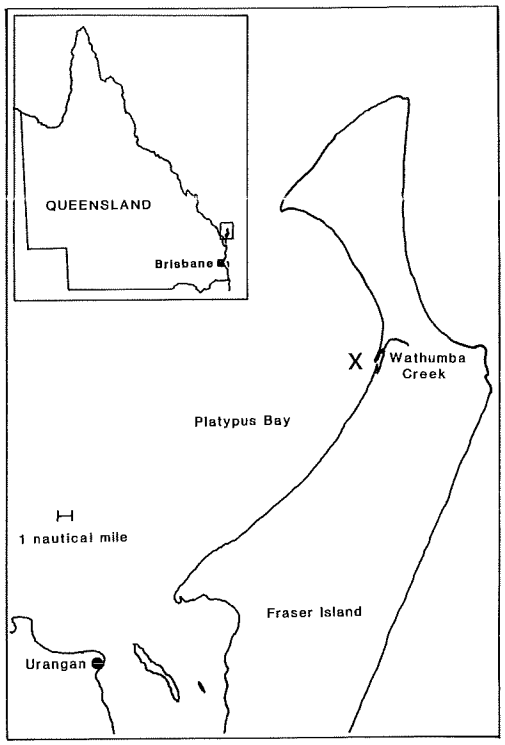

FIG. 1. Map of Platypus Bay, Fraser Island. *Cladophora* samples were collected by scuba divers from the site marked with a cross.

(Fig.1) are perennially covered with several centimetres of a non-attached green macroalgae (*Cladophora* sp.). Samples of *Cladophora* were collected by scuba divers during seven sampling trips between April 1988 and February 1990 from about 1.5 nautical miles E of the mouth of Wathumba Creek in c.15 m of water (Fig.1). Samples were collected in Bitran zip-lock plastic bags, transported to Brisbane, stored at 4°C overnight and then processed. Insufficient material was collected during the July 1988 trip for toxicity studies but this sample was examined for benthic dinoflagellates. The contents of the plastic bags from the remaining six trips were shaken and poured onto 2mm, 500μm, 250μm and 45μm diameter plankton mesh sieves. The filtrate from the 45μm sieve was vacuum filtered on Whatman Number 1 filter paper; the filtrate obtained after this was discarded. The fractions retained by the 500μm, 250μm and 45μm diameter sieves and the filter papers were extracted for toxins. A 590g sample of *Cladophora* (retained on the 2mm sieve) collected in May 1988 was also homogenised and extracted for toxins. Benthic dinoflagellates were identified with a light microscope (Fukuyo,1981). Cell numbers were calculated from counts of 1ml subsamples of sieved plastic bag samples of *Cladophora* (n=2–4 plastic bag samples) using a Sedgewick-Rafter counting chamber. Scanning electron micrographs of formalin (5%) fixed biodetrital fractions were prepared (Holmes et al., 1990). *Coolia monotis* was identified by SEM examination of cultured cells isolated from a *Cladophora* sample collected in July 1988.

Solvent Extraction of Sieved Fractions

Fractions were homogenised for 20 minutes in acetone (3-times, 3:1, v:v, acetone:sample) using a Ystral air powered homogeniser. The 45-250μm fractions were additionally homogenised with methanol (1-time, 3:1, v:v, methanol: sample). The extracts for each size fraction were pooled, vacuum filtered through Whatman GF/A glass fibre filters and dried under vacuum. The dried extracts were resuspended in 9:1 methanol-water and then separated into hexane-, diethyl ether-, butanol- and water-soluble fractions (Holmes et al.,1990). Gambiertoxins (or ciguatoxins) extracted from these samples would partition into the diethyl ether fractions, whereas maitotoxins partition into both the diethyl ether and butanol fractions (Holmes et al.,1990,1991; Holmes & Lewis,1992). Diethyl ether fractions which induced gambiertoxin-like bioassay signs in mice were further characterised after separation by silicic acid column chromatography (Holmes et al.,1990; Holmes & Lewis,1992).

Mouse Bioassay

Fractions were dried under vacuum and finally freed of solvent under a stream of N_2, resuspended in 0.5 ml of 1% or 5% Tween 60 saline and injected intraperitoneally (i.p.) into 18–21g Quackenbush strain mice (either sex) at a maximum dose of 1g of dried fraction weight per kg mouse body weight. One mouse was injected per dose, with 2 or 3 mice injected per fraction. Where appropriate, 10% and 90% of fractions were injected (for example, where 10% was not lethal and 90% was ≤ maximum injectable dose). Mice were observed intermittently over 24 hours and signs and death-times recorded. Fractions where considered non-toxic if injection of a maximal dose was not lethal. Lethal fractions were characterised on the basis of the signs and death-times displayed by mice compared with the responses induced by authentic gambiertoxins,

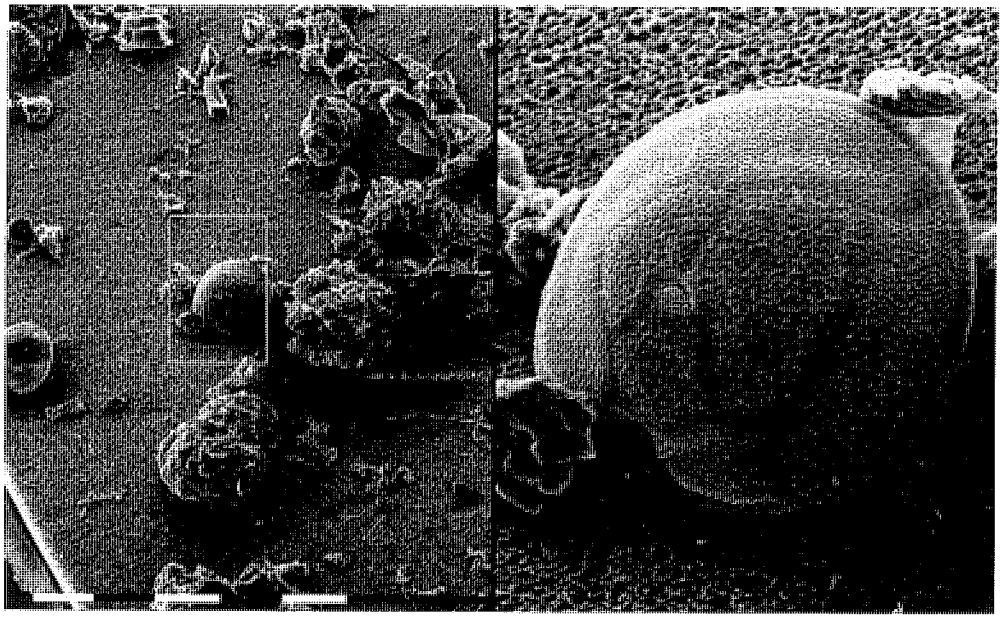

FIG. 2. Scanning electron micrograph of sieved Platypus Bay biodetritus (45-250μm) containing *Gambierdiscus toxicus*. A *G. toxicus* is shown enclosed in the box. Scale bar = 50μm.

ciguatoxins and maitotoxins. Gambiertoxins were quantified using the dose *vs* death-time equation for the major gambiertoxin; log (dose)= 3.2 log $(1+t^{-1})$, where dose is in mouse units (MU) and t = death-time in hours (Holmes & Lewis, 1992). One MU is defined as an i.p. LD_{50} dose.

Gambiertoxins, ciguatoxins and maitotoxins are all slow acting (Holmes et al.,1990; Lewis et al.,1991; Holmes & Lewis,1992). For each, i.p. injection of a dose one-tenth of that which would kill a mouse in 30 minutes, would also be lethal. Fast-acting toxins were therefore defined as fractions which killed mice in 30 minutes (or less) but which were not lethal at one-tenth of this dose. The limit of detection of gambiertoxins from non-toxic fractions was calculated from the maximum amount of material that could be injected into mice which was non-toxic, assuming 0.5 MU of gambiertoxin can be detected from the bioassay signs displayed by a mouse.

RESULTS

Platypus Bay biodetritus contains (Fig.2) cells of *G. toxicus* as described by Fukuyo (1981). *G. toxicus* was found in sieved fractions collected from all seven sampling trips with mean population densities of 4–556 cells/g of *Cladophora* (Fig.3). There was considerable variation in the size of the *G. toxicus* populations, with the May 1988 sample having the highest cell densities and May 1989 the second lowest. There was no obvious relationship between *G. toxicus* population

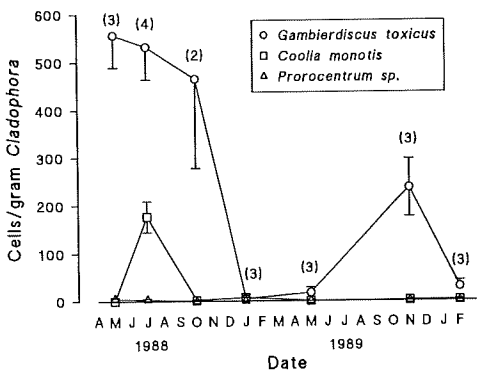

FIG. 3. Population densities of *G. toxicus*, *Coolia monotis* and *Prorocentrum* sp. on *Cladophora* from Platypus Bay. Samples collected May 1988 to February 1990. Shown are means ±1 standard error, 2 to 4 replicate samples as indicated by numbers in parenthesis. Sieved fractions were tested for toxicity for all samples except the July 1988 sample.

of producing considerably greater amounts of gambiertoxins than the mean cell toxicity (10^{-5} MU/cell) of the toxic wild sample found in this study, given that the wild sample would likely contain a mixture of high and low gambiertoxin-producing strains. The high gambiertoxin-producing strains of *G. toxicus* (here after referred to as 'super-producing' strains) may increase the potential for ciguatera. Production of gambiertoxins in these super-producers may be partly controlled by environmental parameters. Holmes & Lewis (1992) showed that the concentrations (or type) of gambiertoxins produced by cultured clones of *G. toxicus* can change during the time they are maintained in culture. Therefore, the size of *G. toxicus* populations does not necessarily reflect their potential to cause ciguatera. The absence of ciguatera at Flinders Reef in southern Queensland, which seasonally harbours more than 1,800 *G. toxicus*/g of macroalgal substrate (Gillespie et al.,1985a,b; Lewis et al.,1988a) could therefore be explained by an absence of gambiertoxin (or super-gambiertoxin) producing strains of *G. toxicus* .

A number of *Prorocentrum* species produce toxins (Yasumoto et al.,1980; Nakajima et al., 1981; Dickey *et al.*,1990) while *C. monotis* is thought to be non-toxic (Yasumoto et al.,1980; Nakajima et al.,1981; Tindall et al.,1984). *Prorocentrum* and *C. monotis* are unlikely to have contributed significantly to the toxicity of Platypus Bay biodetrital fractions tested because of the small numbers of cells (<9cells/g *Cladophora*) present with *G. toxicus*. Additionally, there is no evidence that toxins produced by these species accumulate in fish to cause human illness. Approximately 30% of biodetrital fractions were lethal to mice and about 60% of these fractions induced maitotoxin-like signs, including all butanol-soluble extracts of the 45–250μm fractions. All of the 45–250μm fractions contained *G. toxicus* and therefore butanol-soluble extracts of these fractions would likely contain maitotoxins. Toxins which induce maitotoxin-like signs in mice have been extracted from the viscera of fishes (Yasumoto et al.,1976; Lewis et al.,1988a; Lewis et al.,1991), but there is no evidence for the bioaccumulation of maitotoxin or maitotoxin-like toxins in the flesh of Queensland fishes causing human illness. Mouse bioassay signs alone are unlikely to be diagnostic for maitotoxins as the benthic dinoflagellate *Ostreopsis siamensis* also produces a toxin (more-polar than maitotoxin) that induces similar signs in mice (Holmes et al.,1988). Fast-acting ciguatoxins have been reported from the flesh of ciguateric fishes from the Caribbean (Vernoux & Talha,1989) but authentic ciguatoxins, gambiertoxins and maitotoxins from Pacific Ocean sources have all proved to be slow-acting toxins in mice (Holmes et al.,1990; Holmes & Lewis,1992; Lewis et al.,1991). The dose vs death-time relationship in mice is therefore a useful method for differentiating toxins that produce otherwise similar bioassay signs in mice.

The *G. toxicus* populations in Platypus Bay are the second highest reported from Queensland (Gillespie et al.,1985a). However, these populations are smaller than the 4.5 x 10^5cells/g of macroalgae reported from the Gambier Islands (Bagnis et al.,1985). Bagnis et al. (1990) found 100-fold increases in *G. toxicus* populations can occur in less than two weeks. The environmental parameters which control the size of *G. toxicus* populations and the proportion of these cells which produce gambiertoxins (and possibly super-producers) need to be determined before the potential risk of ciguatera can be predicted from *G. toxicus* populations.

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SURVEY FOR CIGUATERA FISH POISONING IN WEST HAWAII

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Ichinotsubo, D., Asahina, A.Y., Titus, E., Chun, S., Hong, T.W.P., Shirai, J.L. & Hokama, Y. 1994 08 01: Survey for ciguatera fish poisoning in west Hawaii. *Memoirs of the Queensland Museum* **34**(3): 513–522. Brisbane. ISSN 0079-8835.

Approximately 25–30 fishes have caused ciguatera poisoning in >100 individuals in Hawaii, as reported annually by the State Department of Health. Generally, about 6–10 species are involved including herbivores and carnivores. A specific site at Puako, on the island of Hawaii was selected because of persistent outbreaks of fish poisoning in the first few months of nearly every year due to *Cheilinus rhodochrous* (wrasse, po'ou). The survey consisted of (1) algae and *Gambierdiscus toxicus* assessment; (2) fish analysis by immunological assay; (3) following fish extraction testing in mouse toxicity assay; and (4) analysis with guinea pig atrium for an effect on Na^+ channels. The immunological assay showed borderline and positive in more than 50% of species examined (herbivores and carnivores). Several species of algae were found, including *Jania* sp. and *Turbinaria ornata* previously shown to be associated with *Gambierdiscus toxicus* blooms. In 5 areas along 2 miles of shoreline, *Gambierdiscus toxicus* was noted in 2 areas (9–291/g algae). Organic solvent extracts from *Ctenochaetus strigosus* and *Acanthurus sandvicensis* showed inhibition of the Na^+ channel in the guinea pig atrium assay. The inhibition appears to be very similar in action to tetrodotoxin.

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Approximately 115 incidents of ciguatera fish poisonings occurred in the South Kohala area from 1971–1992. The number of illnesses is highly underreported due to the flu-like symptoms of ciguatera (Hokama,1988) which often lead to its misdiagnosis by physicians. On Oahu, at a downtown restaurant in February 1991, approximately 21 individuals became ill (5 hospitalized) after consuming rose colored wrasses *Cheilinus rhodochrous* (po'ou). *Cheilinus rhodochrous* extracts have shown typical ciguatoxin-like activity in the immunological, mouse toxicity and guinea pig atrium analysis (Amra et al., 1990). The active toxins of the po'ou flesh extract were found in the 1:9 (methanol:chloroform) and 1:1 (methanol:chloroform) fractions in the silica gel chromatography analysis. The 10% methanol fraction contained 10M.U. in 100mg crude flesh extract, while the 50% methanol fraction had 5M.U. in 100mg crude flesh extract (1 M.U. (mouse unit), is the amount of fish extract that kills a 20g mouse in 24hrs). The origin of the implicated fish was an area called Puako on the South Kohala Coast of Hawaii (Fig.1).

Puako is on the leeward coast of Hawaii in the district of South Kohala. Before 1957 there was essentially no development along this coast. However, over the years many residential dwellings, a 3.5km accessible coastline road, boat launching ramp and several public right of ways have been developed. Although development has progressed markedly there are still no real beaches in this area. Coarse sand and gravel make up much of the ocean-land interface with its underlying structure composed mostly of lava. Therefore, the land tends to be very porous. Other nearshore areas are bordered mostly by *Prosopis pallida* (kiawe trees) and soil is sparse. The limited soil probably leads to nutrients being distributed in small, restricted quantities along the coast. However, as one nears the boat launching area, soil levels substantially increase which accounts for the often turbid nature of the water as one nears this location and this probably increases its nutrient content. Puako is an extremely arid region with a visible freshwater lens noticeable during the diving expeditions along the coast. This is the underground fresh water which tends to lower the salinity of the ocean water in some areas of Puako.

The Puako study area extends 3.4km from Puako Bay to approximately 1km N of Pauoa Bay; it was divided into areas A-D, each 850m long (Fig.1). The study commenced in April 1992 and visits were made every 2 months. Each visit included collection and identification of fish and

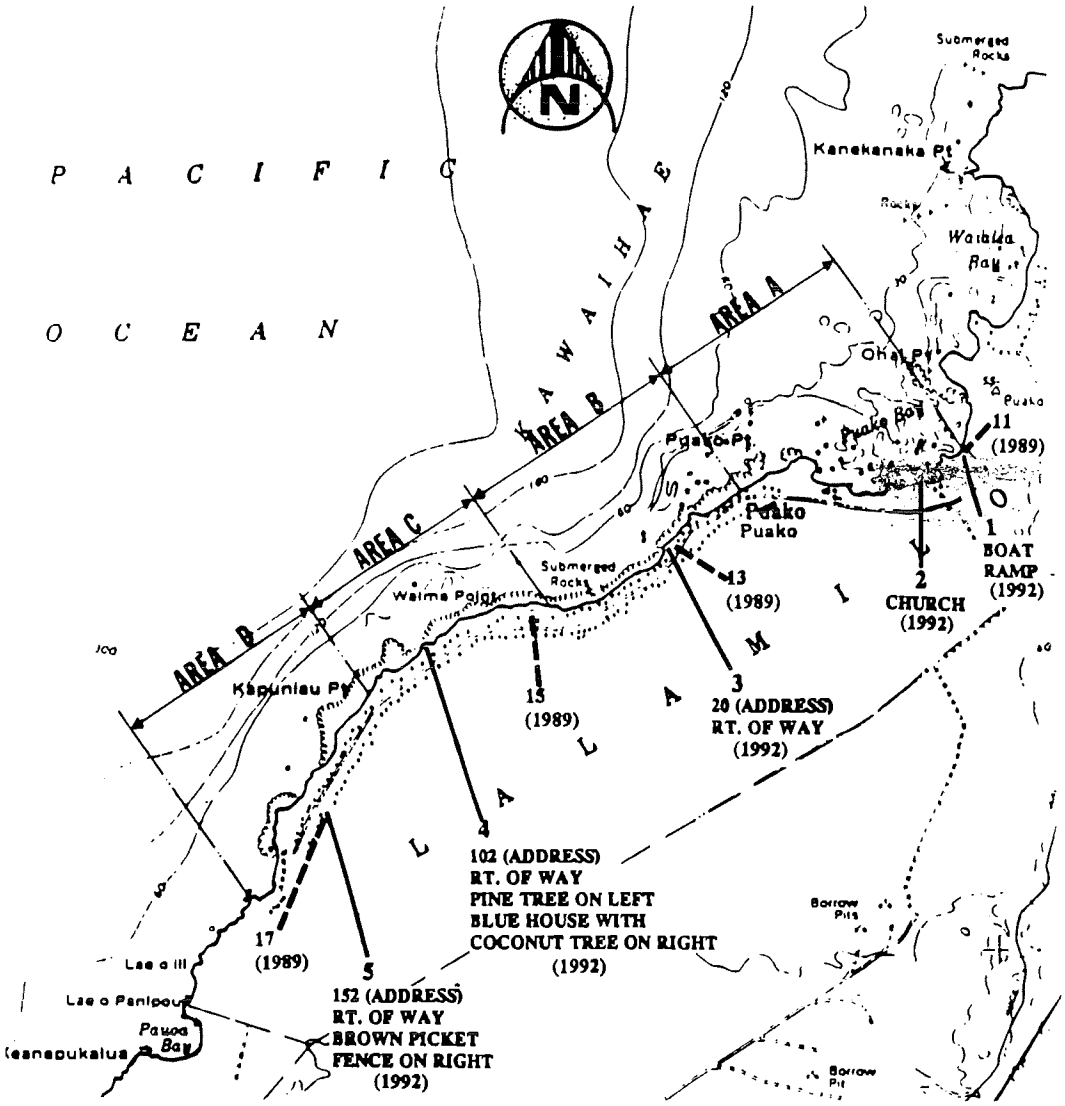

FIG.1. Map of the puako area surveyed for ciguatera. The 2 mile shoreline is transected into 4 segments (A, B, C and D) from north (A) to south (D).

algae, upon which were conducted: 1) identification of various algae types, 2) analysis of fish by the Solid Phase Immunobead Assay (S-PIA) for ciguatoxin and related polyether compounds, 3) analysis of algae for *Gambierdiscus toxicus*, 4) analysis of fish extracts with the mouse bioassay, 5) analysis of fish extracts with the guinea pig atrial assay, 6) water quality analysis. Part of the concept of this approach followed Yasumoto et al. (1980, 1984) and Hokama et al. (1993).

METHODS

IDENTIFICATION OF ALGAE

Algae from each station was placed into 50ml conical centrifuge tubes containing 2% formalin/seawater solution. Samples were taken to Professor Isabella Abbott (Department of Botany, University of Hawaii) for identification.

ANALYSES FOR *GAMBIERDISCUS TOXICUS*

Algal specimens were collected either by hand

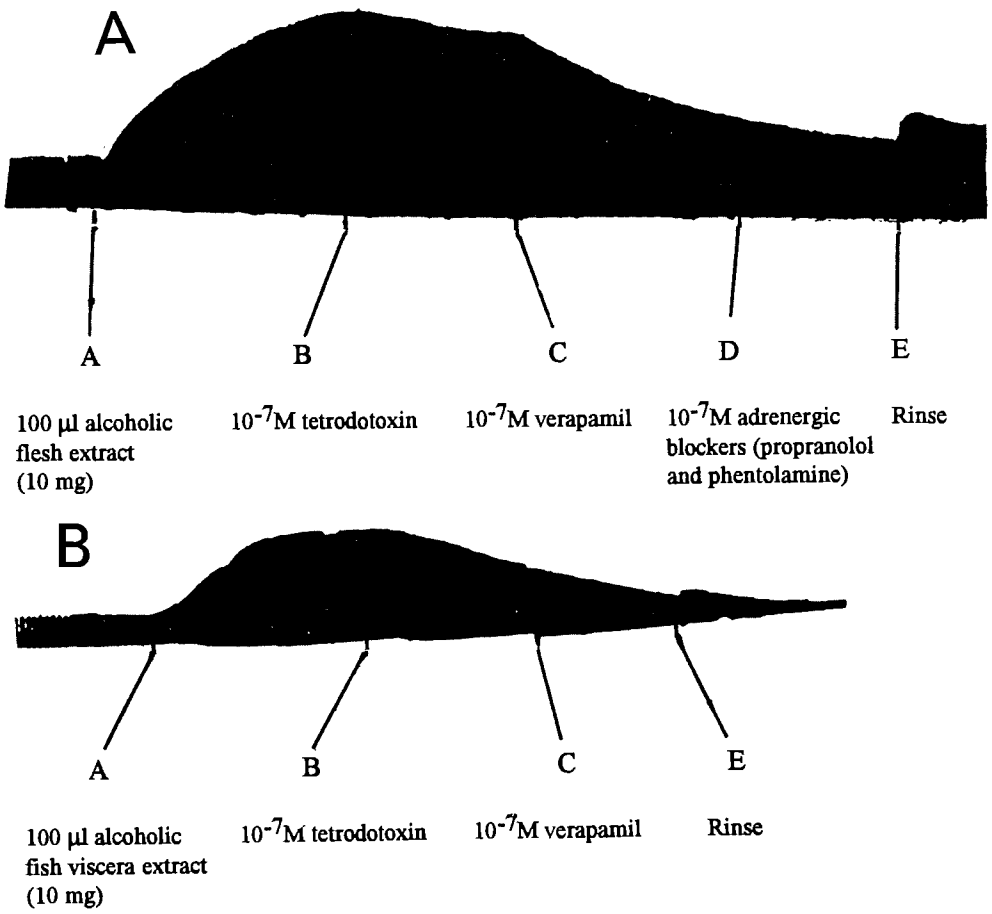

FIG.2. Guinea pig atrial responses to *Ctenochaetus strigosus* (kole) alcoholic extracts and the effects of blockers.

or by gently scraping coral substrates throughout areas A-D. Samples were then placed into 1 gallon ziplock bags containing 0.5–1l of seawater. The contents were shaken for 2min to remove any possible epiphytic dinoflagellates present on the algal substrate. The algae/seawater suspension was then consecutively passed over a 125µm mesh sieve to remove any large algal fragments then through a 38µm sieve. The residue on the 38µm sieve was then backwashed with an enriched seawater media, collected into a 100ml sterile glass bottle and loosely capped to provide aeration. After gentle agitation, 1ml was placed onto a Sedgewick Rafter Cell Counting Slide. Counts were performed in triplicate to determine the average number of cells per ml and the number of cells per gram of algae (Yasumoto et al.,1984).

ANALYSIS OF FISH BY S–PIA

Various herbivorous and carnivorous fishes were collected by spearing and line fishing. A bamboo paddle coated with pentel correction fluid was then inserted into an incision made near the head of each sample and the residue retained on the paddle allowed to air dry for 5–10mins. The paddle was then immersed in methanol for 0.1sec and allowed to air dry. The paddle was then put into a blue latex bead-antibody solution (ciguatoxin antibody) for 5 or 10min intervals. Readings obtained were then scored according to the intensity of the coloring obtained on the pad-

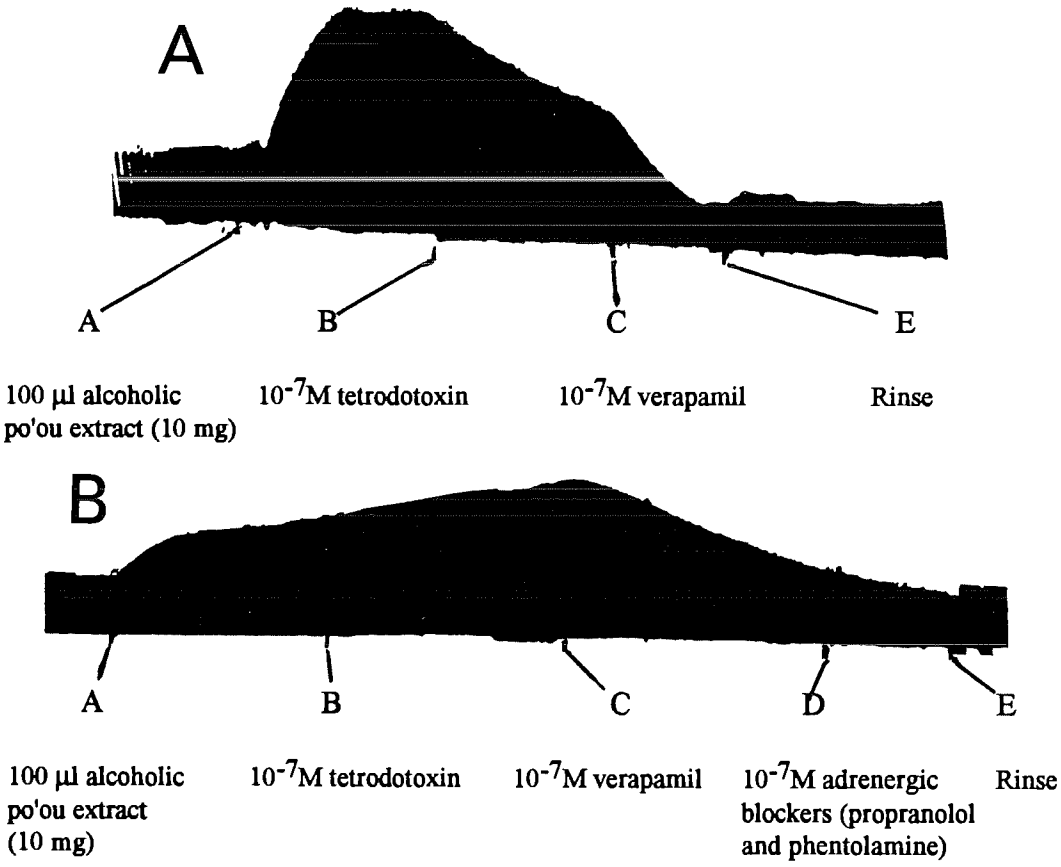

FIG.3. Guinea pig atrial responses to *Cheilinus rhodochrous* (po'ou) alcoholic extracts and the effects of blockers.

dle and rated as negative (no color), borderline (slight blue) or positive (distinct blue). The procedure followed Hokama (1990).

Extraction of Ciguatoxin and Related Polyether Compounds

Fish were separated into flesh and viscera samples and extracted for ciguatoxin and related polyether compounds. Each portion (flesh and viscera) was placed in acetone at a 1:2.5 ratio (flesh:acetone) and allowed to soak overnight. The acetone suspension produced was then decanted over a Whatman 4 filter and the filtrate allowed to collect into a flask. The acetone was then evaporated utilizing a Buchi Rotavapor. The residue retained in the flask was then washed with chloroform and partitioned with a brine solution (2x) at a 4:1 ratio (chloroform:brine) in a separatory funnel. The chloroform was successively evaporated and the residue retained was then brought up in a 80% methanol solution and partitioned with hexane (3x) at a 1:2 ratio (80% methanol:hexane). The 80% methanol was evaporated and the residue was collected and used for further analysis in the mouse bioassay and the guinea pig atrial assay. The method of Kimura et al. (1982), and Miyahara et al. (1989) were used in this study.

Analysis of Fish Extracts with Mouse Bioassay

100mg of the 80% methanol fraction fish extract was diluted in 1ml of a Tween 60/saline solution. The suspension was then injected intraperitoneally (IP) into a 20–25g Swiss Webster mouse and signs observed. Readings were successively taken at 30min, 1,2,4,6,8,24, and 48hrs post-injection and rated on a scale of 0–5 accord-

TABLE 1. Survey of *G. toxicus* at Puako, April to August, 1992. * areas refer to sections shown on Fig.1.

AREA *	DATE	*G. TOXICUS* CELLS/GM ALGAE	ALGAL SPECIES	TOTAL # FISH	S-PIA NEGATIVE	S-PIA BORDER LINE	S-PIA POSITIVE
A	APR 92	291	*Galaxaura fasciculata* *Tolypiocladia glomerulata*	74	18% (13)	55% (41)	27% (20)
	JUN 92	7.2	*Galaxaura marginata* *Galaxaura fasciculata*	76	43% (33)	43% (33)	13% (10)
	AUG 92	0	*Galaxaura marginata* with large clumps of *Biddulphia aurita* (diatom) *Galaxaura fasciculata* *Turbinaria ornata* epiphytic *Tolypiocladia glomerulata* *Dictyota friabilis* (brown) *Chondria polyrhiza, Jania* sp., *Cladophora* sp. (green)	86	30% (26)	63% (54)	7% (6)
B	APR 92	0	*Sargassum* sp.				
	JUN 92	0	*Turbinaria ornata* *Pterocladia caerulescens*				
	AUG 92	0	*Eupogodon iridescens* *Schizothrix calcicola* (bluegreen) *Centroceras clavulatum*				
C	APR 92	0	*Turbinaria ornata*				
	JUN 92	3.4	*Turbinaria ornata* with *Jania* *Pterocladia caerulescens*				
	AUG 92	0	*Schizothrix calcicola*				
D	APR 92	9.0	*Turbinaria ornata* with epiphytic *Jania*				
	JUN 92	1.2	*Turbinaria ornata* with *Jania*, bluegreen and *Biddulphia aurita* (diatom)				
	AUG 92	0	*Phormidium crosbyanum*				

ing to the signs presented. Toxicity ratings are scored according to Kimura et al. (1982).

Analysis of Fish Extracts with Guinea Pig Atrial Assay

Hartley Guinea Pig weighing approximately 300–350g were sacrificed and hearts surgically removed after anesthetization (*Enflurane*). The atria was then isolated from the ventricles and separated further into its left and right components. Each piece of atrial tissue was placed into a 25ml physiological bath (37°C) containing Krebs-bicarbonate solution and constantly aerated (95% O_2, 5% CO_2). Electrical probes stimulated the left atria while the right relied on its sinoatrial node. Subsequently, 100μl of fish extract (80% methanol fraction) at a concentration of 100mg/ml resuspended in Krebs bicarbonate solution was added to the physiological bath chamber. The tissue was then observed for both an inotropic and/or chronotropic responses displayed on a polygraph as reactions to the extract. Additionally, pharmacological characteristics were noted with the addition of 12.5μl of tetrodotoxin (sodium channel blocker), verapamil (calcium channel blocker) and propranolol/phentolamine (adrenergic blocking drugs) following the method of Miyahara et al. (1989). In one experiment (Fig.5) Manini extract from the viscera was used in the atrial analysis. The extract acted as a blocking agent. This action though resembling TTX or PSP was neither of these toxins, since it had a non-polar lipid characteristic.

Water Quality Analysis

Physical parameters of the Puako coastline (Areas A-D) were taken by the Department of Health Clean Water Branch in December 1989 and August 1992. Measurements including salinity, nitrate-nitrite, ortho-phosphates, ammonia, silica, total dissolved nitrogen, total dissolved phosphorous, temperature, conductance, dissolved oxygen and pH were taken utilizing a

TABLE 2. Puako fish assessment with S-PIA (April 1992). * Area refers to sections shown in Fig.1.

AREA *	SPECIES	TOTAL	S-PIA RESULTS		
			–	+/–	+
A	Hawaiin kole	1	0	1	0
	Kole	9	0	8	1
	Po'ou	6	2	3	1
	Roi	6	0	4	1
	Total	21	2	16	3
	Percent		(10)	(76)	(14)
B	Kole	9	1	4	4
	Po'ou	4	0	4	0
	Roi	2	0	0	2
	Total	15	1	8	6
	Percent		(7)	(53)	(40)
C	Hawaiin kole	6	0	6	0
	Kole	7	0	4	3
	Po'ou	4	1	2	1
	Roi	2	1	1	0
	Total	19	2	13	4
	Percent		(11)	(68)	(21)
D	Kaku *(S. barracuda)*	1	0	0	1
	Kole	10	0	10	0
	Po'ou	1	1	0	0
	Roi	7	2	4	1
	Total	19	3	14	2
	Percent		(16)	(74)	11
TOTAL (%)		74	8 (11)	41 (69)	15 (20)

Surveyor 2 water quality monitoring instrument. Testing areas for those parameters measured in December 1989 and August 1992 were within the areas of A through D (Fig.1).

RESULTS

IDENTIFICATION OF ALGAE

Puako contains a variety of algal types (Table 1), some of which have been found associated with *G. toxicus* previously.

ANALYSIS OF ALGAE FOR *G. TOXICUS* (Table 1)

The highest level of cells (per g of algae) occurred in area A during April 1992. *G. toxicus* was found at all stations (in limited quantities), except for Area B which was devoid of any dinoflagellates over the survey period. The moderate to high levels of *G. toxicus* were especially shown in April of 1992 in Area A and this was associated with *T. glomerulata*. Area B had no *G. toxicus* despite a variety of algae. Areas C & D had mild levels of *G. toxicus* probably associated with the epiphytic *Jania* sp.

TABLE 3. Puako fish assessment with S-PIA (June 1992). * Area refers to sections shown on Fig.1. # Not defined in Fig.1, but represents an area of similar size north of A. A is the first transect which originates at the boat ramp.

AREA	SPECIES	TOTAL	S-PIA RESULTS		
			–	+/–	+
A	Hawaiin kole	2	0	2	0
	Kole	6	6	0	0
	Po'ou	4	3	1	0
	Roi	3	0	2	1
	Total	15	9	5	1
	Percent		(60)	(33)	(7)
AA#	Hawaiin kole	1	1	0	0
	Kole	8	5	3	0
	Po'ou	3	3	0	0
	Roi	3	2	1	0
	Total	15	11	4	0
	Percent		(73)	(27)	(0)
B	Hawaiin kole	1	0	0	1
	Kole	7	3	4	0
	Po'ou	3	1	1	1
	Roi	4	0	2	2
	Table boss (spp.)	1	1	0	0
	Total	16	5	7	4
	Percent		(31)	(44)	(25)
C	Hawaiin kole	5	1	4	0
	Kole	2	0	2	0
	Po'ou	1	1	0	0
	Roi	3	1	2	0
	Total	11	3	8	0
	Percent		(27)	(73)	(0)
D	Hawaiin kole	5	5	0	0
	Kole	6	0	4	2
	Po'ou	4	0	3	1
	Roi	4	0	2	2
	Total	19	5	9	5
	Percent		(26.3)	(47.4)	(26.3)
TOTAL (%)		76	33 (43.4)	33 (43.4)	10 (13.2)

ANALYSIS OF FISH WITH S-PIA (Tables 2–4)

The targeted species in this Puako Survey included a herbivore *Ctenochaetus strigosus* (kole) and 2 carnivores *Cephalopholis argus* (roi);

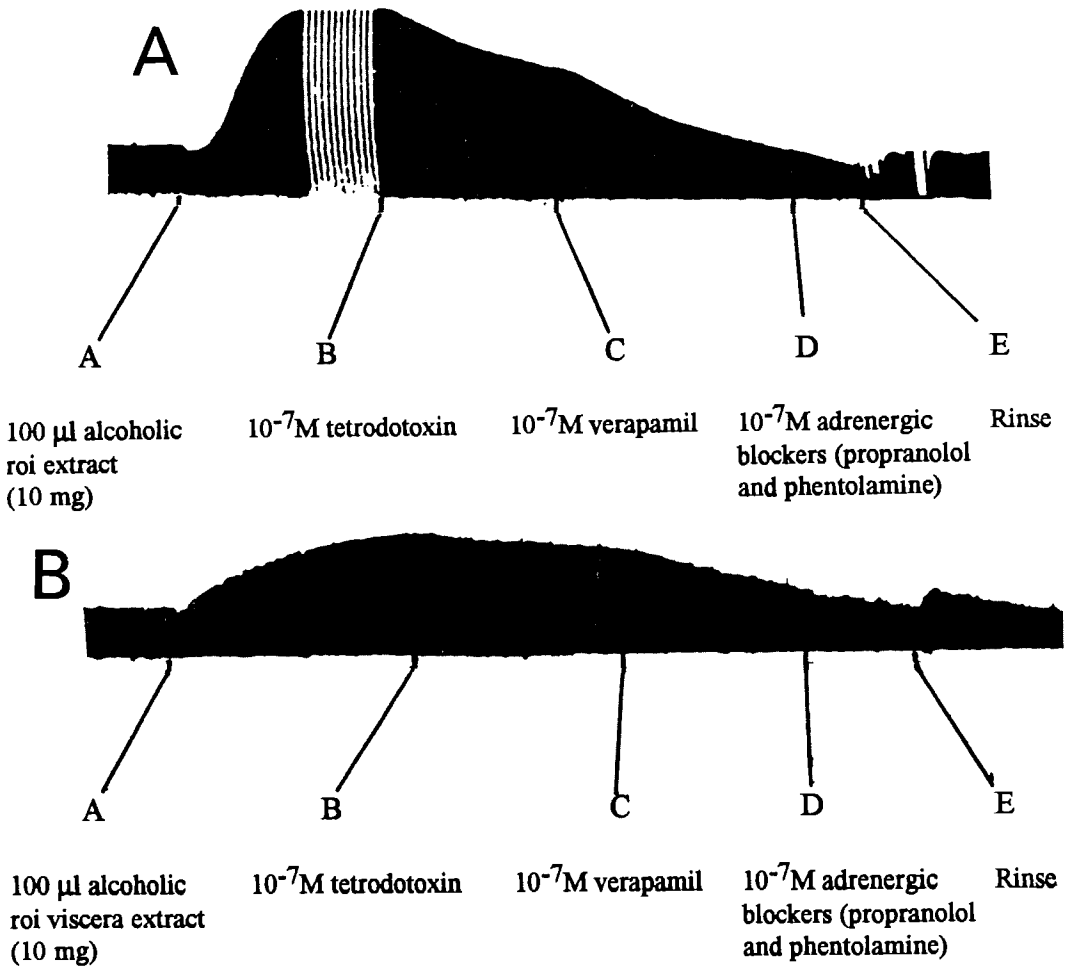

FIG.4. Guinea pig atrial responses to Cephalopholis argus (roi) alcoholic extracts and the effects of blockers.

Cheilinus rhodochrous (po'ou). The highest overall percentage of fish falling into the rejection category of S-PIA (borderline and positive) occurred during April (1992) in Areas A, B, and C. Area D had the greatest percentage of fish in the rejection range in August 1992.

ANALYSIS OF FISH EXTRACTS WITH THE MOUSE BIOASSAY (Table 5)

Both flesh and viscera contained compounds that induce abnormal symptoms and death in mice. Although all fish tested showed both high and low levels of toxicity, kole viscera on the average gave consistently the highest toxicity ratings. In all species, toxicity seemed to be higher in the viscera as compared to the flesh extract. There is a slight difference in the flesh and viscera toxicities in mice. The viscera extracts tended to be most consistently toxic and are noted in all areas and periods examined. The mouse toxicities of po'ou extracts from Areas A and D appear less severe than kole extracts. Again, within the species tested the viscera were more toxic especially in Areas B and C. Similarly, the roi extracts tended to be less toxic than the kole extracts. Again, the flesh extracts of roi appear to be less toxic than the roi viscera extract.

TABLE 4. Puako fish assessment with S-PIA (August 1992). * Area refers to sections shown on Fig.1. # Not defined in Fig.1, but represents an area of similar size north of AA, as described in Table 3.

AREA *	SPECIES	TOTAL	S-PIA RESULTS		
			–	+/–	+
A	Kole	20	6	12	2
	Papio	1	1	0	0
	Po'ou	3	2	1	0
	Roi	5	2	3	0
	Table boss	1	0	1	0
	Tang (*A. achilles*)	1	0	1	0
	Total	31	11	18	2
	Percent		(35.5)	(58.1)	(6.4)
B	Kole	10	3	7	0
	Po'ou	2	2	0	0
	Roi	3	1	2	0
	Total	15	6	9	0
	Percent		(40)	(60)	(0)
C	Kole	10	3	7	0
	Po'ou	2	1	1	0
	Roi	3	1	2	0
	Total	15	5	10	0
	Percent		(33)	(67)	(0)
D	Kole	10	0	6	4
	Po'ou	1	0	1	0
	Roi	4	0	4	0
	Total	15	0	11	4
	Percent		(0)	(73.3)	(26.7)
F#	Kole	10	4	6	0
	Total	10	4	6	0
	Percent		(40)	(60	(0)
TOTAL (PERCENT)		86	26 (30.2)	54 (62.8)	6 (7)

TABLE 5. Compilation of mouse toxicity of three species of fish: viscera and flesh for the months of April, June and August, 1992. * mouse toxicity values ranged from 1 (little or no toxicity) to 5 (not toxic). NS = no sample.

AREA	SPECIES	TISSUE	MOUSE RATING*		
			APR	JUN	AUG
A	*Ctenochaetus strigosus*	flesh	3	4	5
		viscera	5	5	5
B	*Ctenochaetus strigosus*	flesh	1	5	5
		viscera	5	5	5
C	*Ctenochaetus strigosus*	flesh	3	5	5
		viscera	5	5	5
D	*Ctenochaetus strigosus*	flesh	5	2	4
		viscera	5	5	4
A	*Cheilinus rhodochrous*	flesh	3	NS	3
		viscera	5	NS	3
B	*Cheilinus rhodochrous*	flesh	2	NS	5
		viscera	5	NS	5
C	*Cheilinus rhodochrous*	flesh	1	NS	4
		viscera	NS	NS	4
D	*Cheilinus rhodochrous*	flesh	1	NS	3
		viscera	NS	NS	3
A	*Cephalopholis argus*	flesh	2	3	5
		viscera	5	3	5
B	*Cephalopholis argus*	flesh	2	1	5
		viscera	4	2	5
C	*Cephalopholis argus*	flesh	1	2	5
		viscera	5	5	5
D	*Cephalopholis argus*	flesh	2	2	3
		viscera	4	5	NS

Analysis with the Guinea Pig Atrial Assay

Testing of fish extracts with the Guinea Pig atrial Assay indicated at least 2 pharmacologically different toxins. The inotropic effect elicited by one of these compounds is blocked by tetrodotoxin, indicating that the observed reaction has characteristics similar to ciguatoxin by allowing a greater influx of sodium ions through the membrane channel. Likewise, the second reaction observed is an inhibition by verapamil in response to the inotropic effect elicited by the fish extract. Both types of reactions (TTX and verapamil inhibitions) are found in the herbivores as well as the carnivores.

Inotropic patterns and their inhibition by tetrodotoxin, verapamil or both are demonstrated (Figs 2–4) for kole, po'ou and roi, respectively. Kole flesh and viscera extracts show typical inhibition by TTX and verapamil (Fig.2A,B). However, in some kole viscera extracts only verapamil or TTX showed inhibition.

Po'ou (whole fish) extract induced positive inotropy is inhibited by TTX and verapamil (Fig.3A). The latter inhibitor appears to give a stronger reaction. A pooled po'ou extract scored negative to the S-PIA test (negative for polyether); this is reflected by no inhibition with TTX channel blocker, but inhibition by verapamil(Figure 3b).

The major difference between the inotropic responses of whole roi (Fig.4A) and separated viscera extracts (Fig.4B) is in the initial rise. Both extracts are affected by TTX and verapamil, al-

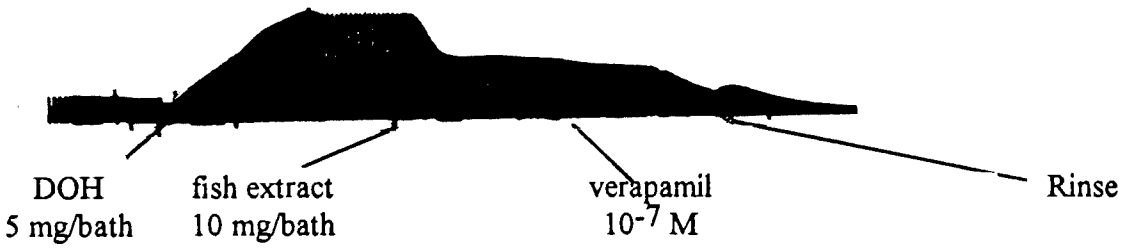

FIG.5. Inotropic response of pooled Department of Health (DOH) fish extract implicated in ciguatera poisoning and the negative inotropic effect of manini (*Acanthurus sandvicensis*). This DOH extract was toxic for mice (Value of 5) and its inotropic pattern showed strong block with tetrodotoxin (pattern not shown).

though the viscera extract appears to be affected less than the whole extract. There is a suggestion that the toxin in the viscera is slightly different from whole roi extract.

Fig.5 shows the inhibition of the inotropic effect of known ciguateric pooled fishes (Department of Health samples) by a sample from *Acanthurus* sp. extract (manini) giving a TTX or verapamil like block. This has been shown on occasion with kole extracts also.

WATER QUALITY ANALYSIS

The Puako area is associated with underground freshwater infusion into the ocean (Kay et al.,1977), especially in Areas B and C . Salinity has varied from a low of 2.2% to 3.4% in Areas B and C. This fluctuation in part may account for the variation in *G. toxicus* numbers.

DISCUSSION

There appears to be no relationship between high levels of toxicity in fish and presence or absence of *G. toxicus*; even in the absence of *G. toxicus*, levels of SPIA borderline and positive fishes were noted. April,1992 showed the least number of negative fish in all species and areas (18%) followed by August (30%) and then June (43%) (Tables 2,3,4). In general, the herbivores appeared to be more toxic (±and +) than the carnivores in all three months examined.

The mouse toxicity showed that the herbivore (kole) was the most toxic with higher number of mouse values of 5. The carnivores roi and po'ou were also toxic but less than the kole, except for the viscera extracts. Analysis of the guinea pig inotropic response suggested the ciguatoxin-like, maitotoxin-like (Figs 2,3,4) and a sodium channel blocking toxin (polyether-like) similar to TTX or verapamil (Fig.5).

It is difficult to correlate immunological results with extracts used in the mouse and atrial assays, since the immunoassay is carried out in individual fishes whereas the mouse and atrial assays are done with pooled extracts though of the same species. Nevertheless in general, those fishes with borderline and positive SPIA results appeared to be more toxic than the negative fishes.

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TEST OF THE EFFECT OF DISTURBANCE ON CIGUATERA IN TUVALU

URSULA L. KALY AND GEOFFREY P. JONES

Kaly, U.L. & Jones, G.P. 1994 08 01: Test of the effect of disturbance on ciguatera in Tuvalu. *Memoirs of the Queensland Museum* **34**(3): 523–532. Brisbane. ISSN 0079-8835.

We describe a field study on the potential link between the occurrence and intensity of ciguatera outbreaks and human disturbance of coral reefs. We focused on the atolls Niutao, Nui and Nanumea in the Tuvalu group; each having a different history of ciguatera. Relatively small-scale disturbances associated with ship wrecks; and moderate disturbance associated with blasting for boat channels were examined. Increases in *Gambierdiscus toxicus* abundances were detected around channels at Nanumea and Niutao (prior history of outbreak), but not at Nui (historically ciguatera free). At Niutao, the overriding pattern of *G. toxicus* density around the island was independent of either form of human disturbance. Fish toxicity (Hokama Stick Test) data indicated a similar pattern unrelated to human disturbance. We suggest that some forms of human disturbance might affect (or even precipitate) outbreaks of ciguatera but that other factors are likely to play a larger role.

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Ciguatera poisoning from coral reef fishes is a problem for Pacific Island countries, both for expansion of subsistence fishing to support increasing populations, and for inshore commercial fisheries (Dalzell,1992; Lewis, in press). While knowledge has increased on the toxins involved (Murata et al.,1990; Lewis et al.,1991), the dinoflagellates responsible for ciguatoxins (*Gambierdiscus toxicus* and others) (Lewis et al. 1991; Holmes & Lewis,1991; Holmes et al.,1991), and the fish responsible for intoxications (Anderson & Lobel,1987; Dalzell,1992), the causes of outbreaks elude us. The spatial and temporal patterns in the abundance of *G. toxicus* and ciguatera poisonings paint a complex picture. Some areas appear never to have outbreaks. Other areas show severe short-term but unpredictable outbreaks lasting a few years (Kaly et al.,1991), hereafter 'acute outbreaks'. Other 'hot spots' have had ciguatera problems for decades, sometimes with a marked seasonality (Carlson & Tindall,1985; Gillespie et al.,1985a), hereafter 'chronic outbreaks'. Field data of dinoflagellate numbers relative to ciguatera outbreaks are rare.

Speculations on the cause(s) of ciguatera outbreaks include natural and human-induced phenomena. Anecdotal and correlative studies have implicated high salinity (Yasumoto et al., 1980b; Carlson,1984; Taylor,1985), low rainfall (Carlson & Tindall,1985), low storm or wave activity (Taylor & Gustavson 1985), high levels of nutrients (Carlson,1984), physical destruction of reefs, both natural and man-made (Randall, 1958; Banner,1976) coral death or bleaching (Yasumoto et al.,1980a,b; Kohler & Kohler, 1992) and even nuclear testing (Bagnis,1969). However, no one factor provides a universal explanation and there have been few attempts to test specific hypotheses.

The notion that the physical destruction of reef areas promotes ciguatera, is based on the idea that freshly denuded surfaces are colonised by certain opportunistic macroalgae that are the preferred hosts for the epiphytic dinoflagellates responsible for ciguatera (Randall,1958; Banner,1976). Support comes from studies on a variety of disturbances including the construction of boat channels (Kuberski,1979; Banner,1974; Tebano,1984, 1992), the locations of wrecks or anchorages (Cooper,1964), and storm-induced and other disturbances to the reef system (Bagnis,1969; Bagnis et al.,1985). However, testing the importance of these factors requires the simultaneous sampling of disturbed and undisturbed sites, and preferably, sampling both before and after the disturbance has occurred.

Our study examines potential effects of boat channels and shipwrecks on incidence of ciguatera in Tuvalu. We used 2 field indicators to test the potential effects of these disturbances. Firstly, abundance of *Gambierdiscus toxicus* was measured on a range of host algae. Secondly, we used an index of toxicity of muscle tissue from a surgeonfish (*Ctenochaetus strigosus*) implicated in ciguatera, using the Hokama 'stick' test (Hokama et al.,1983,1987). We assumed that these techniques may independently provide a measure of the levels of ciguatera at different sites or times without there necessarily being a direct

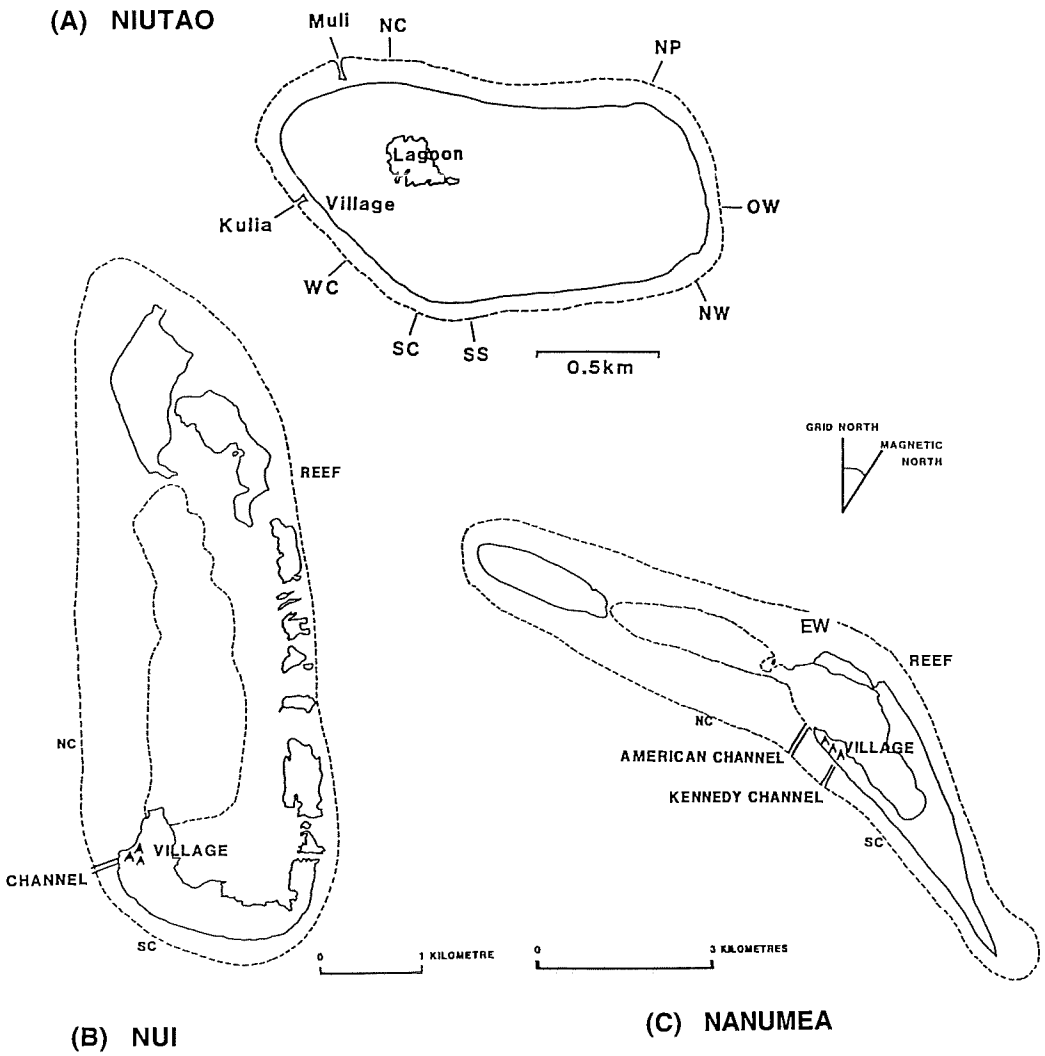

FIG. 1. Maps of (A) Niutao, (B) Nui and (C) Nanumea showing channels, controls and wrecks surveyed. Muli and Kulia are channels, NC=North Control, WC=West Control, SC=South Control, SS=Sagasaga, NP=North Point, NW=New Wreck, OW=Old Wreck, EW=East Wreck.

link between dinoflagellate numbers and fish toxicity.

Sampling protocols to test the effect of disturbance were: (1) a comparison between areas adjacent to pre-existing channels and shipwrecks with control areas, for numbers of *G. toxicus* and for fish toxicity; and (2) a short-term study of changes occurring in response to the construction of two new boat channels, and distant control areas. By sampling different distances from these boat channels and control areas over an 18 month period, we attempted to demonstrate any successional effects on ciguatera that could be attributed unequivocally to boat channels. Since an outbreak of ciguatera began just prior to the construction of the channels on Niutao (Kaly et al., 1991), the study provided a useful test of the

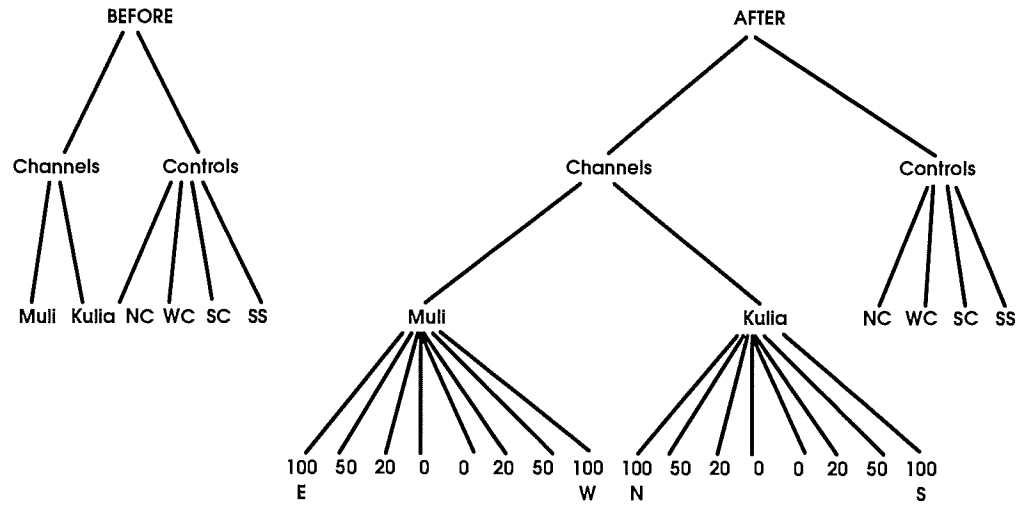

FIG.2. Design of the sampling programme used to detect any impacts of channels on indicators of ciguatera at Niutao. Numbers under each channel *after* construction, are distances away from the channel in metres.

effects of disturbance during a period when fish toxicity was high.

METHODS

Study Areas and Sampling Regimes

Construction of boat channels and ship wrecks represent two of the most obvious forms of human disturbance to coral reef platforms and the shallow subtidal zone in Tuvalu. This study focussed on pre-existing boat channels on Nanumea (2 channels) and Nui (1 channel), pre-existing ship wrecks on Nanumea (1 wreck) and Niutao (2 wrecks), and two new boat channels constructed on Niutao during the study. These islands differed in history of recorded ciguatera outbreaks, with none 'ever recorded' from Nui, an area on Nanumea which appears to have been toxic for many years, and a severe outbreak which occurred on Niutao and recovered over a 2-4 year period.

The Nui channel and two control sites on that island were sampled during early September 1989 (Fig.1B). The two channels (American and Kennedy), two control sites and the wreck site on Nanumea were sampled in late September 1989 (Fig. 1C). On Niutao, 2 wreck sites (NW and OW) and 4 control sites (NC, Muli, WC and NP) were sampled prior to construction of any boat channels during May 1989 (Fig.1A).

The 2 future channel sites (Kulia and Muli) and 4 controls (NC, WC, SS, SC) (Fig.1A) were sampled prior to construction of the channels during January 1989 (Fig.2 for sampling design). Sampling at these sites was repeated during channel construction (May 1989), 3 months after channel construction (September 1989) and 15 months after channel construction (September 1990). Sampling was carried out adjacent to each channel, 20m, 50m and 100m in both directions away from the channels, to assess spatial extent of any impacts on the abundance of *G. toxicus*.

Measurement of *Gambierdiscus* Abundance and Cover of Host Algae

The distribution and abundance of *Gambierdiscus toxicus*, the only dinoflagellate potentially responsible for the toxin that was found in appreciable numbers in our study, was sampled using a method similar to Yasumoto et al. (1980a). This is a simple washing procedure to separate dinoflagellate from host macroalga. It has been used successfully in field studies, with minor modifications, by other workers (Tebano & McCarthy,1984; Tebano,1984). Weighed samples of 100g of each algal species were placed in a plastic container to which filtered seawater was added. The contents of the container were then shaken vigorously for 2 min (c.250 shakes) before being sieved at 1mm and 38μm. The residue on the 38μm sieve was then washed into a 50ml vial to which 5ml of concentrated formalin

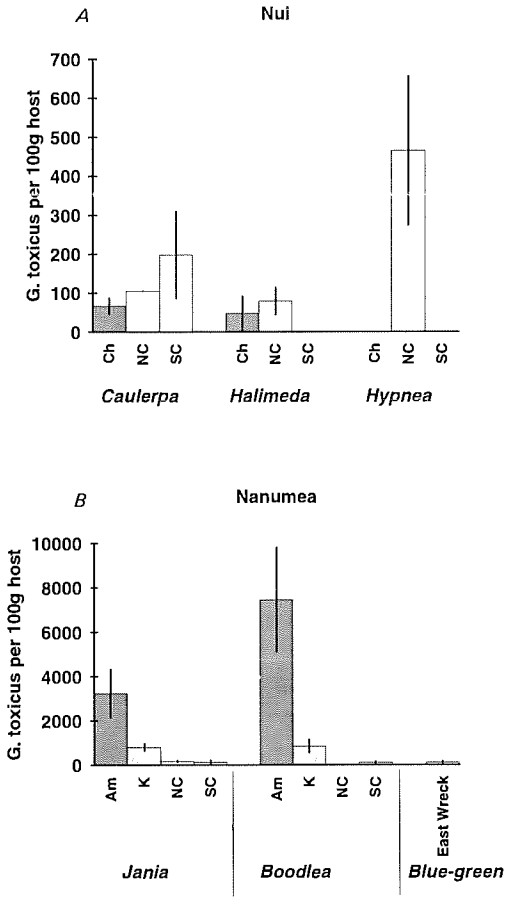

FIG.3. Mean abundance of *G. toxicus* at Channels and Controls at (A) Nui and (B) Nanumea (Time 3 = October 1989). Values are mean cells counts ± standard error. Ch=Nui Channel; NC=North Control; SC=South Control; Am=American Channel; K=Kennedy Channel.

had been added. Samples were brought back to the laboratory, quickly shaken to resuspend particles, and allowed to settle for several days. After settlement, each vial was found to contain a basal sediment layer, a layer of partially suspended mainly organic matter and an uppermost layer of transparent formalin and seawater solution. Neither the formalin solution nor the sedimentary layer were found to contain *G. toxicus* that would have been alive at the time of collection (there were some skeletons in the sedimentary layer).

To estimate the abundance of *G. toxicus* in a sample, the thickness of the primarily organic layer was measured in the pre-settled but undisturbed sample jar using vernier callipers (correct to 0.1mm) for later determination of volume. Five replicate 0.1ml subsamples of the organic layer were drawn from each jar using a micro-pipette and mounted individually on a microscope slide. All cells of *G. toxicus* on each slide were counted with the 5 replicates and volume measurement being used to obtain an estimate of the total number of cells in the organic layer of the sample (and hence the whole jar). This value gave abundances of cells per 100g of host alga.

Three replicate samples of 100g of 2–3 species of intertidal host algae were collected from each sampling site and time. The identity of the host species varied among islands and sites according to what was available. On Nui, *Caulerpa*, *Halimeda* and *Hypnea* were sampled; on Nanumea, *Jania*, *Boodlea* and an unidentified blue-green alga were sampled. On Niutao, *Jania*, *Caulerpa* and an unidentified green turfing alga were collected. To convert estimates of cell numbers per 100g of individual host algae to relative densities per 100g of all hosts, measurements of the percentage cover of the hosts were obtained for all sites at Nui and Nanumea. Five replicate readings of cover were made using a rope which was marked with 20 random points, recording the algal species found under each point.

MEASUREMENTS OF *CTENOCHAETUS STRIGOSUS* TOXICITY

Ten to fifteen *Ctenochaetus strigosus* were speared at the shallow subtidal areas at the site and time of sampling on all islands, except Nui. Fifty grams of muscle was dissected from each fish and stored in 100% ethanol. Ciguatoxins in these fish samples were confirmed by mouse bioassay (undertaken by E. Shang, Sept,1990). All tissue samples were analysed in the laboratory by the 'Stick-EIA' immunoassay method (Hokama et al.,1983,1987).

ANALYSES

Cell abundance and fish toxicity data were analysed using analyses of variance (ANOVA) with planned comparisons. The main factors examined were: Locations (which were subdivided to examine differences among channels, among controls, between channels and controls, between the wreck and controls, and distances away from channels); Island; Times and Host algae (all fixed). Assumptions of homogeneity of variances were tested using Cochran's test (Winer,1971) and data were transformed $\sqrt{(x+1)}$ as required.

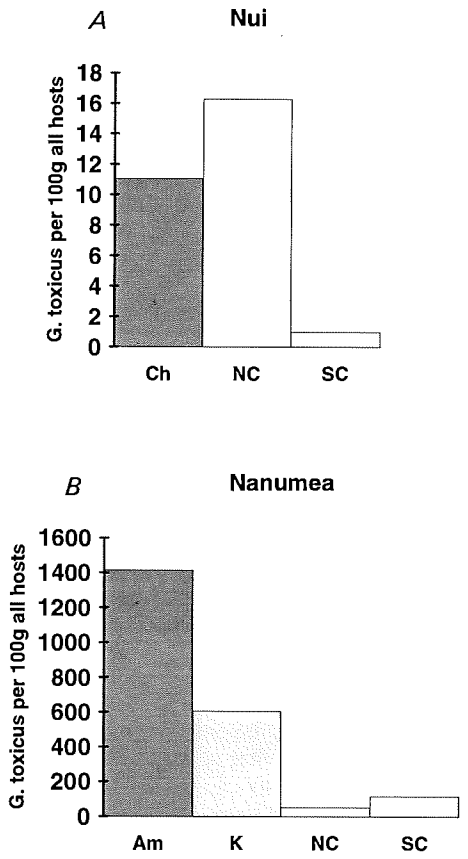

Fig.4. Composite abundances of *G. toxicus* at (A) Nui and (B) Nanumea. Values are calculated as cells per 100g of all hosts (i.e. the relative abundance of each macroalga is taken into account). Ch=Nui Channel, NC=North Control, SC=South Control, Am=American Channel; K=Kennedy Channel.

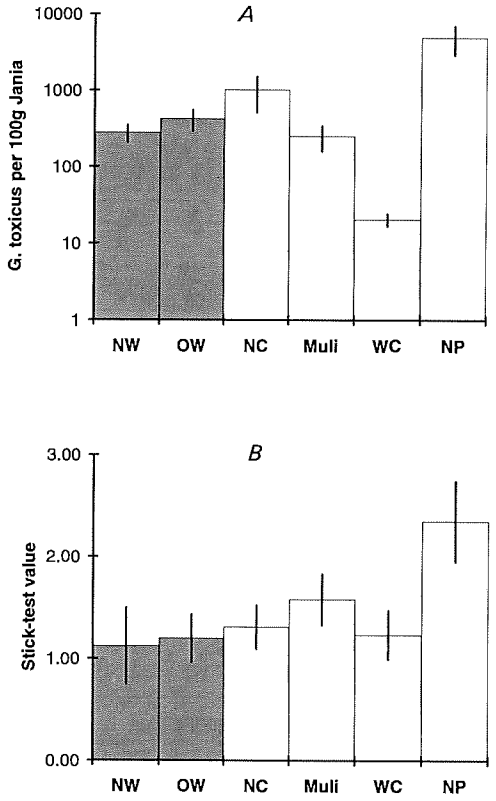

FIG.5. Abundance of *G. toxicus* and fish toxicity at Niutao (Time 2): A comparison of existing wrecks with controls (Note that 'Muli' is regarded as a control for this comparison: at Time 2 channel construction had not yet begun). (A) *G. toxicus* density; (B) Stick test values for fish toxicity. NW=New Wreck, OW=Old Wreck, NC=North Control, Muli=Future site of Muli Channel, WC=West Control, NP=North Point.

RESULTS

PRE-EXISTING BOAT CHANNELS AND WRECKS

Abundance of G. toxicus. There was no difference between the Nui channel and controls in the abundance of *G. toxicus* on *Caulerpa*, *Halimeda* and *Hypnea* (Fig.3A). The numbers of cells recorded was low, with a maximum of 500 cells per 100g of algae on one host at one site only. However, at Nanumea, both channels examined had greater densities of cells than the control areas on *Boodlea* and *Jania* (Fig.3B). For the American passage, densities of cells reached 7000 per 100g of algae. The only alga that could be found at the wreck site was a blue green alga not represented at the channels or controls. Only a few *G. toxicus* were associated with this species at the wreck site.

When cell densities per individual host were adjusted to account for the different percent cover of the algal species, and host algae pooled to provide relative densities of cells per 100g of 'all hosts', a similar pattern was evident. The channel on Nui was intermediate between the two con-

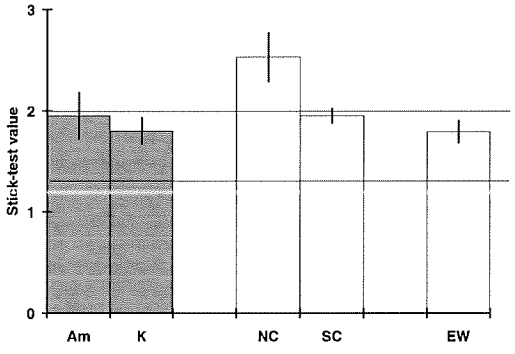

FIG.6. Fish toxicity readings from Nanumea (September 1989=Time 3). Values are means of 10 fish ± standard errors.

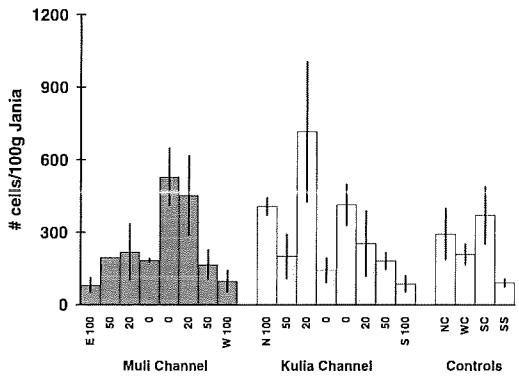

FIG.7. Mean abundance of *G. toxicus* at Channels and Controls at Niutao (Time 3 = October 1989). Values are mean cells counts ± standard errors. At Muli and Kulia, samples were taken at four distances on either side of the channels. At Muli, these were to the East and West of the channel and at Kulia to the North and South. Hence E100 =100m to the East of the Muli Channel etc.

trols (Fig.4A). On Nanumea, densities were considerably higher at channel sites compared to controls (Fig.4B). On average, densities were higher on Nanumea than Nui.

No significant difference could be detected between the 2 wreck sites on Niutao, and 4 controls prior to construction of boat channels (Fig.5A). Cell numbers reached 5000 per 100g of *Jania* at North Point (NP), a concentration similar to the American Passage on Nanumea.

Fish toxicity. There was no effect of boat channels or the wreck on toxicity of *C. strigosus* at Nanumea (Fig.6). Marginally higher toxicity was recorded at the north control site (NC).

Fish collected at wreck sites were no more toxic than control areas on Niutao (Fig.5B). If anything, the means were lower at these sites. The most striking feature of this survey was that the highest level of fish toxicity was recorded at North Point (NP), which corresponded to the highest concentrations of *G. toxicus* in the host alga *Jania*. This was the only site that we consistently found fish giving a positive result to the Hokama immunoassay.

TABLE 1. Analyses of variance of *G. toxicus* cell abundances at Niutao: planned comparisons of channels, controls and distance from channels during Time 3. NW=new wreck; D=distance; Ch=channels; C=controls; NS=non-significant F test; *=p<0.05. Data transformed sqrt(x+1).

FACTOR	DF	SS	MS	DENOM	F	f1,f2	SIG
All cells	62	11270.0	181.77	Res	10.27	62,240	*
All locations	20	7350.68	367.53	S(L)	3.94	20,42	*
Channels	1	191.75	191.75	S(L)	2.05	1.42	NS
Distance	7	4000.21	571.46	S(L)	6.12	7,42	*
Ch x D	7	1990.42	284.35	S(L)	3.05	7,42	*
Controls	3	872.97	290.99	S(L)	3.12	3,42	*
NW vs C	1	163.43	163.43	S(L)	1.75	1,42	NS
Ch vs NW vs C	2	131.94	65.97	S(L)	0.71	2,42	NS
Sample (L)	42	3921.79	93.38	Res	5.28	42,240	*
Residual	240	4246.17	17.69				
Total	302	15516.10					

Effects of New Boat Channels on Niutao

No consistent effect of new boat channels on the abundance of *G. toxicus* was detected (Fig.7, Table 1). While some of the highest densities were recorded at the 0m and 20m sites on the down current side of the channels, most of the channel sites fell within the range recorded at the controls. Patterns of change away from channels indicate that there are major changes in abundance over a 200m distance, but no obvious gradients which could be attributed to channels. Patterns of abundance were so localised that *G. toxicus* abundance on opposite sides of the channels (the 0m distances, only 10m apart) differed at both channels sites.

No significant difference be-

tween channel and control sites could be found in the toxicity of *C. strigosus* (Fig.8, Table 2). During channel construction (May 1989), 3 months after (September 1989) and 15 months after channel construction, toxicity levels at channels were well within the range exhibited at the controls. In terms of Hokama's thresholds, most fish were within the negative to borderline categories in terms of edibility.

Temporal Patterns in the Outbreak on Niutao

Major changes in the abundance of *G. toxicus* were recorded over the period of this study (Fig.9A, Table 3), being highest at December 1988, and dropping to near 0 at the September 1990. In contrast, fish toxicity levels were lowest at the first sampling date and rose to a peak in September 1989 (Fig.9B, Table 4). This coincided with a severe outbreak of ciguatera on this island (Kaly *et al.* 1991). Toxicity began to decline again in 1991. Although we were not able to continue sampling beyond this date, we have received reports that the outbreak of ciguatera had started to abate by the end of 1992 and fish were being consumed by late 1993. Although data are minimal, there is a suggestion of a lag phase of approximately one year in peak *G. toxicus* numbers and peak toxicity, at least in an indicator herbivorous fish.

DISCUSSION

This study provided no clear support for the view that physical disturbances, such as shipwrecks and boat passages, promote outbreaks of ciguatera fish poisoning. The boat channel on Nui, and the shipwrecks on Nanumea and Niutao all exhibited *Gambierdiscus toxicus* densities and *Ctenochaetus strigosus* toxicity in the range observed at sites several km from these disturbances. However, the results for Nanumea boat channels and trends at Niutao suggest that disturbance may play a role at some times. *G. toxicus* numbers were elevated in the vicinity of boat channels, particularly the American passage, despite the fact that fish toxicity was not. It is noteworthy that the channel area, although not toxic at the time of this study, has been toxic at irregular intervals over the last 20–30 years and at those times was avoided by local fisherman. The channel bisects the reef crest into what was once a ponding lagoon and is a region of extreme currents, and continual disturbance due to wave action. An area considered extremely toxic at the time of this study (on the eastern side of the island) was well-removed from the boat channels.

The before and after study of two new channels

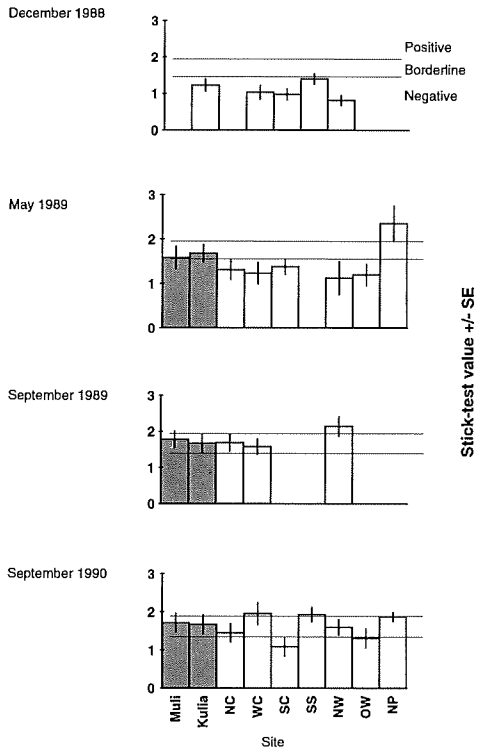

FIG.8. Toxin levels in *Ctenochaetus strigosus* collected at Niutao at 4 times between December 1988 and September 1990. Values are means of stick-test results obtained from 10 fish tested at each site and time ± standard errors. Muli and Kulia are channels, NW and OW are wrecks, remaining sites are controls. Missing bars are unsampled sites, not zero values.

TABLE 2. Analyses of variance of fish toxicity: comparison of channels and controls during T3 at Niutao and Nanumea (Cochran's Q=0.1645, v=14, k=11, NS); NS=non-significant F test; *=p<0.05.

FACTOR	DF	SS	MS	DENOM	F	f1,f2	SIG
Island	1	3.06	3.06	L(IxCh)	4.24	1,4	NS
Ch vs C	1	0.78	0.78	L(IxCh)	1.08	1,4	NS
I x [Ch vs C]	1	1.81	1.81	L(IxCh)	2.50	1,4	NS
Location (I x Ch vs C)	4	2.89	0.72	Res	1.41	4,77	NS
Residual	77	39.44	0.51				
Total	84	47.98					

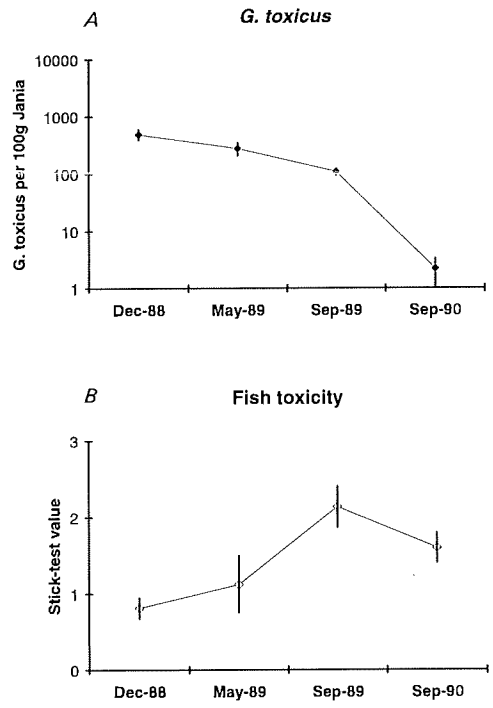

FIG.9. (A) abundance of cells and (B) levels of fish toxicity through time at 'New Wreck', Niutao.

on Niutao did not indicate a successional change in macro-algal hosts and an early successional outbreak in *G. toxicus*, although numbers appeared to be slightly elevated on the down-current side of each channel. However, cell numbers in the vicinity of channels were not unusually high relative to control areas at any stage during the study. Channel construction on this island was preceded by a bloom of *G. toxicus* which must have been caused by some other phenomenon (Kaly et al. 1991). This may have made any effect of channels difficult to detect.

Physical disturbance to reefs may or may not lead to ciguatera. 'Disturbance' encompasses a host of phenomena which may affect the habitat and population dynamics of *G. toxicus* in different ways. Whether or not ciguatera is induced by disturbance may depend on the intensity, timing, frequency and scale of the disturbance, all factors known to affect the trajectory of successional patterns on hard substrata (Connell & Keough,1985). The time-scale for recovery may depend on the regime of disturbance and chance factors in the recolonisation of damaged areas. Disturbance itself may interact with other phenomena to explain the observed patterns of outbreaks. Factors such as wave exposure, fresh water run-off and coral destruction are likely to be closely linked, making such interactions likely and single-cause scenarios extremely unlikely.

Different intensities of disturbance may have opposite effects on ciguatera. If coral habitat is damaged and the growth of host algal species promoted, then densities of *G. toxicus* could be elevated. Seasonally high wave exposure and wind at North Point (NP) on Niutao appeared to correlate with high numbers of *G. toxicus*, which also coincided with a higher average fish toxicity. In other studies, moderate wave exposure has acted to reduce cell densities, presumably by dislodging cells without disrupting the habitat (Anderson & Lobel 1987).

A promising area for research lies in the potential relationship between *G. toxicus* numbers and fish toxicity. While there was some evidence that the distribution of *G. toxicus* around Niutao correlated with fish toxicity, toxic fish were caught all round the island during the peak of the outbreak (Kaly et al. 1991). Temporal monitoring during this outbreak was limited but suggested a lag phase of approximately one year between peak *G. toxicus* numbers and the highest toxicity levels. More comprehensive sampling is needed to confirm this pattern. However, if such a pattern proves to be of general significance there are obvious implications with regard to the forecasting and managing of ciguatera outbreaks.

TABLE 3. Analyses of variance of *G. toxicus* cell abundances at Niutao: comparison of 2 hosts (*Jania* sp. and unidentified green) through time at New Wreck (NW). NS=Non-significant F test; *=p<0.05. Data transformed sqrt(x+1).

FACTOR	DF	SS	MS	DENOM	F	f1,f2	SIG
Times	3	4453.72	1484.57	S(Ti x H)	77.83	3,12	*
Host	1	968.58	968.58	S(Ti x H)	50.78	1,12	*
Ti x H	2	500.31	250.15	S(Ti x H)	13.11	2,12	*
Sample (Ti x H)	12	228.91	19.08	Res	3.46	12,69	*
Residual	69	380.35	5.51				
Total	86	3718.46					

TABLE 4. Analysis of variance of fish toxicity: comparison through time of Kulia, New Wreck and West control at Niutao (Cochran's Q=0.1399, v=9, k=12, NS). NS=non-significant F test; *=p<0.05.

FACTOR	DF	SS	MS	DENOM	F	f1,f2	SIG
Time	3	14.66	4.89	Res	8.99	3,103	*
Location	2	0.94	0.47	Res	0.86	2,103	NS
T x L	6	4.70	0.78	Res	1.44	6,103	NS
Residual	103	55.95	0.54				
Total	114	76.25					
Tukey's Test:	T3 (Sep 89)	T4 (Sep 90)	T2 (May 89)	T1 (Dec 88)			

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MAITOTOXIN INDUCES MUSCLE CONTRACTION AND A NON-SELECTIVE CATIONIC CURRENT IN SINGLE SMOOTH MUSCLE CELLS OF THE GUINEA-PIG PROXIMAL COLON

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Lang, R.J., Vogalis, F., Holmes, M.J. & Lewis, R.J. 1994 08 01: Maitotoxin induces muscle contraction and a non-selective cationic current in single smooth muscle cells of the guinea-pig proximal colon. *Memoirs of the Queensland Museum* **34**(3), 533–540. Brisbane. ISSN 0079-8835.

We have investigated the mechanisms of action of maitotoxin-2 (MTX), a marine toxin isolated from the toxic dinoflagellate, *Gambierdiscus toxicus*, on the contractility of the intact circular smooth muscle of guinea-pig proximal colon and on the membrane currents recorded in enzymatically-dispersed single cells from this muscle, using standard contraction and patch clamp recording techniques. MTX (0.005-5.0nM) induced an initial phasic contraction and a subsequent cessation of all spontaneous contractile activity, which was sometimes associated with a sustained increase in muscle tone. The initial contraction to MTX was blocked by atropine (2μM), the muscarinic receptor antagonist. Contractions to acetylcholine (0.5μM) were reduced approximately 75% after 7 minutes exposure to MTX (0.5nM) (n=5), this blockade was resistant to washout. MTX (5nM) completely abolished the contractions to acetylcholine, but reduced contractions to raised external concentrations of K^+(40mM) only 54 ±9% (n=4).

Single colonic smooth muscle cells were perfused with K^+- filled patch pipettes and voltage clamped at a holding potential of -80 mV. MTX (5nM) added to the bathing solution induced a large inward current (1-3 nA) after 15-45 minutes. Development of this current was not prevented by a number of K^+ channel blockers, including tetraethylammonium (TEA; 2–126 mM), 4-aminopyridine (5mM), quinidine (5mM) or glibenclamide (10μM); nor when the Ca^{2+} was removed, or replaced with Ba^{2+} (7.5mM). This current was, however, blocked by Cd^{2+} (0.1-1mM) and reduced by nifedipine (10μM) and La^{3+} (1mM). The MTX-activated current had an almost linear current-voltage relationship with a reversal potential near –30 and 0 mV when cells were respectively filled with K^+ or Cs^+. When most of the extracellular Na^+ (126mM) was replaced with TEA^+, this current reversed near –60mV. These results suggest that MTX induces the appearance/opening of voltage-insensitive channels which allow the flow of Na^+, K^+ and Cs^+, but not TEA^+, and which are blocked by Cd^{2+}.

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Ciguatoxins(CTX) and maitotoxins(MTX) are potent marine toxins isolated from the dinoflagellate *Gambierdiscus toxicus*. Ciguatoxins are lipid soluble and accumulate in the flesh and viscera of reef fish; they are the principal toxins responsible for cigatera. Maitotoxins are more polar and extracted in a number of forms from cultures of *G. toxicus* (Yokayama et al.,1988; Holmes et al., 1990). They have positive inotropic effects on cardiac muscle (Kobayashi et al.,1985) and cause contraction in smooth muscle (Ohizumi & Yasumoto,1983). They also induce a rise in the internal Ca^{2+} levels in BC_3H_1 muscle cells (Sladeczek et al.,1988) and aortic smooth muscle cells in culture (Berta et al.,1988) associated with phosphoinositide metabolism (Gusovsky et al., 1987,1988; Sladeczek et al.,1988; Meucci et al., 1992), and promote release of transmitters from neurones and hormones from a number of secretory cells (Kim et al.,1985; Gusovsky et al., 1988). Almost all of these effects of MTX depend on the presence of extracellular Ca^{2+} and can be, depending on the tissue, blocked by both organic (verapamil, some dihydroprydines) and inorganic Ca^{2+}-channel entry blockers (Cd^{2+}, Ni^{2+} and Co^{2+}). This rise in intracellular Ca^{2+} induced by MTX has therefore been suggested to arise from (*i*) modulation of voltage-activated Ca^{2+} channels (Kobayashi et al.,1987, Yokayama et al.,1988), (*ii*) the mobilization of Ca^{2+} from internal stores (Meucci et al.,1992), or (*iii*) from the influx of Ca^{2+} through MTX-activated pores or channels (Yoshii et al.,1987; Sladeczek et al., 1988). Here, we describe that MTX-2 inhibited

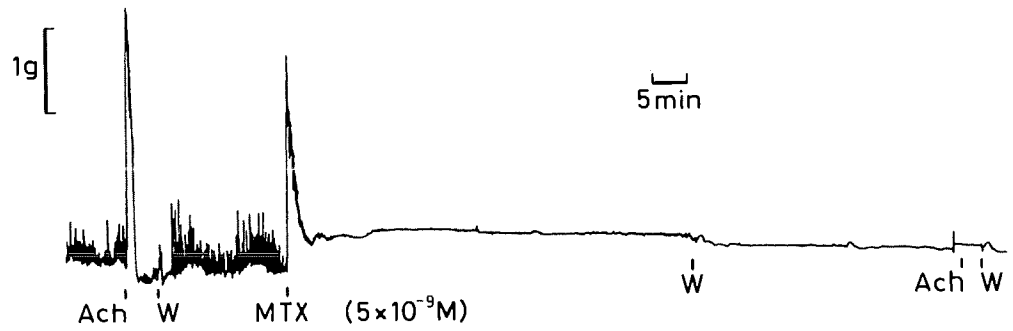

FIG. 1. Effects of acetylcholine and MTX-2 on the contractile activity of circular muscle strips of the guinea-pig proximal colon. MTX (5nM) induced a transient contraction sensitive to atropine (2μM). Contractions to acetylcholine (Ach, 5μM) were abolished irreversibly, even after extensive washout (W) of MTX.

the spontaneous and acetylcholine-induced contractile activity in the intact guinea-pig proximal colon. These effects are likely to be related to the large inward current observed in voltage clamped single cells exposed to MTX, arising from the induction of MTX channels selective for K^+ and Na^+. Some of these results have been presented previously in brief (Lang et al.,1992).

METHODS

MTX-2 used in this study was isolated from cultures of the NQ1 strain of *G. toxicus* and purified to homogeneity on HPLC as previously described (Holmes et al.,1990). This MTX has a molecular weight of 3290 Daltons for the sodium salt, a LD_{50} of 0.08μg/kg in mice (applied intraperitoneally) and was dissolved in a small volume of methanol: water (1:1).

Contraction Studies

The proximal colon (6cm long, 1–2cm aboral of the caecum) of the guinea-pig was excised and cut into 0.5cm rings Preparations were placed in 5ml organ baths and suspended under 0.5g tension between two stainless steel rods to allow recording of circular muscle contraction.

Isometric recordings (F-60 Narco Biosystems) were made at 37°C. Tissues were maintained in oxygenated (95% O_2: 5% CO_2) physiological saline solution containing (mM): NaCl 137; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 1.0; KH_2PO_4 0.5, $NaHCO_3$ 11.9; glucose 5.5. Preparations were equilibrated for 30 min and then used after obtaining reproducible responses to acetylcholine (5μM).

Cell Dissociation

The proximal colon (3–4cm long), 1–2cm aboral of the caecum, of the guinea pig was excised, cut open longitudinally and pinned out, mucosal surface uppermost, in a dissecting dish filled with a nominally Ca^{2+}-free physiological saline (PS)(see below). After removal of the mucosa, the circular muscle layer was peeled from the underlying longitudinal layer, cut into small pieces ($2mm^2$) and rinsed in low-Ca^{2+} (30 μM) PS for 2 min (at 37°C). The muscle pieces were then transferred to low-Ca^{2+} PS containing: collagenase Type 1(0.6mg/ml; Worthington); bovine serum albumin (2mg/ml; Sigma) and trypsin inhibitor (0.2mg/ml; Sigma). After a 60 minute incubation period, the muscle pieces were re-suspended in low-Ca^{2+} PS and gently agitated for 10 min (at 37°C). Single cells were obtained by gentle trituration with a wide-bore glass pipette. Cells were allowed to settle for 5-10 min to the glass bottom of the recording chamber mounted on an inverted microscope; the solution was then exchanged for normal Ca^{2+} (1.5mM) PS (Vogalis et al.,1993).

Whole-cell and Single Channel Current Recordings

Patch pipettes were drawn from glass capillary tubing (1.5–1.8mm; Kimax-51, Kimble, USA) on a programmable micro-pipette puller (Sachs-Flaming PC-84, Sutter Instruments) and their tips fire polished (MF-84 Narishige). Pipettes resistances ranged from 2–7MΩ when filled with pipette solution. Single channel and whole-cell membrane currents were recorded at room temperature using an Axopatch 200 (Axon In-

struments) and conventional patch-clamp techniques (Hamill et al.,1980). Current and voltage signals from the patch-clamp amplifier were digitized with a Labmaster TM125 analog-to-digital device (Scientific Solutions) interfaced to an Arrow-AT desktop computer using p-CLAMP software (Axon Instruments). Digitized data were stored and analyzed using this p-CLAMP software.

The physiological saline (PS) was of the following composition (mM): NaCl 126; KCl 6; HEPES 6; d-glucose 11; $MgCl_2$ 1.2; $CaCl_2$ 1.5; adjusted to pH 7.4 with 5M NaOH. The pipette solution contained (mM): KCl 126; HEPES 6; Na_2ATP 3; EGTA 3; $MgCl_2$ 3; d-glucose 11; pH was adjusted to 7.4 with 5M KOH. In some cells, current flow through all K^+ channels was blocked by replacing the KCl in the pipette solution with an equimolar concentration of CsCl, pH was set with 5M NaOH (Vogalis et al.,1993).

RESULTS

Contraction Studies

A typical isometric recording of the spontaneous contractions in a strip of circular muscle from the guinea pig proximal colon is illustrated in Fig.1. Acetylcholine (Ach, 5μm) caused a transient increase in muscle tension. MTX (5nM) produced a similar transient increase in tension which was sometimes followed by a maintained increase in muscle tone associated with a loss of the spontaneous contractile activity. This increase in muscle tone decreased upon washout (W) of the MTX, the spontaneous contractile activity, however, did not return. These effects of MTX were concentration dependent. Threshold phasic contractions (0.07-0.22g) to MTX were evoked with concentrations between 0.005 and 0.5nM MTX (n=3-5), substantial contractions (>2g) were only recorded with 5 nM MTX (n=7). In four muscle strips, these effects of MTX (5nM) were blocked by atropine (2μM), the muscarinic receptor antagonist.

The contractions to Ach (5μM) were completely abolished by MTX (5nM) and never recovered, even 145 minutes after the removal of MTX (n=6). In contrast, contractions elicited by direct muscle depolarization with 40mM K saline were decreased only 54.6±8.7% (n=4) by MTX (5nM). These effects of MTX were also concentration dependent, the concentration of MTX which half-maximally inhibited the Ach contractions was approximately 0.2nM MTX. These effects of MTX were mimicked in part by monensin, the Na^+ ionophore, which blocked the acetylcholine contractions dose-dependently (0.1–10μM), half-maximal reduction was achieved with approximately 0.2μM monensin (n=4). Monensin, however, did not induce any muscle contraction. These data suggest that MTX, monensin and acetylcholine may well be sharing a common mechanism of action. In view of this, the experiments below describe our preliminary investigations of the action of MTX on the membrane channel currents recorded in single smooth muscle cells of the guinea-pig proximal colon.

Control Whole-cell Current Recordings

Resting membrane potentials of –40 to –60 mV were recorded when a 6mM: 126 mM K^+ gradient was established across the cell membrane of single cells of the proximal colon. Depolarizing currents triggered action potentials which peaked between –10 and 0mV and had durations of 100-200ms at their half-maximal amplitude. Under voltage clamp, depolarizations, from a holding potential of –80mV, triggered complex membrane current responses (Fig. 2A). At potentials positive to –60mV, a rapidly-activating and inactivating outward current was triggered. At more positive potentials (–20mV) the transient current, in most cells, was followed by a second slowly developing and decaying outward current. The pharmacological identification of three K^+-channel currents and one Ca^{2+}-channel current underlying these current responses to membrane depolarization has been demonstrated (Vogalis et al.,1993) and will be briefly summarized.

A substantial portion of the second slowly-decaying outward current recruited at positive potentials is blocked by the addition of tetraethylammonium (TEA) (2–5mM) and a Ca^{2+}- entry blocker (0.1 mM Cd^{2+}) to the bathing solution, suggesting that this current flows through the large conductance Ca^{2+}-activated ('maxi' or 'BK') K^+ channels (I_{KCa}) which have been recorded in all smooth muscles so far examined (Vogalis et al.,1993). The Ca^{2+}-insensitive current remaining in the presence of low TEA (2–5mM): Cd^{2+} (0.1mM) activates rapidly and then decays slowly to a sustained current after 400ms, and can be further divided into two K^+ channel current components. A slowly-activating, non-inactivating K^+ current (I_{Kdel}), which has characteristics similar to delayed rectifier K^+ currents found in many electrophysiological preparations (Rudy,1988), is revealed when 4-aminopyridine (4-AP) (5 mM) blocked the initial

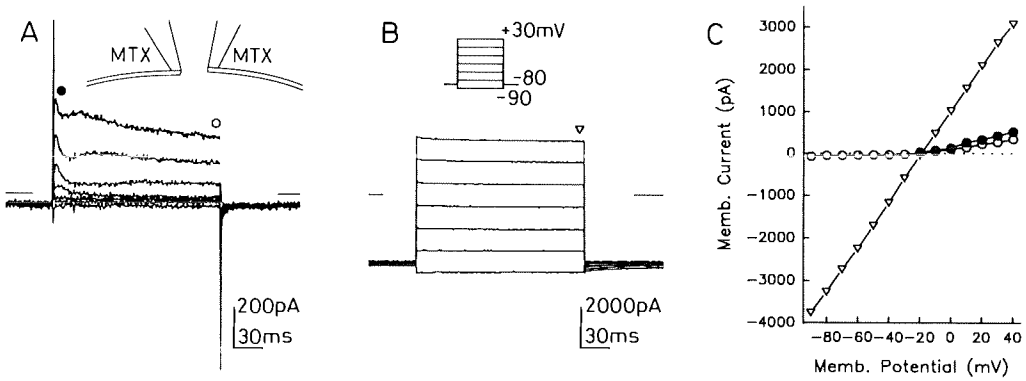

FIG. 2. Effects of MTX (5nM) on the whole-cell membrane currents recorded in single cells of the guinea-pig proximal colon. Cells were voltage clamped at a holding potential of -80mV with a K^+-containing pipette. Stepped changes in potential (100-400ms in duration) were applied to cells to evoke voltage-gated membrane channel currents in control solutions (A) and 34 minutes after exposure to MTX (5nM)(B); short lines on either side of these panels represent the zero current level. Note the ten-fold change in the vertical scale in B. C, current voltage plots of the initial peak amplitude (filled circles) in control saline and the end of pulse current before (hollow circles) and after MTX exposure (filled triangles).

itial transient component of the Ca^{2+}-insensitive K^+ current. On the other hand, blockade of the sustained component of the Ca^{2+}-insensitive current with TEA (12–20mM) reveals the time course of the rapidly activating and inactivating transient outward current, (I_{Kto}) which was blocked by 4-AP (5mM) (Rudy,1988). In cells recorded with pipettes containing Cs-saline, stepped depolarizations elicits an inward current at potentials positive to –40mV which peaks near +10mV, reverses in direction near +50mV and decays slowly over 400ms. This inward current was increased when Ba^{2+} replaced Ca^{2+} in the bathing solution and blocked by the Ca^{2+}-entry blockers, nifedipine (10μM) or Cd^{2+} (0.1mM), indicating that it represents current flow through 'high-voltage activated' or 'L-type' Ca^{2+} channels (I_{Ca}) (Vogalis et al.,1993).

ACTION OF EXTERNALLY-APPLIED MTX

MTX (5nM) induced a massive increase in the holding current (at a holding potential of –80 mV), from about 50–100pA to about 1000-3000 pA, but only after a delay of some 15–45 minutes (n=14 cells). When the whole-cell membrane currents (elicited every 20mV between –90 and +30mV) recorded before (Fig.2A) and 34 minutes after (Fig.2B) the application of MTX (5 nM) are illustrated, note the ten-fold change in the vertical scale in Fig.2B. The time-dependent whole-cell currents in control saline were totally swamped by the MTX-induced current (I_{MTX}) which shows little time dependence. During the development of this inward current, however, MTX had little effect on the three whole cell K^+ channel currents or on the Ca^{2+} channel current which underlie the currents in Fig.2A (data not shown). In fact, development of I_{MTX} was little affected by a number of known K^+ channel blockers such as TEA (2–126mM), quinidine (0.5 mM), 4-AP (5mM), glybenclamide (10–20μM) or Ba^{2+} (7.5mM).

The current-voltage (I-V) relationship of the initial peak amplitude (I_{Kto}) and the current at the end of these depolarizing steps in control saline (I_{KCa} + I_{Kdel}) and 34min after MTX (5nM) (Fig.2C) shows that I_{MTX} has no voltage dependence (linear) and reverses in direction near –20 mV, suggesting that MTX induces an increase in the membrane conductance to the cations Na^+ and K^+, or to Cl^-, possibly by the opening of membrane channels.

The ionic selectivity of these MTX-activated channels was investigated by substituting the extracellular Na^+ and intracellular K^+ with other cations known to have different permeating properties. In the TEA^+ (126mM) saline, I_{MTX} induced by 5nM MTX (Fig.3A,top panel; B) was linear between –90 and +40mV and reversed in direction at –60mV, positive of E_K (–78mV), suggesting that current flow was mainly carried by K^+. This I_{MTX} was blocked upon the addition

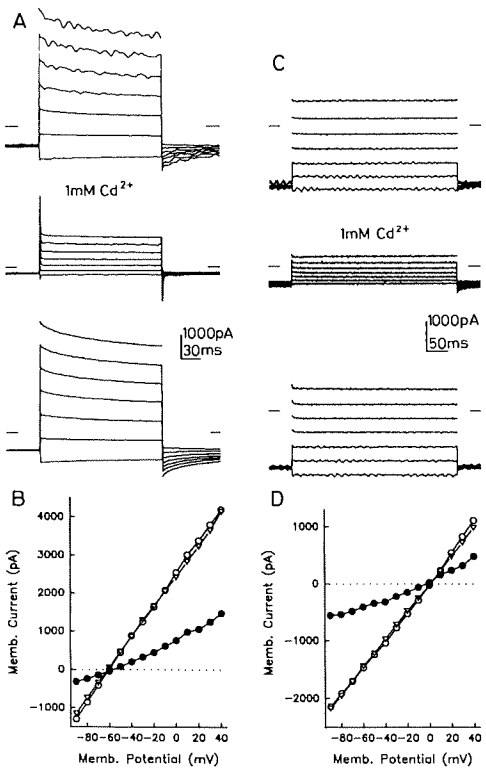

FIG. 3. MTX-induced currents in colonic cells recorded with K^+ or Cs^+-containing pipettes. MTX-induced whole-cell currents (I_{MTX}) every 20 mV between –90 and +30mV (from a holding potential of –80mV) in colonic cells filled with K^+-containing (A, B hollow circles) or Cs^+-containing (C, D hollow circles) pipette solutions. I_{MTX} was blocked by Cd^{2+} (1mM) (A,C middle panels; C,D filled circles), these effects of Cd^{2+} were quickly and readily reversible upon Cd^{2+} removal (A,C lower panels; B,D hollow triangles). Short lines on either side of these panels represent the zero current level. In *A,B*, the external Na^+ concentration had been mostly replaced with the impermeant TEA^+ (126mM), I_{MTX} reversed in direction near –60mV. In Cs^+-filled cells I_{MTX} reversed in direction near 0mV (C,D).

of Cd^{2+} (1mM) to the bathing solution (Fig.3A,middle panel; B). These effects of Cd^{2+} were completely reversible upon its removal (Fig.3A,B,lower panels; C,D). When the intracellular K^+ (126 mM) is replaced with Cs^+ (Fig.3C), a cation known to be permeant through many Ca^{2+} and non-selective cationic channels, but mostly impermeant through K^+ channels (Hille,1984), the reversal potential of I_{MTX} was near 0mV (Fig.3D), a reversal potential 10 to 20mV positive of the reversal potential obtained when K^+ was the main intracellular monovalent cation. The data suggest that I_{MTX} under normal physiological gradients is carried by Na^+ and K^+ and that Cs^+, but not TEA, will also freely pass through these MTX-induced channels

The channels activated by MTX may also allow the flow of, or be modulated by Ca^{2+}. The development of I_{MTX} was not prevented if Ca^{2+} in the bathing solution was omitted. However, I_{MTX} increased in amplitude if the Ca^{2+} concentration was raised to 6.25mM Ca^{2+}. I_{MTX} was also reduced but not blocked by nifedipine (1–10μM) or La^{3+} (1mM).

Effects of Internally-Applied MTX

When MTX (5nM) was added to the pipette solution (containing 3mM EGTA) I_{MTX} developed slowly after formation of the whole-cell seal. Effects of MTX on the instantaneous whole-cell current (Fig.4B) activated by a ramp depolarization (to potentials between –60 and +100mV) showed this current reversed near 0mV and was sensitive to blockade by Cd^{2+} (0.1mM) (Fig.4C). This I_{MTX} was only 200–300pA in amplitude (at –60mV) compared with the 2000–3000 pA current (at –80mV) observed when MTX was applied externally (Fig.2). However, if the Ca^{2+} chelating agent, EGTA, was omitted from the pipette solution the I_{MTX} induced by internally applied MTX was larger and reversed at more negative potentials (near –60mV)(n=2). The addition of TEA (5mM) to bathing solution substantially reduced this I_{MTX} and shifted its reversal potential to near 0mV, suggesting that in the absence of EGTA the internal Ca^{2+} concentration is relatively high so that I_{KCa} contributes to the measured increase in current. These data also suggests that MTX can form/activate its channels from the internal surface of the membrane.

Single Channel Recordings

Recordings of the current flow through single MTX channels were made in the cell-attached patch clamp mode with patch pipettes filled with normal PS containing 10mM TEA and 5nM MTX. The membrane patches were depolarized with ramp depolarizations to obtain the instantaneous I-V relationship of any open channels. After seal formation the slope of the ramped-evoked current increased with time until discrete single channel openings and closings were observed. The current flow through these channels

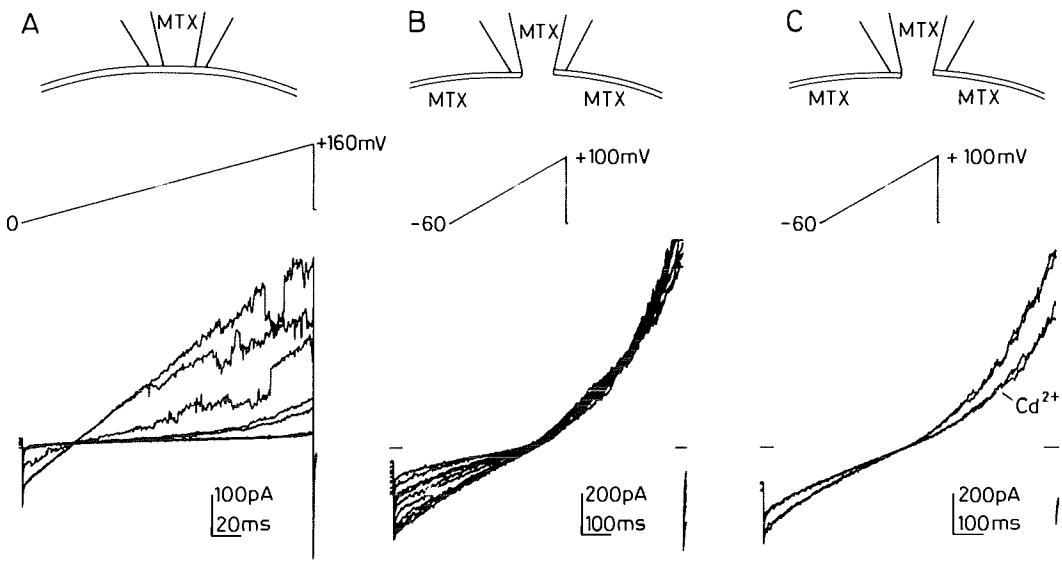

FIG. 4. Influence of pipette-applied MTX. A, cell-attached recordings of the development of MTX-activated single channels. Pipette solution contained normal saline plus 10mM TEA and 5nM MTX. B, development of whole-cell I_{MTX} after introduction of MTX (5nM) into the cell interior. Pipette solution contained high K^+ saline plus 3mM EGTA. I_{MTX} activated by internal MTX was also sensitive to extracellular Cd^{2+} (1mM) blockade (C).

was inward at the resting membrane potential (0mV added to the patch pipette). As the membrane patch was depolarized, however, the single channel amplitudes decreased until zero current flow was recorded at a potential some 30mV positive of the resting membrane potential. Outward current flow was recorded with further depolarization of the membrane patch. Such a reversal potential 30mV positive of the resting membrane potential is consistent with the reversal potential of I_{MTX} (–20 to –10mV) measured under whole-cell voltage clamp (Figs 2A,3A).

DISCUSSION

The spontaneous contractions of the circular muscle of the proximal colon and the contractions to acetylcholine were inhibited concentration-dependently by MTX (0.005-5nM). These effects of MTX followed an initial transient contraction to MTX (Fig.1) which was sensitive to blockade by the muscarinic antagonist, atropine. Given that MTX can stimulate rises in internal Ca^{2+} levels and neurotransmitter release from nerves and glands (Kim et al.,1985; Gusovsky et al.,1988), we suggest that this initial contraction arises from the release of acetylcholine from cholinergic motor neurones known to be present in this colon preparation. The subsequent blockade of the spontaneous activity and the contractions to acetylcholine were mimicked by monensin (0.1-10μM), the Na^+ ionophore, suggesting that a rise in the intracellular Na^+ is induced by MTX.

At the single cell level, MTX triggered a whole-cell inward current, I_{MTX}, that was some 50–100 times larger than the holding current (at –80mV) in control saline (Figs 2,3). The channels opened by MTX appeared equally permeable to K^+ and Na^+ as the reversal potential of I_{MTX} (–30 mV) was midway between E_K and E_{Na}. Confirmation of this reversal potential comes from the cell-attached single channel data which showed that the reversal potential of the single channel currents was some 30mV positive of the cell's resting membrane potential (likely to be –40 to –60mV) (Fig.4A). Replacing most of the external Na^+ with TEA^+ shifted the reversal potential of I_{MTX} to near –60mV, suggesting that, under these conditions, current flow was now mostly carried by K^+. Replacing the internal concentration K^+ with Cs^+ moved the I_{MTX} reversal potential some 20mV positive (to 0mV), even though it had a greater driving force (no added Cs^+ in the bath would mean that its Nernst potential would be very negative), suggesting that Cs^+ does not flow through these MTX channels as readily as K^+.

Preliminary calculations from the amplitudes of current flow through the MTX channels with voltage suggest that these channels have a conductance of up to 100pS. As MTX (5nM) activated an inward current of about 2000–3000 pA (at –80mV), this suggests that 330–500 channels/cell were activated. These channel currents do not arise from the recruitment of normal voltage-operated 'L-type' Ca^{2+} channels since the time course of I_{Ca} (or any of the K^+ currents) activated by membrane depolarization was not altered during the development of I_{MTX} (Yoshii et al.,1987); concentrations of nifedipine (10μM) which would block I_{Ca}, only slightly reduced I_{MTX}, and the reversal potential of I_{MTX} was approximately 50mV negative of the reversal potential of I_{Ca} in Cs^+-filled cells (Vogalis et al.,1993). It has been suggested, however, that MTX may well modulate Ca^+ channels, removing their voltage sensitivities for activation and inactivation and their ionic selectivity (Kobayashi et al.,1987; Yoshii et al. 1987). Perhaps a more attractive hypothesis is that MTX is triggering the opening of cation-selective channels normally opened by acetylcholine. These channels allow the flow of small cations and Ca^{2+}, have a reversal potential near 0mV and are opened by muscarinic agonists which stimulate phosphoinositide hydrolysis and IP_3-induced release of stored Ca^{2+} (Sims,1992). These channels, however, show a marked outward rectification at negative potentials and have a single channel conductance of 20–25pS (Inoue et al., 1987). If MTX is indeed opening these channels it must also be modifying them, removing their rectifying properties and perhaps inducing some sort of 'channel clustering'. If this is correct, our results indicate that 4–5 cholinergic channels would be needed to form a 'single' conducting pore with a conductance of 100pS. Other explanations are that MTX is an ionophore, or that it induces a pore in association with a membrane protein not necessarily involved in ion conductance. The delay in the action of MTX may also suggests that several MTX might be acting cooperatively.

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IMMUNOLOGICAL, BIOCHEMICAL AND CHEMICAL FEATURES OF CIGUATOXINS: IMPLICATIONS FOR THE DETECTION OF CIGUATERIC FISH

RICHARD J. LEWIS

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A major advance in the management of ciguatera will come with the development of a validated, cost-effective assay that detects ciguatoxins contaminating fish. Progress towards such a goal is summarised and the implications for detection of the toxins involved in ciguatera are discussed. Ciguatera results predominantly from CTX-1 which is present at >0.1ppb (10^{-10} mole/kg) in the flesh of carnivorous fish. Other toxins in ciguateric fish are likely to have no more than a minor role in ciguatera. Consequently, CTX-1 should be the principal target of any assay for ciguateric fish. However, significant levels of the less potent ciguatoxins, particularly ciguatoxin-2 and -3, may also accumulate in fish and such toxins could potentially interfere with the response of an assay. Ciguatoxins-1, -2 and -3 have an affinity for voltage-dependant sodium channels (ED_{50} = 0.2 –0.9nM) that is proportional to their i.p. LD_{50}s in mice. Assays (biosensors) that measure this binding or perhaps the ciguatoxin induced sodium channel opening may provide a sensitive assay for ciguatoxins with a response proportional to toxin potency. Ciguatoxins also bind to a range of other proteins, which may interfere with the response of some assays. Alternatively, such interactions may be utilised in the development of novel sandwhich-type assays. Ciguatoxins-1, -2 and -3 do not possess a useful chromophore for selective spectroscopic detection; however, each possesses a relatively reactive primary hydroxyl through which a label could be attached (after appropriate clean-up) prior to detection. Detectors (e.g. fluorescence or mass spectrometry) coupled to optimised HPLC may provide the required sensitivity for analytical detection of derivatised ciguatoxins in crude extracts of fish. Such assays could replace the mouse bioassay for the validation of responses obtained by more rapid screening assays.

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Ciguatera is a disease with a wide array of gastrointestinal and neurological symptoms. It stems mostly from the effects of ciguatoxin-1 (Fig.1), the most potent of the ciguatoxins (Murata et al.,1990; Lewis et al.,1991; Lewis & Sellin,1992). The disease can be debilitating and slowly resolving but is seldom fatal. In many Western countries outbreaks of ciguatera often attract media attention with a consequent negative impact on the marketing of seafood and victims of ciguatera may seek compensation through the courts. A cost-effective means of detecting ciguateric fish prior to consumption remains one of the few management options that can directly reduce the adverse impacts of ciguatera. Antibody-based assays appear to hold most promise since they are able to detect, under favourable circumstances, targeted compounds to 10^{-12} M and can be developed as cost-effective screens (Gazzaz et al.,1992). This paper reviews antibody-based assays for the identification of ciguateric fish and discusses immunological, biochemical and chemical features of the ciguatoxins relevant to their detection.

DEVELOPMENT OF ANTIBODY-BASED ASSAYS FOR CIGUATERIC FISH

The potential of an antibody-based screening assay for detecting ciguatoxin in fish flesh was first indicated by Hokama et al. (1977). Hokama has since led efforts to develop a rapid screen for ciguateric fish (Hokama,1991). The original radioimmunoassay screened 88% of moray eel and 38% of other fish as toxic (>3.5 x 10^5cpm/g; Kimura et al.,1982) despite these fish rating as non-toxic by the mongoose assay. Despite this high false positive result, all fish rating toxic by the mongoose rated as toxic by the antibody assay, indicating the potential for this approach to detect ciguateric fish. This assay was subsequently employed to screen 5,529 *Seriola dumerili* captured in Hawaiian waters (Kimura et al.,1982). This study found 15% of *S. dumerili* tested positive, with the remaining fish, including those >9kg which normally are not marketed owing to their perceived higher risk of ciguatera, being consumed without incident. The quantity of additional *S. dumerili* entering the market in-

creased 68% as the direct result of the study. However, Kimura *et al.* (1982) found that 7% of fish (3 of 42) clinically implicated in ciguatera tested negative by the radioimmunoassay.

In 1984 the radioimmunoassay for ciguateric fish was replaced by a simpler enzyme immunoassay that in binding inhibition assays was sensitive to as little as 5pg of free ciguatoxin (Hokama et al., 1983,1984). The cross-reactivity of this assay with other polyether toxins was also documented. An enzyme labelled polyclonal antibody was subsequently used to develop a further simplified 'stick-test' that rapidly distinguished toxic from non-toxic flesh samples (Hokama, 1985); however six tests per fish appeared necessary for accurate determination of ciguateric fish that tested close to the borderline level. With the stick-test the rejection rate for *S. dumerili* was only 11% and *S. dumerili* testing non-toxic were consumed without incident. This assay responded directly to >1.0ng of pure ciguatoxin (Hokama,1985).

These early studies all employed a polyclonal antibody raised to ciguatoxin in sheep with the disadvantage that for long-term antibody production a continual supply of antigen is required for booster injections. Monoclonal antibodies, on the other hand, can provide a continuous supply of a selected antibody. Hokama et al. (1985,1989b) reported production of monoclonal antibodies to a related polyether toxin okadaic acid as well as to ciguatoxin (likely CTX-1). Using a monoclonal IgG to ciguatoxin in a stick enzyme immunoassay, Hokama et al. (1989a) found that 98% of fish implicated in ciguatera (50 of 51) tested positive, while 55% of a random mix of fish, 55% of *Ctenochaetus striatus* and 44% of *S. dumerili* tested positive. A further simplified solid-phase immunobead assay for detection of ciguateric fish (Hokama,1990) appeared more sensitive than previous stick tests.

The monoclonal antibody to ciguatoxin used in these studies has been assessed for cross-reactivity to other polyether toxins (Hokama *et al.*, 1989b,1992). The assay employing the antibody raised to ciguatoxin detected similar concentrations of pure ciguatoxin, okadaic and a synthesised fragment of okadaic acid (EC_{50} = ~ 0.5 ng of toxin per ml methanol). The cross-reactivity of an antibody for ciguatoxin and okadaic acid is somewhat unexpected, given recent molecular modelling studies which show that in solution the structure of okadaic acid is quite different from that of ciguatoxin (Norte et al.,1991; Lewis and Ramsdale, unpublished observations). It is also intriguing that a monoclonal antibody obtained specifically to okadaic acid was less sensitive at detecting okadaic acid (EC_{50} ~15ng/ml) than the ciguatoxin antibody using the same assay format. Somewhat different cross-reactivity was reported in an earlier study (Hokama et al.,1989b). Importantly, addition of pure ciguatoxin (EC_{50} ~1 ng/ml) and okadaic acid (EC_{50} ~3ng/ml) to a ciguatoxin antibody inhibited the subsequent binding of this antibody to stickscoated with an extract from a fish implicated in ciguatera. This result suggests these polyether toxins compete at a specific, saturable site on the IgG. Since ciguatoxin alone can bind to correction fluid coated sticks (the Hokama poke stick method is based on the ability of sticks dipped into an alchohol-based typist's correction fluid to extract and immobilise CTX prior to detection of this immobilised CTX with labelled antibody) and subsequently bind antibody but the preformed antibody-CTX complex is no longer able to bind to such sticks through the CTX link, it is possible that CTX binding sites on the coated sticks are also saturable.

Development of a commercial screening assay for ciguateric fish is being undertaken by Hawaii Chemtec International who purchased development rights for the Hokama stick test from the University of Hawaii. Recent findings are given in other articles in this memoir.

IMMUNOLOGICAL FEATURES

An antibody response is elicited in an animal following injection of an immunogenic antigen. The ciguatoxins (m/z = 1,094–1,110) are relatively small haptens that are likely to have only low immunogenicity. The absence of any protection in people repeatedly exposed to ciguatoxins through their diet supports this suggestion. A hapten will become immunogenic when covalently conjugated to a carrier protein possessing high immunogenicity (Erlanger,1980). However, non-covalent conjugation and/or Fruends' complete adjuvant are unable to significantly enhance the immunogenicity of native haptens (Layton et al.,1987; Gazzaz et al.,1992). One draw-back with having to use a hapten conjugated to a carrier protein is that the antibodies obtained often do not have high specificity for the unconjugated (native) form of the hapten.

Ciguatoxins possess a relatively reactive primary hydroxyl (Murata et al.,1990; Lewis et al.,1991; Lewis et al.,1993) which can be reacted with succinic anhydride to yield a hemisuccinate.

The hemisuccinate so formed has an available carboxyl group through which ciguatoxin can now be linked to a carrier protein (e.g. bovine serum albumin, keyhole limpet haemocyanin, ovalbumin) using a water soluble carbodiimide cross-linking reagent. Ciguatoxin cross-linked to a carrier protein in this way is expected to have considerably enhanced immunogenicity compared with the native ciguatoxin. Such a complex may additionally have reduced potency, an important consideration for *in vivo* immunisation. The availability of only one easily accessible site on ciguatoxin for conjugation to a protein limits (Mandal & Latif, 1988) the possibilities for producing a range of antibodies possessing differing selectivities for the various ciguatoxin analogues. To-date antibodies to ciguatoxin have not been obtained using an immunogen covalently attached to a carrier protein. However, the use of such an immunogen is presently under consideration in a number of laboratories. Attempts in our laboratory to produce a hemisuccinate of ciguatoxin-1 have met with little success, despite the use of succinic anhydride in dried pyridine at 80°C (Baden et al.,1984), a method that we successfully used to produce a hemisuccinate of brevetoxin-3 (Lewis unpubl. data).

To obtain a maximal *in vivo* immune response with small quantities of immunogen, the immunogen should be emulsified in Fruends' complete adjuvant prior to injection (Vaitukaitis, 1981; Smith et al.,1992). Blood from suitably immunised animals is then collected and assessed for selective antibody titre against the compound of interest. For monoclonal antibody production, the spleen cells of immunised mice are fused with a murine myoloma cell line, with hybridoma(s) secreting ciguatoxin-specific immunoglobulins subsequently isolated using an appropriate screen. This approach follows well described procedures (Galfrè & Milstein,1981; Goding,1986; Peters & Baumgarten,1992). Alternatively, monoclonal antibodies can be obtained through *in vitro* immunisation procedures (Brazeau et al., 1982; Van Ness et al.,1984; Buchman et al.,1985; Borrebaeck,1986; Brams et al.,1987; James & Bell,1987; Borrebaeck & Glad,1989). This approach allows the production of high specificity antibodies (including human antibodies) with small quantities of immunogen and compared with *in vivo* immunisation is less susceptible to immunogen toxicity. Modifications to standard *in vivo* immunisation protocols can also reduce the quantity of immunogen required (Vaitukaitis,1981; Forest & Ross,1993). Such *in vitro* and *in vivo* approaches may be useful for production of antibodies to ciguatoxin.

To allow detection of potentially useful antibodies, a screen must be developed that is selective only for those antibodies that combine with high affinity to the compound being targeted. Such a screen may utilise a hapten-protein conjugate that is attached, through non-specific interactions, to the plastic surface of microtitre plates. To avoid potential problems of cross-reactivity to carrier protein or to epitopes extending beyond the hapten itself, a protein *and* cross-linking reagent should be used in screening that is different from that used in the preparation of the immunogen. Additionally, any method employed to couple ciguatoxin to a carrier protein should not alter the structure of the targeted compound, otherwise antibodies may be produced that will not recognise the native compound. This is equally important for compounds labelled for use as the competitor in competitive antibody binding assays. The careful choice of blockers (Tween 20, fetal calf serum, albumins etc) must be made to ensure that the response is specific for the compound of interest (e.g. ciguatoxin). One pitfall is the ability of certain clones to express antibody which binds promiscuously to plastic (Conger *et al.*,1988). We have noted that exposing plastic microtitire plates to methanol-water (1:1) increases the plate's affinity for IgG, an effect that could not be blocked by traditional blockers and gave rise to a number of false postive results.

Ciguatoxin recognising antibodies obtained in an appropriate manner, and with confirmation that they are specific for only the CTX-class of polyethers, could then be used in the detection of ciguateric fish. Screening assays employing antibodies that have both high affinity and selectivity for an epitope on ciguatoxin-1 are essential if routine detection of the low levels of ciguatoxins (10^{-10} to 5 x 10^{-9}g CTX-1/g) contaminating the flesh of ciguateric fish is to be achieved. Cross- reactivity to other ciguatoxin congeners may also be acceptable. Ideally the chosen antibodies should have an affinity that is directly proportional to the oral potency (to humans) of the contaminating toxins and should not cross-react with compounds normally present in non-toxic fish. The high cross-reactivity of ciguatoxin antibodies to less potent and structurally dissimilar polyether toxins such as okadaic acid (Hokama et al.,1989b,1992) may in part explain the high number of false positive results obtained with assays employing such antibodies. Recent studies using steroid-antibody

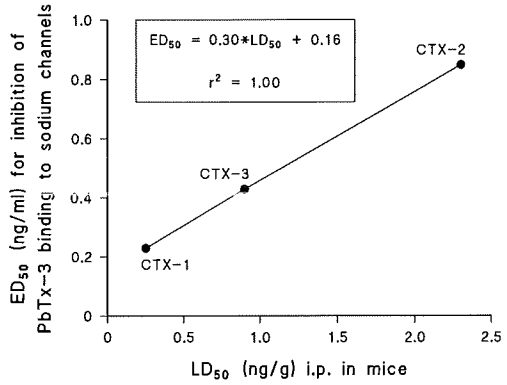

FIG. 1. Relationship between sodium channel binding affinity and mouse lethality for three ciguatoxins.

interactions as a model, have revealed that antibodies recognising apolar and functionally inert molecules (such as ciguatoxin) can have a high afinity binding site but that this site apparently can't be engineered with high specificity to avoid its cross-reactivity with related ligands (Arevalo et al.,1993). In contrast to antibodies that recognise ciguatoxin, and somewhat surprisingly given the previous statement, antibodies raised to brevetoxin and okadaic acid have been found to possess low cross-reactivity with other polyethers including CTX-1, -2, and -3 (Levine et al.,1988; Lewis et al.,1991; Poli et al.,1992) and it remains to be confirmed if it is possible to obtain brevetoxin-antibodies that cross-react with ciguatoxin. Non-selective bindings of ciguatoxin to IgG and non-selective binding of IgG to fish tissue (Parc et al.,1979; Chanteau et al.,1981; Emerson et al.,1983) may present additional obstacles to the development of a successful screening assay for ciguateric fish. The limited experience of other laboratories with several prototype assays utilising polyclonal (Berger & Berger,1979) and monoclonal antibodies (Lewis, unpubl. data) developed by Hokama have at times given less than satisfactory results that are difficult to explain.

BIOCHEMICAL FEATURES

Ciguatoxin binding to sodium channels

Ciguatoxins are characterised by high affinity binding (ED_{50} = 0.23–0.85ng/ml) for voltage sensitive sodium channels (Lewis et al.,1991). The binding affinity of each ciguatoxin for the sodium channel (ED_{50}) is proportional to its i.p. LD_{50} in mice (Lewis et al.,1991) indicating that the lethal effect of the ciguatoxins likely stem from their action on sodium channels. Interestingly, this relationship is found to be linear (ED_{50} = 0.30 x LD_{50} + 0.16) for CTX-1, -2 and -3 (Fig. 1). That this relationship has a slope of 0.3 indiates that binding affinity is not directly proportional to lethality and additional factor(s), presumably pharmacokinetic (Lewis et al.,1991) attenuate the lethality of the ciguatoxins with lower binding affinity. Since these ED_{50}s are a measure of the on-rate for binding, differences in off-rate may in part account for the above result. However, recent pharmacological studies indicate that the off-rates for ciguatoxins-1, -2 and -3 are similarly slow (Lewis & Wong Hoy,1993).

Ciguatoxins are sodium channel activator toxins that bind to site 5 on sodium channels, a site overlapping the brevetoxin binding site (Lombet et al.,1987; Lewis et al.,1991). Ciguatoxin binding leads to the opening of sodium channels which in turn results in an influx of sodium ions, cell depolarisation and the appearance of spontaneous actions potentials. The high affinity binding and subsequent alteration of voltage dependent sodium channels by ciguatoxins could form the basis of biosensor-type assays able to screen for the ciguatoxins. The technology for developing such biosensors is progressing rapidly (Ogert et al.,1992; Malmqvist,1993) but there are few commercial biosensor products available (Griffiths & Hall,1993). The advantage of biosensor technology is that the response can be proportional to level of sodium channel activator in a mixture. The mouse bioassay could be considered a crude form of biosensor. This assay has been validated for detection of ciguatoxins in up to 20mg of lipid extract from fish flesh (Lewis & Sellin, 1993). Cell based assays also show potential for detection of sodium channel blocking toxins (Gallacher & Birbeck,1992) and hold potential for detecting sodium channel activator toxins (Hungerford, 1993). Such assays also have the potential to be automated and miniaturised (Goguen & Kedersha,1993).

Ciguatoxin binding to other proteins

Ciguatoxins also have affinity for various proteins including IgG from a variety of sources and fish liver and fish flesh proteins (Parc et al., 1979; Emerson et al.,1983; Vernoux et al., 1985; Hahn & Capra,1992). The affinity of ciguatoxin for these proteins has not been quantified and these studies have failed to exclude the possibility that the binding they are measuring is not simply

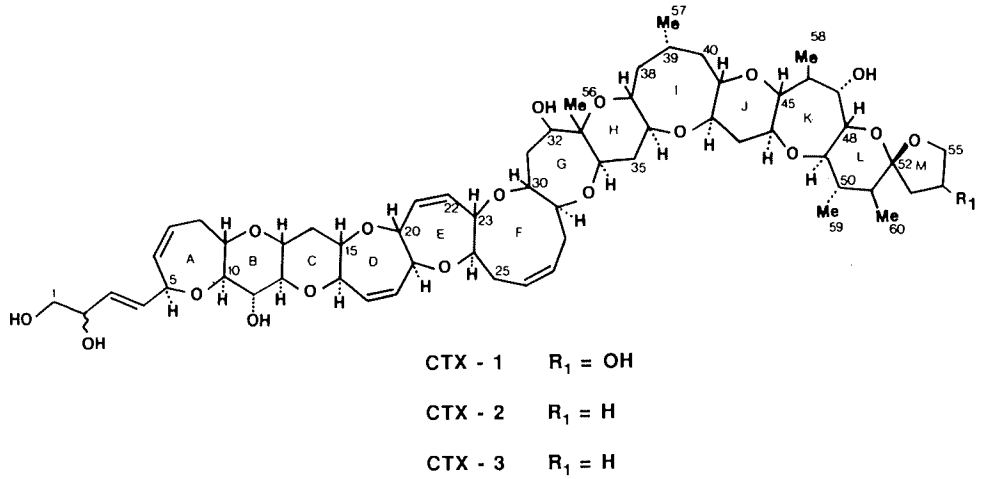

FIG. 2. Structures of the major ciguatoxins present in the flesh of carnivorous fish in the Pacific. CTX-2 is 52-epi CTX-3 (stereochemistry shown for CTX-1 and CTX-3).

the binding of ciguatoxin to the sodium channels present in the protein-containing extracts. Such an interaction is expected to be considerable in tissues rich in excitable cells (e.g. flesh). A putative ciguatoxin binding protein has been detected in fish flesh and its appearance has been proposed to result from the exposure of fish to ciguatoxin (Hahn & Capra,1992). Such a protein in fish may explain why fish are relatively less susceptible to the ciguatoxins than the closely related brevetoxins (Lewis,1992). An assay to detect such a protein in fish could provide the basis for a novel screening assay for ciguateric fish.

Any high affinity binding of ciguatoxins to proteins (including sodium channels) present in fish tissue could be used to immobilise ciguatoxin to a solid phase prior to detection with a labelled antibody specific for ciguatoxin-1. This approach can be considered a type of sandwich assay and could allow rapid detection of ciguatoxin in fish flesh without the need for a solvent extraction step. Such an approach warrants further investigation but requires an antibody that can 'see' the portion of ciguatoxin molecule not obscured as a result of its binding to the fish protein.

CHEMICAL FEATURES OF THE CIGUATOXINS

Structural features of the ciguatoxins

Ciguatoxins (Fig. 2) are lipid-soluble toxins consisting of 13 rings fused by ether linkages into a mostly rigid, ladder-like structure (Murata et al.,1990; Lewis et al.,1991,1993a); they are relatively inert molecules that can accumulate in fish to levels that are toxic to humans, a feature that distinguishes them from chemically and biochemically related brevetoxins which are lethal to fish before levels can be accumulated that cause human poisoning. Structurally related ciguatoxins-1, -2 and -3, are present in carnivorous fish (Lewis et al.,1991; Lewis & Sellin,1992; Lewis et al.,1993a). However, CTX-1 contributes most to the toxicity of ciguateric fish (Lewis & Sellin,1992) so an assay selective for CTX-1 would have general utility for detection of ciguateric fish. Involvement in human poisonings of other toxins (CTX-2, -3, gambiertoxins etc) in ciguateric fish remains unsubstantiated. The role in ciguatera for toxins other than site 5 sodium channel activator toxins is remote.

Extraction of ciguatoxins

Ciguateric fish contain 0.1–5ppb CTX-2 (Lewis & Sellin,1992; Lewis,1992). Whilst direct detection may be possible, extraction and clean-up procedures can be used to concentrate the ciguatoxins. The ciguatoxins are sufficiently hydrophobic to be insoluble in water but can be extracted from fish flesh with organic solvents such as methanol, chloroform or acetone. However, even with a several step clean-up procedure involving removal of low polarity lipids and water-soluble material, the ciguatoxins are still

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IMPACT OF A VALIDATED, COST EFFECTIVE SCREEN FOR CIGUATERIC FISH

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Ciguatoxins contaminating ciguateric fish may be detected by a range of *in vivo* (e.g. mouse, cat, mosquito or chicken), *in vitro* (ELISA, atria) and chemical assays. Current research seeks a selective screen to detect low levels of ciguatoxin-1 (0.05–5.0ppb) in fish flesh or in an easy-to-prepare extract. This review summarises requirements for a validated, cost effective screen for ciguateric fish. Implementation of such a screen will reduce adverse health effects. An attendant benefit will be the improved marketability of reef fish.

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One important goal of present-day ciguatera research is the development of a cost-effective screen for the toxins contaminating ciguateric fish (Lewis,1993). The range of toxins involved in ciguatera has been the subject of some debate. It appears that only the ciguatoxins and analogues (e.g. gambiertoxins) are involved (Murata et al., 1990; Legrand et al.,1992; Lewis et al.,1991). These toxins are closely related in structure and all activate voltage-dependent sodium channels. Of these, CTX-1 typically contributes ~90% of the toxicity of ciguateric carnivorous fish in the Pacific (Legrand et al.,1992; Lewis & Sellin, 1992) and should be considered the primary target of a screen for ciguateric fish. Other ciguatoxin analogues and toxins such as okadaic acid, maitotoxins or *Trichodesmium* toxins are likely to play a minor role in human illness. However, these lower potency toxins could interfere with the response of a screen. The challenge for researchers is to develop a method that can rapidly and selectively screen CTX-1 which is present between 0.1 and 5ppb in the flesh of fish that cause ciguatera (Lewis,1992). Using a 2-fold risk factor to ensure public health is protected necessitates that the screen be capable of reliably detecting CTX-1 in fish flesh at 0.05ppb (50ppt) and above.

A number of assays have been used to detect ciguatoxin in fish. These include a range of *in vivo* assays (e.g. mouse, cat, chicken, mosquito: Banner et al.,1960; Kimura et al.,1982; Lewis & Endean,1984; Bagnis et al.,1985; Vernoux et al., 1985), a number of *in vitro* assays utilising antibodies (Hokama,1991) or isolated tissues (Kimura et al.,1982) and chemical assays involving derivatisation and HPLC separation with fluorescence detection (Legrand et al.,1992; Dickey et al.,1992). Biosensor-type assays are also under development and this approach holds much promise for the detection of ciguateric fish. These assays remain to be validated for their ability to selectively detect ciguatoxins in crude extracts of fish. We validated the mouse assay for ciguatoxin in up to 20mg of ether extract (Lewis & Sellin,1993). This assay was only able to detect CTX-1 at >0.5ppb in fish flesh. Importantly, this study found that 63± 14% of spiked ciguatoxin was recovered using a standard extraction procedure, thereby establishing its suitability for confirming whether a fish sample was toxic or not. The cost of such an assay, as well as its insufficient sensitivity and ethical considerations, preclude the use of the mouse assay for routine seafood monitoring programmes. This paper summarises requirements for a validated, cost-effective screen for ciguateric fish and discusses some of the impacts of such a test on fisheries and fisheries products.

FEATURES OF A USEFUL SCREEN

A useful screen for detecting ciguateric fish needs to possess the following features: 1, simple implementation; 2, ready availability and long shelf life; 3, toxin selectivity proportional to the human oral potency of the ciguatoxin analogues (ie highest affinity for CTX-1); 4, it should yield a linear response over the range of toxin concentrations encountered in ciguateric fish; 5, acceptable accuracy (±20%) at the level of 0.05ppb CTX-1 that is independent of the fish tested; 6, high recovery of CTX-1 (>60%) during extraction and clean-up (>30% in exceptional circumstances); and 7, total cost of screen must be acceptable to the consumer.

Prototype stick tests (Hokama,1985; Hokama et al.,1985; Hokama,1991) have many of the

above features, but this or modified versions of the test are still not readily available. Published results of screening of fish suggest (but do not prove) that these tests have sufficient sensitivity (i.e. few false positive results reported). However, the apparent high frequency of false positive results (i.e. non-toxic fish rejected) suggests that the antibody employed may have a relatively low specificity for CTX-1.

A screen that utilises high affinity binding of ciguatoxin to a cheap protein substrate (e.g. IgG) and couples this interaction to a simply measured response (e.g. a colour change) has a high likelihood of achieving an adequate compromise between accuracy and cost. Such antibody-based screens have the potential to detect levels of analyte as low as 10^{-12}M in food (Gazzaz et al., 1992). However, matrix effects associated with the type of sample screened can often dramatically reduce assay sensitivity. The extent of such matrix effects can also vary with the solubility characteristics of the analyte. For solid food matrices, enzyme linked immunosorbent assays (ELISA) detected okadaic acid in shellfish of 10-300ppb (DSP-check, Ube Industries, Ltd, Tokyo) and detected aflatoxins above 10ppb (Dorner & Cole,1989; Trucksess et al.,1989) or more recently above 1 ppb in a range of solid foods (Agri-Screen test for aflatoxins, Neogen Corporation, Michigan). The challenge is to develop a rapid screen for ciguatoxins that has one to two orders of magnitude greater sensitivity than presently available ELISA assays. A variety of approaches may be used to improve the sensitivity of antibody-based assays: (i) production of higher affinity antibodies (ii) optimising assay conditions in which the antibody is used (iii) amplifying the assay signal (not always accompanied by improved signal to noise) (iv) optimising sample extraction and clean-up (can add a significant cost). The potential of the latter approach is indicated for ciguatoxin which can be concentrated from the levels in flesh of 0.1-5ppb to levels of ~30–5,000ppb in a crude lipid extract with a two-step clean-up procedure that has 63% efficiency (Lewis & Sellin,1993).

Toxins responsible for ciguatera arise through the biotransformation of a number of gambiertoxins produced by *Gambierdiscus toxicus* (Murata et al.,1990; Holmes et al.,1991; Legrand et al.,1992). So a range of low potency forms of the ciguatoxins and gambiertoxins (including CTX-2 and CTX-3) could accumulate in fish. These low potency forms may be detected with antibodies raised to CTX-1 and if so they could give rise to false positive results. To-date the cross-reactivity of the antibodies in use for the various ciguatoxin analogues has not been established.

An acceptable cost for ciguatera screening has not been determined. This will relate to the added value screened fish will attract in the marketplace. An add-on cost of less than 10% of the value of the product may be reasonable. The 'cost' of a screening programme should incorporate an estimate of the cost of discarding non-toxic fish as a result of false positive results. It may be possible to reduce the cost of screening by pooling fish samples prior to screening. Such an approach requires a highly sensitive screen but could work where ciguatera is a low level problem (e.g. Australia). This approach could not work where non-toxic fish have levels of ciguatoxin within an order of magnitude of levels that cause human poisoning.

A blind screening of ciguateric fish from Australia (specimens confirmed ciguateric by human and mice assays) with a prototype of the Ciguatect™ test kit (November,1991) revealed that there were few, if any, false positive results. However, there was a strong possibility that some ciguatoxic samples went undetected by the test. In fact five of six of the ciguateric fish samples are likely to have given a false negative result. Further testing of these fish using later modifications of the Ciguatect™ test are not reported and no satisfactory explanation has been given for the conflicting results obtained. These results were obtained at a time when the test was being considered for commercial release. Since this time modifications to the test have been made but a final format for the Ciguatect™ test remains elusive.

TYPES OF ANTIBODY ASSAYS AVAILABLE

Several approaches are available for incorporating antibodies (or similar proteins) into an assay format for detecting haptens such as ciguatoxin (Fig.1). In all cases a label, be it a radioisotope, an enzyme or a luminescent or fluorescent probe, is used to detect the targeted compound. Each approach has strengths and weaknesses but all require a high affinity antibody that is selective and specific for the targeted hapten. The first assay considered is the indirect hapten assay which requires that the targeted hapten (e.g. ciguatoxin) is first non-selectively immobilised to a solid support (along with

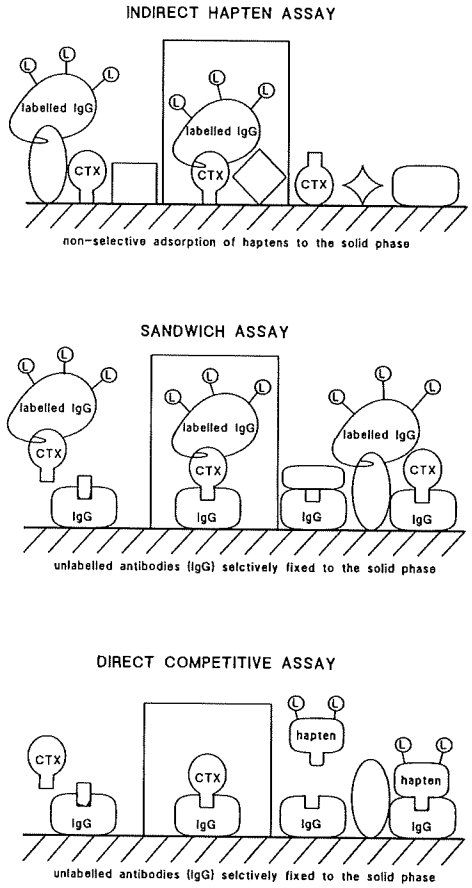

FIG.1. Antibody assays available for the detection of haptens such as ciguatoxin (CTX). Either the IgG or hapten are labelled (L) with probe to indicate the presence of the targeted hapten. Other shapes represent the range of compounds present along with the targeted hapten. The IgG could be replaced with other proteins possessing a high affinity for the targeted hapten (eg the sodium channel could be used for detecting ciguatoxin). The large box in each diagram surrounds the interaction responding to the presence of targeted hapten, while interactions beyond this box result in a reduced (false negative) or enhanced (false positive) assay response that is unrelated to the presence of the targeted hapten.

numerous other contaminants) prior to its detection with a labelled antibody specific for the part of the hapten left exposed following binding. In practice, it is often not possible to obtain an antibody that can still 'see' small haptens bound to a solid phase, but this approach can allow rapid detection of a hapten without a time-consuming extraction step. Tests developed for ciguatoxins by Hokama (1991) and more recently by Hawaii Chemtect (Pasadena) have used this approach in the development of a screen for ciguateric fish.

The second approach is to develop a sandwich assay. This requires development of two antibodies which can mutually bind the targeted hapten. This assay can be more selective than the direct assay although binding reactions in two-site immunoassays are complex and not easily predicted (Boscato et al.,1989). This assay is limited by the difficulties associated with obtaining two antibodies which don't interfere with each others binding to a small hapten such as ciguatoxin.

The third approach is the direct competitive assay which requires that a second hapten is available that competes with the targeted hapten for binding to an antibody. With this approach either the hapten or the antibody can be fixed to the solid phase. Unlike the first two assays, the response is inversely proportional to the targeted hapten, but such a response is likely to be more specific for the targeted hapten than the former assays. The direct competitive assay likely holds most promise for an accurate screen but requires a competing hapten to be found. While it is possible that the sandwich and competitive assays could be developed for the direct sampling of fish flesh, it is likely is that extraction and clean-up steps will be required. The solubility requirements of the hapten and stability of antibody in solvent need to be considered if the assay is designed to detect solubilised hapten.

SCREEN VALIDATION

A number of criteria need to be met before a screen for ciguateric fish can be considered useful, including: 1, the screen must detect all fish samples confirmed ciguateric following human consumption; 2, the screen must have an acceptably low rate of false positives (i.e. a false positive rate within an order of magnitude of the reported ciguatera incidence for the species tested) and the cost of false positive results must be included in the cost of screening; 3, the screen must detect spiked CTX-1 in crude fish extracts at levels occurring naturally and should detect CTX-1 spiked in a fish flesh homogenate; 4, the screen should produce appropriately accurate results for toxic and non-toxic fish both within and between laboratories. Wherever appropriate, negative controls should be run in a pair-wise design and results for these should be negative.

Absence of readily available reference toxins of the ciguatoxin class and lack of a validated analytical method to quantify the level and types of toxins present in samples of fish hinder attempts to validate screening tests for ciguateric fish. The use of the mouse bioassay to assess ciguatoxin levels (Lewis & Sellin in press) is presently the best available alternative to an *in vitro* approach. It could prove misleading to use other *in vivo* bioassays to validate screening tests at this time, especially the unreliable brine shrimp assay (R.J. Lewis unpubl. data) which has recently been used by D.L. Park to characterise the toxins present in fish screened by the S-PIA version of the Ciguatect™ test (Hungerford,1993).

To ensure reliability of a screen, any limitations of a screen with regard to species, sample preparation and storage etc should be well documented. Ideally, the screen should 'work' for all potentially ciguateric fish, irrespective of how they are caught or handled prior to sale. Accuracy must be regularly evaluated with reference to toxic and non-toxic specimens to ensure reliability over time. A sound basis needs to be established for any variation in methodology between control and test procedures.

IMPACT OF A SCREEN FOR CIGUATERIC FISH

Implementation of a useful screen will result in improved marketability of seafood captured in ciguatera endemic areas. Removal of toxic fish before consumption will lead to improved community health standards. With the availability of a screen come possibilities for opening fisheries for species which are presently restricted because of ciguatera. In Queensland new and potentially lucrative fisheries for red bass and perhaps chinaman fish and paddletail could be established once an effective screen is available.

Screening for ciguatera could be conducted at a number a levels in the chain of marketing of fish that includes fisherpersons, wholesalers, commercial companies, government agencies or consumer. Problems are likely to exist for the consumer seeking compensation to prove that the test was indeed performed on the fish involved in the poisoning according to the manufacturers instructions. It may even be necessary to show that the toxin involved was indeed a ciguatoxin. Who ends up conducting the test will depend on the final format of the test, government requirements and the level of risk of ciguatera associated with the fish being screened. Cost of screening will increase if more than one point of testing is required. It is interesting to speculate on the fate of a screening product that is shown to have failed to detect a toxic fish, especially if the fish results in a poisoning episode.

Another issue to be considered is what fish are to be screened. In some areas it might be appropriate to screen all potentially ciguateric reef fish, whereas in other areas only the high risk species presently marketed may need to be screened. Other classes of fish that may require a differential approach include (i) fish presently banned as a result of the threat of ciguatera they pose (ii) fish destined for export and (iii) large whole fish in one of the above categories.

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THE CHANGING FACE OF CIGUATERA PREVALENCE. *Memoirs of the Queensland Museum 34(3) 554. 1994*:- Ciguatera cases in Queensland (recorded mostly by the Queensland Department of Health) between 1965-1992 are compiled into a database of 920 cases attributable to 343 outbreaks. Pelagic fish, mainly mackeral, account for 65% of all recorded cases while reef fish account for 35% of cases. Pelagic fish were found to have a significantly higher prevalence of 8 of the 27 surveyed symptoms than reef fish, these being temperature reversal, diarrhoea, nausea, vomiting, abdominal pain, joint pain, dental pain and ataxia. Northern fish (≤24°S catch location) accounted for 33% of recorded cases while southern fish accounted for 67% of cases. Northern fish were more likely than southern fish to be associated with a neurological symptom profile (odds ratio=2.0; 95% CI [1.38, 2.82]. Neurological profiles (neurological symptoms only) accounted for 18.2% of recorded cases. This symptom profile has become more common over the last decade, reflecting a significant shift in toxic fish consumption from southern pelagic to both northern and reef fish.

A subset of the 920 cases (N=657) were used to model temporal and geographical shifts from 1976-1992 in major responses such as time to onset of first symptom (ONSET) and prevalence of a neurological profile. Statistical modelling included robust regression modelling (generalised additive modelling) and statistical graphics. Significant and complex shifts in temporal and spatial prevalence were found. Results and implications of this modelling are discussed.

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ORAL AND INTRAPERITONEAL ADMINISTRATION STUDIES OF TOXINS DERIVED FROM FISH TISSUES AND EXTRACTS OF CULTURED *G. TOXICUS* IN THE HUMBUG (*D. ARUANUS*), DAMSEL-FISH (*P. WARDI*) AND THE STRIPEY (*L. CARPONOTATUS*). *Memoirs of the Queensland Museum 34(3): 554. 1994*:– Toxin administration experiments were designed to compare effects of ciguatoxin(s) (CTX) and toxin(s) in extracts of *G. toxicus* (GDT) between teleost fish, and between species of teleosts; to quantify bioaccumulation of toxins in fish skeletal muscle; and to obtain evidence of bioconversion of GDT to CTX in treated fish.

Based on interpretation of signs and death-times, CTX and GDT administered i.p. are potent teleost neurotoxins. A comparison of dose effect of *G. toxicus* extract in *D. aruanus* and *P. wardi* shows variable susceptibility to *G. toxicus*-related toxins in fish that may be related to trophic niche.

Feeding and subsequent extraction and quantification of CTX in *L. carponotatus* defined approximate oral effective dosages and rates of incorporation in skeletal muscle. Feeding experiments in *L. carponotatus* indicated that the potency of GDT is at least half that of CTX. *L. carponotatus*, *D. aruanus* and *P. wardi* were unable to bioaccumulate or bioconvert GDT to CTX under these experimental conditions in quantities sufficient for detection in the mouse bioassay of residues derived from the skeletal muscle of experimental fish.

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INVERSE-DETECTED NMR OF CIGUATOXIN: QUATERNARY CARBON LOCATIONS CONFIRMED IN CTX-1

RICHARD J. LEWIS AND IAN M. BRERETON

Lewis, R.J. & Brereton, I.M. 1994 08 01: Inverse-detected NMR of ciguatoxin: quaternary carbon locations confirmed in CTX-1. *Memoirs of the Queensland Museum* **34**(3), 555–559. Brisbane. ISSN 0079-8835.

Short-range (HMQC, $^{1}J_{CH}$) and long-range (HMBC, $^{2,3}J_{CH}$) 2-dimensional inverse-detected heteronuclear nuclear magnetic resonance spectra of 0.45mg of ciguatoxin-1 are shown. These spectra provide independent support for the structure proposed for ciguatoxin-1 and confirm the ^{13}C assignments and the location of the two quaternary carbons in ciguatoxin-1. The presence of four ether linkages was also confirmed from the HMBC experiment.

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A major advance in ciguatera research was the determination of the structure of ciguatoxin (Murata et al.,1989,1990). Ciguatoxin-1 (=CTX-1, Fig.1) is the most toxic ciguatoxin isolated to-date (Lewis et al.,1991) and is dominant in ciguateric fish flesh (Lewis & Sellin,1992). CTX-1 is probably responsible for the clinical syndrome that follows consumption of ciguateric fish; especially carnivorous species.

The structure of CTX-1 was proposed on the basis of one-dimensional ^{1}H NMR and nOe spectroscopy, two-dimensional homonuclear scalar coupled spectroscopy ($^{2\text{-}6}J_{HH}$) and mass spectroscopy (Murata et al.,1989,1990). Short-range (one bond) inverse-detected heteronuclear experiments (HMQC) supported the structure proposed for CTX-1 (Murata et al.,1992). The absolute stereochemistry of CTX-1 (Fig.1) has been proposed (Suzuki et al.,1991). Short-range (HMQC) and long-range (HMBC) inverse-detected spectra of CTX-1, determined using the method of Martin & Crouch (1991), provide independent support for this structure, including confirmation of the location of the two quaternary carbons in the molecule.

METHODS

CIGUATOXIN-1 (CTX-1)

CTX-1 was purified in 1991 (Lewis et al., 1991). NMR experiments were performed on a 0.45mg sample of CTX-1 in 0.45ml of pyridine-d_5 (99.96%, Cambridge Isotope Laboratories).

NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

The formation of heteronuclear multiple quantum coherence between protons and ^{13}C nuclei provides a powerful tool for molecular structure determination. Two dimensional experiments

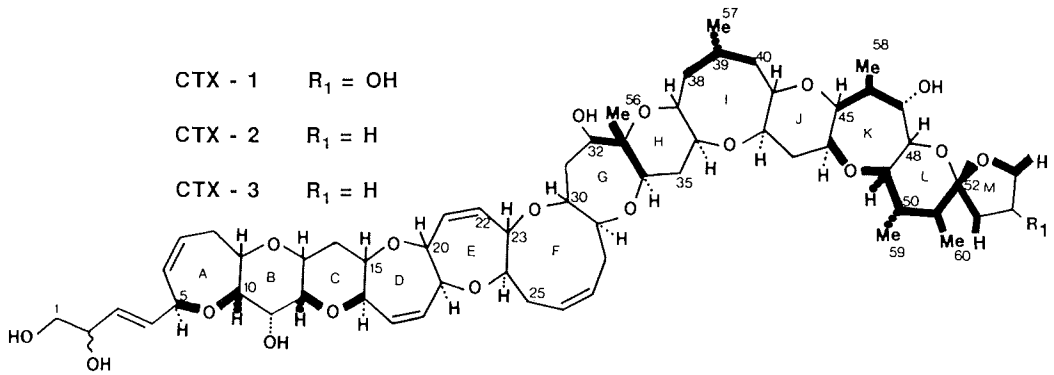

FIG.1.Structure of ciguatoxin-1 (R_1 = OH) proposed by Murata et al. (1990). Connectivities confirmed from the HMBC spectrum are shown bolded.

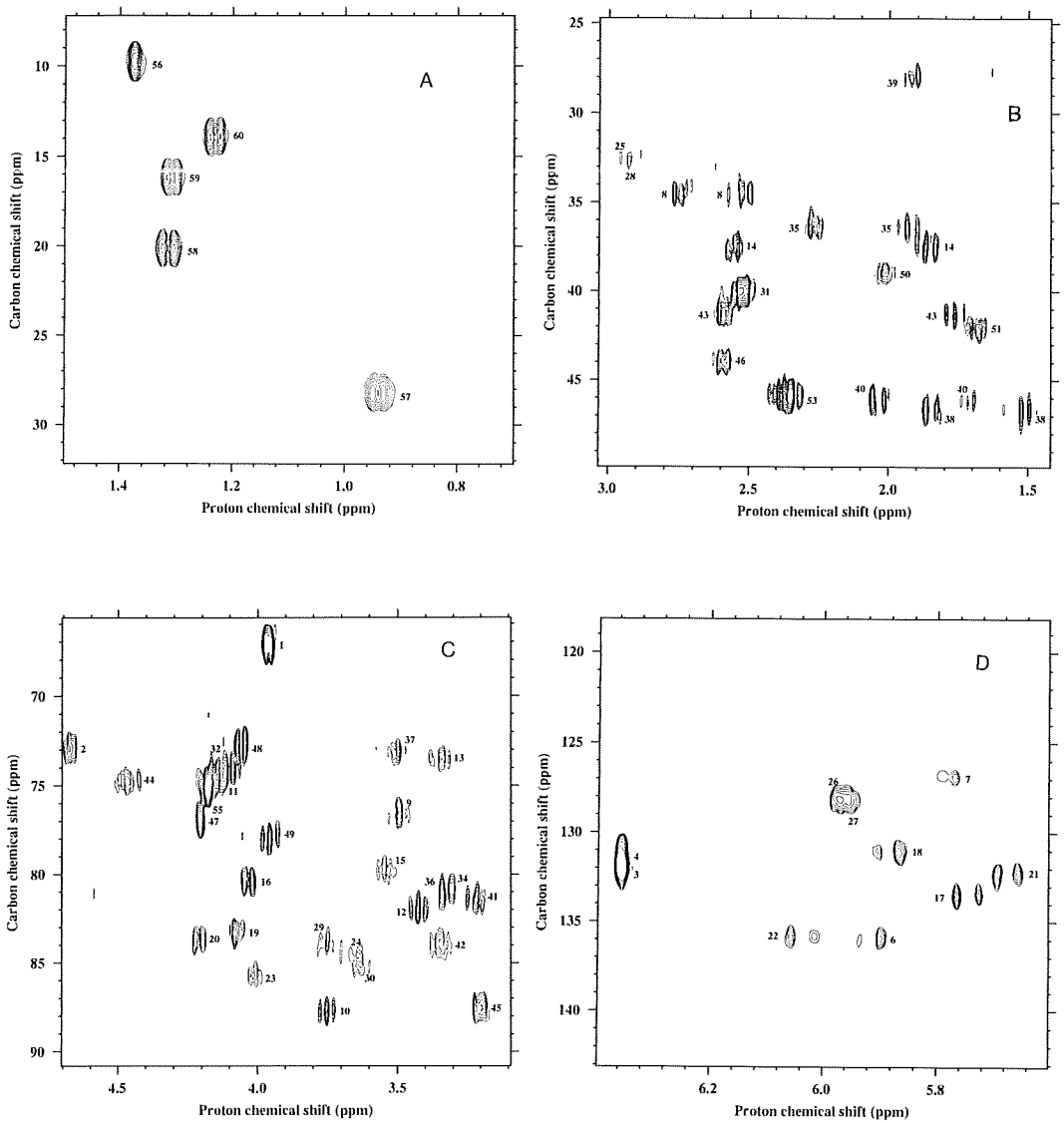

FIG.2. HMQC spectrum of ciguatoxin-1 (CTX-1) at 400 MHz in pyridine-d_5 (30°C). $^1J_{CH}$ clustered into four regions shown in detail in panels A to D. Carbon number of the carbons giving rise to $^1J_{CH}$ are labelled according to the structure of CTX-1 (Fig.1). Data were zero filled to give a matrix consisting of 4 K x 1 K points.

provide extensive proton to carbon connectivities, both direct via one bond scalar coupling ($^1J_{CH}$) with the HMQC experiment and long range via 2 and 3 bond scalar couplings ($^{2,3}J_{CH}$) with the HMBC. The long-range experiment allows correlations across and to quaternary carbons (i.e. carbons without attached protons) and across heteroatoms (e.g. oxygens). Whether short- or long-range correlations are determined depends on the evolutionary manipulation of heteronuclear coherences according to the value of the respective heteronuclear coupling constants (~140Hz for one bond couplings and 5–10 Hz for 2 and 3 bond couplings). The sensitivity of these experiments can be maximised if the generated heteronuclear multiple quantum

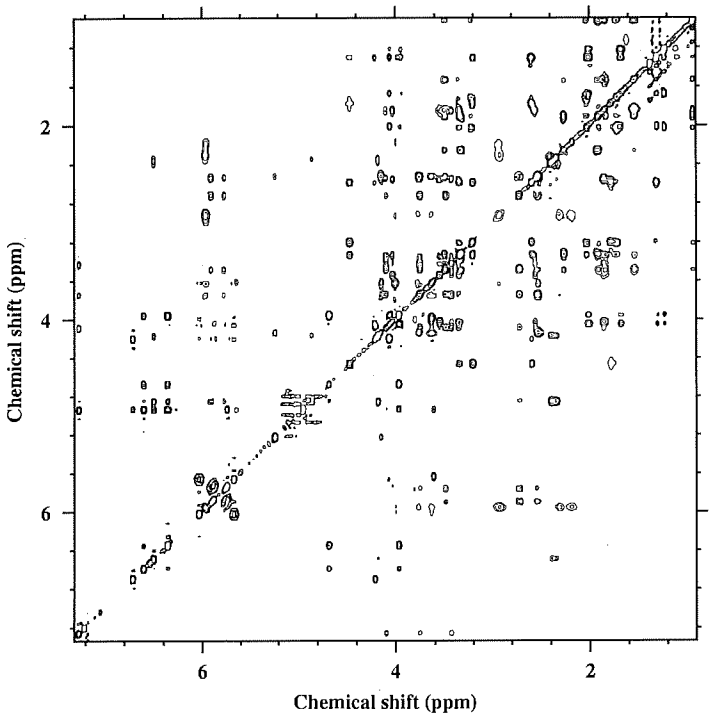

FIG.3.HOHAHA spectrum of ciguatoxin-1 (CTX-1) at 500MHz in pyridine-d_5 (30°C). Chemical exchange between hydroxyl protons of CTX-1 and H_2O was detected as direct and relayed cross-peaks from each hydroxyl proton to the water resonance at 4.94ppm (mixing time 55msec). Data were zero filled to give a matrix consisting of 4 K x 1 K points.

coherences are converted to observable proton signals, rather than using magnetisation from the less sensitive heteronucleus, in the so-called "inverse" or proton detected correlation experiment. The one-bond correlation variant of this experiment is known as the inverse-detected HMQC and the somewhat less sensitive long-range variant as the inverse-detected heteronuclear multiple bond correlation (HMBC) experiment. These procedures were applied to the ciguatoxins as described below.

The short-range ($^1J_{CH}$) inverse-detected (HMQC experiment) two-dimensional NMR spectrum of CTX-1 was obtained at 400MHz on a Bruker AMX-400 at 30°C (206 t_1 values, each with 2048 points (512 scans averaged) over a spectral width of 4000Hz). The long-range ($^{2,3}J_{CH}$) inverse-detected (HMBC) two-dimensional NMR experiment on CTX-1 was performed at 500MHz on a Bruker AMX-500 at 30°C (495 t_1 values, each with 2048 points (96 scans averaged) over a spectral width of 4310Hz) and was optimised for 8.3Hz couplings. The two- dimensional homonuclear Hartman Hahn experiment (HOHAHA) was performed according to Bax & Davis (1985) at 500MHz on an AMX-500 at 30°C (246 t_1 values, each with 2048 points (88 scans averaged) over a spectral width of 4505Hz). No water suppression was used for these experiments. The NMR data were processed using FTTOOL and spectra analysed on a SUN SPARCstation-2 also using this software. For each 2-D experiment, a gaussian deconvolution with line broadening was applied to t_2 data (data zero filled to give 4K points in F2) and a Hamming filter applied to t_1 data (zero-filled to give 1K points in F1) to obtain optimal signal to noise in each dimension. Chemical shifts are given in ppm downfield of the pyridine resonance (^1H at 7.21ppm and ^{13}C at 123.5ppm).

RESULTS

The HMQC spectrum of CTX-1 (Fig.2) detected all $^1J_{CH}$ except those associated with the proton resonances at 4.86 ppm which were obscured by the H_2O resonance which was not suppressed in this experiment. This spectrum allowed us to independentlydetermine the ^{13}C chemical shifts for each carbon on CTX-1 and the ^1H chemical shifts of the attached proton (Table 1). These data correspond to the assignment of Murata et al. (1992), with the minor exception of carbon 36 to which we assign a ^{13}C chemical shift of 81.0ppm, as opposed to 83.69ppm. These data confirm all methines in CTX-1 and the assignment of the double bond in the flexible portion of CTX-1 (ring F). In addition, all carbons assigned as attached to oxygen had ^{13}C chemical shifts characteristic of such a chemical environment. The ^{13}C assignments were confirmed by careful comparison of the coupling patterns of protons observed in the HMQC, HOHAHA and reference

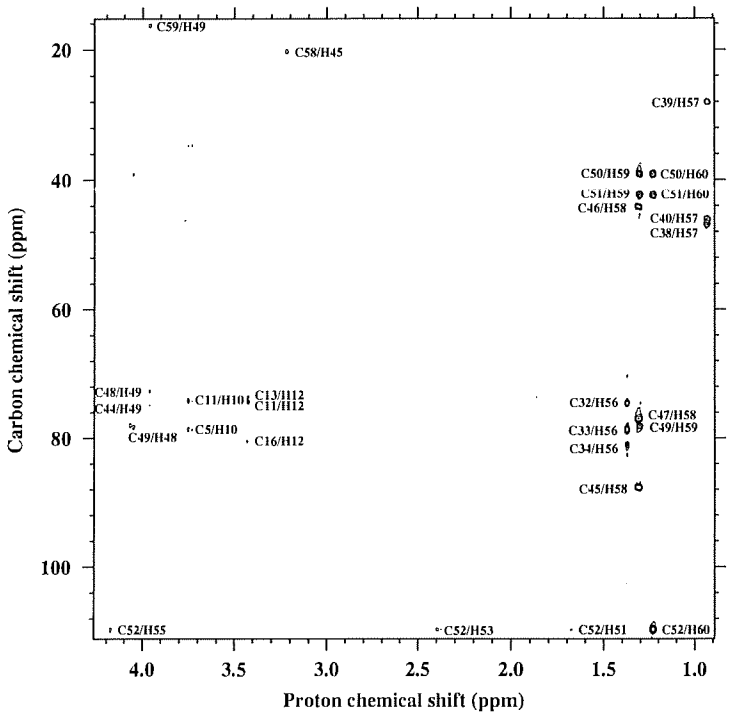

FIG.4.HMBC spectrum of ciguatoxin-1 (CTX-1) at 500 MHz in pyridine-d_5 (30°C). $^{2,3}J_{CH}$ are labelled according to the proposed structure of CTX-1 (Fig. 1). Data were zero filled to give a matrix consisting of 4 K x 1 K points.

1-D ^{1}H NMR spectra. All ^{1}H chemical shifts assigned from the HMBC experiment overlapped the chemical shifts assigned from the HOHAHA experiment also obtained at 30°C (Fig.3). In addition to scalar coupled connectivities, the HOHAHA (55msec mixing time) also detected the chemical exchange between hydroxyl protons of CTX-1 and residual H_2O in the solvent. Similar exchange was detected previously for CTX-2 (Lewis et al.,1993). Such signals provide a useful means of identifying the exchangeable hydroxyl protons in molecules.

The HMBC spectra of CTX-1 (Fig.4) includes $^{2,3}J_{CH}$ for all methyl protons. Fortunately, the two quaternary carbons in CTX-1 (carbons 33 and 52) were within three bonds of a methyl, allowing us to confirm unambiguously the ^{13}C chemical shift (Table 1) and location (Fig.1) of these carbons. Most other long-range couplings expected for the proposed structure of CTX-1 were not detected, at least in part owing to the small quantity of CTX-1 available (experiments were performed on a 1 mM solution). However, we were able to confirm the location of four ether linkages by this experiment (Figs 1,4).

DISCUSSION

We obtained HMQC and HMBC spectra of a 1mM solution of CTX-1 in pyridine-d_5 at 30°C. The HMQC spectrum independently confirmed the ^{13}C assignments of CTX-1 given by Murata et al. (1992). The HMBC spectra confirmed the position of carbons 33 and 52, the two quaternary carbons present in CTX-1 (Fig.1). This spectrum also confirmed the location of four of the 13 ether linked rings (Fig. 1). The location of these quaternary carbons and 12 of the 13 ether linkages was previously inferred from ^{13}C chemical shifts and one-dimensional nOe experiments (Murata et al.,1990).

The HMBC experiment detected all carbons two or three bonds from methyl protons but owing to the relatively poor signal to noise of this experiment (compared to the HMQC experiment) it was not sufficiently sensitive to detect many of the $^{2,3}J_{CH}$ couplings. These couplings may also be either larger or smaller than 8.3Hz, the coupling size for which the experiment was optimised. In conclusion, our data support the structure proposed originally for CTX-1 by Murata et al. (1989).

ACKNOWLEDGEMENTS

We thank Peter Barron (Bruker, Australia) for running the HMQC experiment and Ray Norton and David Doddrell for encouragement and support.

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TABLE 1. ^{13}C and ^{1}H NMR chemical shifts for CTX-1 at 30°C (400MHz, pyridine-d_5) from the HMQC experiment.

Position	^{13}C (ppm)	^{1}H (ppm)	Position	^{13}C (ppm)	^{1}H (ppm)
1	67.1	3.96	31	40.0	2.52,2.52
2	72.9	4.68	32	74.5	4.16
3	132.0	6.36	33	78.6[b]	-
4	131.0	6.36	34	80.8	3.31
5	78.0[a]	4.86	35	36.4	1.92, 2.26
6	136.0	5.91	36	81.0	3.34
7	126.8	5.78	37	73.1	3.50
8	34.6	2.54, 2.73	38	46.8	1.54, 1.84
9	76.6	3.50	39	27.8	1.91
10	87.8	3.75	40	46.1	1.71, 2.03
11	74.1	4.10	41	81.5	3.21
12	82.0	3.43	42	84.0	3.35
13	73.6	3.34	43	41.4	1.78, 2.59
14	37.5	1.85, 2.56	44	74.8	4.47
15	79.7	3.55	45	87.6	3.20
16	80.5	4.03	46	44.0	2.59
17	133.6	5.74	47	77.0	4.21
18	131.1	5.89	48	72.6	4.06
19	83.3	4.07	49	77.9	3.96
20	83.6	4.21	50	39.0	2.01
21	132.3	5.67	51	42.2	1.68
22	135.9	6.04	52	109.7[b]	-
23	85.7	4.02	53	45.9	2.34, 2.40
24	85.0	3.64	54	70.7[a]	4.86
25	32.5	~2.2, 2.96	55	75.1	4.18, 4.18
26	128.2	5.97	56	9.7	1.37
27	128.2	5.96	57	28.3	0.94
28	32.7	~2.3, 2.93	58	20.2	1.32
29	83.9	3.76	59	16.2	1.31
30	85.4	3.63	60	13.9	1.23

[a] 1H chemical shifts at 4.86ppm (2 protons) were obscured by the water resonance (^{13}C values from Murata et al.,1992).

[b] ^{13}C chemical shift values for quaternary carbons from the HMBC experiment at 30°C (500 MHz, pyridine-d_5).

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SUZUKI, T., SATO, O., HIRAMA, M., YAMAMOTO, Y., MURATA, M., YASUMOTO, T. & HARADA, N. 1991. Enantioselective synthesis of the AB ring fragment of gambiertoxin 4B. Implication for the absolute configuration of gambiertoxin 4B and ciguatoxin. Tetrahedron Letters 35: 4505–4508.

ON THE GLOBAL INCREASE OF HARMFUL ALGAL BLOOMS. *Memoirs of the Queensland Museum 343(3): 560. 1994*:– Harmful algal blooms have occurred throughout recorded history but during the past two decades they have increased in frequency, intensity, and geographic distribution; their effects on human health and economics have increased accordingly. To some extent, this reflects our increased awareness of toxic species and the enormous expansion in aquaculture efforts. Evidence is accumulating, however, that human activities contribute significantly to this increase through the stimulation of exceptional blooms by cultural eutrophication (e.g. from domestic, industrial and agricultural wastes; acid precipitation, deforestation and increased runoff from cleared land) and by the spreading of nuisance organisms in ships' ballast water. The global distribution of these phenomena is illustrated with examples from Japan, North America, Europe, South-East Asia and Australia, and involving dinoflagellates, diatoms, prymnesiophytes, raphidophytes and cyanobacteria.

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CIGUATOXIN-1 INDUCES SPONTANEOUS SYNAPTIC ACTIVITY IN ISOLATED SYMPATHETIC GANGLIA OF GUINEA PIGS *Memoirs of the Queensland Museum 34(3): 560. 1994*:– An electrophysiological study has been undertaken of the actions of purified ciguatoxin-1 (CTX-1) on the neurones of guinea pig sympathetic ganglia isolated in vitro, using conventional intracellular microelectrode techniques. Low concentrations of CTX-1 (0.2-0.8nM) applied even briefly (<15min) via the perfusing solution induced a dramatic increase in the spontaneous occurrence of excitatory synaptic potentials (ESPs) which persisted for many hours. The amount and pattern of activity varied between neurones and occurred in the absence of any change in passive or active electrical properties of the neurones themselves. Single supramaximal preganglionic stimuli evoked a summed response which was unaltered after exposure to CTX-1, but was followed by a variable duration high frequency burst of ESPs. These bursts resembled those occurring spontaneously in the same cell, and apparently arose from individual preganglionic axons. The effects were abolished by reduced Ca^{++}, ω-conotoxin, low doses of TTX or raised divalent cation concentrations. The results indicate that some preganglionic axons have CTX-binding sites that open Na^{+} channels causing spontaneous depolarization and initiating repetitive discharges.

Paul Hamblin, Department of Physiology & Pharmacology, University of Queensland, St Lucia, Queensland 4067, Elspeth M. McLachlan & Richard J. Lewis, Southern Fisheries Centre, Department of Primary Industries, P.O. Box 76, Deception Bay, Queensland 4508; 12 April, 1993.

INVERTEBRATES IMPLICATED IN THE TRANSFER OF GAMBIERTOXINS TO THE BENTHIC CARNIVORE *POMADASYS MACULATUS*

RICHARD J. LEWIS, MICHAEL J. HOLMES AND MICHELLE SELLIN

Lewis, R.J., Holmes, M.J. & Sellin, M. 1994 08 01: Invertebrates implicated in the transfer of gambiertoxins to the benthic carnivore *Pomadasys maculatus*. *Memoirs of the Queensland Museum* **34**(3): 561–564. Brisbane. ISSN 0079-8835.

The food chain hypothesis for the transfer of ciguatoxins (CTX) to carnivorous fish has gained widespread acceptance. This study was undertaken to determine the vector(s) transferring gambiertoxins to the often ciguateric blotched javelin fish (*Pomadasys maculatus*) in Platypus Bay, Queensland. *P. maculatus* is a benthic carnivore which in Platypus Bay was found to feed predominantly on small shrimps and crabs that live amongst *Cladophora* sp. that also harbours *Gambierdiscus toxicus*. Of the potential prey of *P. maculatus* in Platypus Bay, only the shrimps (mostly *Alpheus* sp.) contained detectable levels of ciguatoxin-like toxins, implicating shrimps as an important vector in the transfer of gambiertoxins to carnivorous fish. Any toxic effects of *G. toxicus* on shrimps may facilitate the selective feeding of fish on shrimps containing the highest toxin levels. Such selective feeding provides a mechanism for the funnelling of toxins from *G. toxicus* to *P. maculatus*. It remains to be established if shrimps are capable of biotransforming the gambiertoxins to ciguatoxins or whether biotransformation of the gambiertoxins is accomplished exclusively by fish. Given that *P. maculatus* is at times highly toxic, and within a year can be non-toxic, it is likely that the gambiertoxins enter the food chain as intense bursts that perhaps last for only several weeks. Depuration and/or detoxification are likely to account for the apparent rapid loss of gambiertoxins and ciguatoxins from shrimps, crabs and *P. maculatus*.

Richard J. Lewis, Michael J. Holmes, and Michelle Sellin, Southern Fisheries Centre, Queensland Department of Primary Industries, PO Box 76, Deception Bay, Queensland 4508; 22 November, 1993.

The food chain hypothesis for the transfer of ciguatoxins (CTXs) to carnivorous fish has gained widespread acceptance through the results of numerous studies (Randall,1958; Yasumoto et al.,1971,1977a,b,1979; Banner,1974; Murata et al.,1990; Holmes et al.,1991; Lewis et al.,1991; 1992). Key steps in the food chain hypothesis include (i) the uptake by herbivorous fish of gambiertoxins (GTXs) produced by *Gambierdiscus toxicus* and (ii) the transfer of the toxins from herbivorous to carnivorous fish. Grazing molluscs (Yasumoto & Kanno,1976) and fish that feed on invertebrates (Banner,1974) have also been implicated in ciguatera. The involvement of invertebrates in the ciguatera food chain has been speculated upon (Kelly et al.,1992) following laboratory observations that brine shrimp were capable of feeding on *G. toxicus*. However, evidence that invertebrates play an important role in the transfer of CTX or their precursors remains circumstantial.

Platypus Bay, Queensland, regularly produces ciguateric fish including the piscivorous Spanish mackerel (*Scomberomorus commersoni*) and barracuda (*Sphyraena jello*) (Lewis & Endean, 1983,1984). To reduce the adverse impacts of ciguatera, a ban has been imposed on capture of these species in Platypus Bay. Another common fish, *Pomadasys maculatus* (blotched javelin fish; Fig. 1), can also be toxic in this area (Lewis et al.,1988) and may be a link in the transfer of CTXs to Spanish mackerel and barracuda (Lewis & Sellin,1992). *P. maculatus* are often more toxic than Spanish mackerel and toxic individuals of both species are contaminated with CTX-1, -2 and -3 at similar relative levels (Lewis & Sellin,1992). In this paper we report that *P. maculatus* probably accumulates CTX through feeding on invertebrates (especially the shrimps) living in the macroscopic algae (*Cladophora* sp.) that harbours *G. toxicus* in Platypus Bay.

METHODS

POMADASYS MACULATUS IN PLATYPUS BAY

P. maculatus were captured in Platypus Bay (24° 58'S, 153° 10'E) by line or trawl net in ~15 m of water (Holmes et al., this memoir). Feeding preferences were determined from visual assessment of the stomach and intestinal content of *P. maculatus* collected intermittently over a year (n = 40). Small benthic fish that could be potential prey of *P. maculatus* could not be confirmed by scuba diver observations in Platypus Bay. The

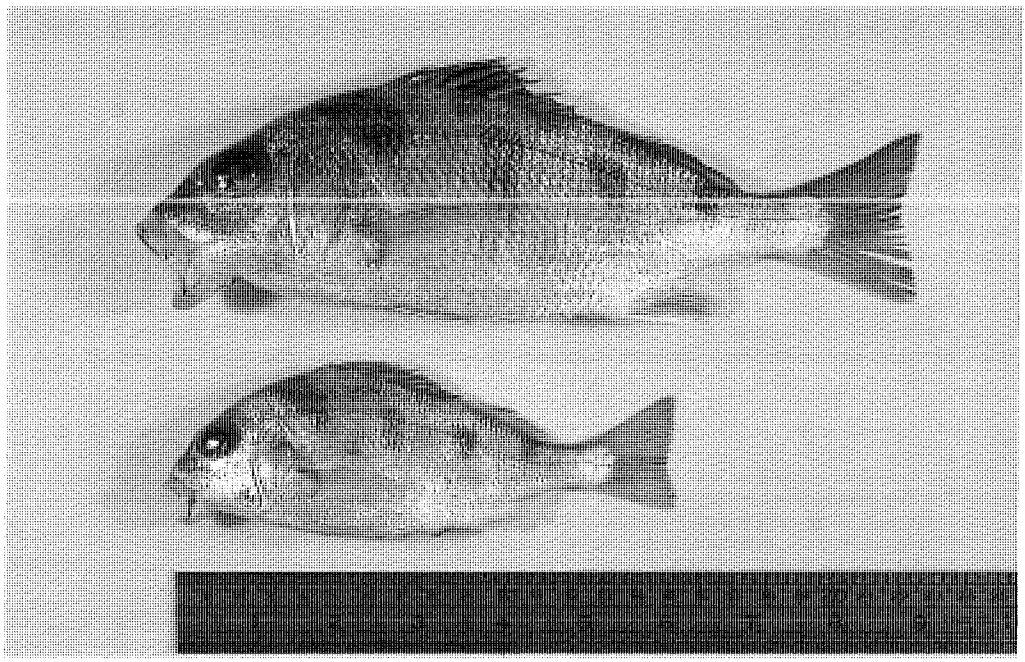

FIG.1. Examples of *Pomadasys maculatus* captured from Platypus Bay. Note the downward deflection of the mouth that indicates this species is specialised for bottom foraging.

relative intestine length and pH (after dilution with water) of pooled stomach and intestinal contents (n=5) were measured to assess the digestive strategy.

Invertebrates from Platypus Bay

Invertebrates (alpheid shrimps, crabs, nematodes, polychaetes, gastropods) living in association with the green macroalga, *Cladophora* sp., carpeting the sandy substrate of the study site were collected by small dredge, beam trawl or diver. From 8–10 May 1991 a small dredge was used to collect several species of benthic 'worms', in addition to shrimp and crab samples. The diver-collected (9 May 1988) *Cladophora* (9.2kg) was processed exclusively for the small gastropods present. A beam trawl was used to obtain (22 October 1991 and 20 March 1992) two further *Cladophora* samples of 191 and 154kg from which additional shrimps and crabs were collected and extracted for toxins. The visible invertebrates in each of the above samples were sorted by hand.

Assessment of Toxin Levels in Invertebrates in Platypus Bay

Invertebrates were extracted for ciguatoxin-like toxins with acetone and the acetone-soluble material partitioned as previously described (Lewis et al.,1992). The ether-soluble and selected butanol-soluble fractions (up to 30mg) were dried, suspended in Tween 60/saline and assayed in 20±2g mice (Quackenbush strain, either sex). Signs in mice (n=2) following intraperitoneal (i.p.) injection of these fractions were used to characterise the toxicity of each fraction (Holmes et al.,1991; Lewis et al.,1991).

RESULTS

Toxicity was detected in the ether-soluble fraction of shrimps but not in the ether-soluble material of other invertebrates (Table 1). Mouse bioassay signs induced by the ether-soluble toxin in the shrimps included severe diarrhoea and laboured respiration, signs consistent with an injection of a sub-lethal dose of ciguatoxin-like toxins. Two additional samples of shrimps (178 and 163g) and crabs (78 and 11g), collected by beam trawl, had levels of gambiertoxins below the limit of detection of the mouse bioassay. The butanol-soluble material from the gastropods as well as from the shrimps and crabs from these latter two collections were also assayed by mouse

TABLE 1. Yield (mg) and toxicity (MU) of ether extracts of invertebrates collected by dredge from Platypus Bay, Queensland.

Invertebrate	Wet Weight (g)	Average Size g (range)	Ether extract mg (MU)[a]
Shrimps (mostly *Alpheus* sp.)	42.5	0.15 (0.02-1.2)	30(0.5)
Crabs (mostly *Thalamita* sp.)	21.9	0.2 (0.04-1.7)	36 (0)
Small nematodes	38.1	0.05	94 (0)
Large tube-dwelling polychaetes	7.9	0.2	27 (0)
Gastropods	-	~0.1	(0)

[a]Ciguatoxin-like activity quantified in mouse units (MU). One MU = 1 LD_{50} dose for a 20 g mouse.

bioassay. Toxins resembling the maitotoxins were detected in the butanol fraction but these were not characterised further.

P. maculatus in Platypus Bay which ranged from 30-300g (fork length 13.5-24.0cm) has a thin walled stomach (pH=7.0) and a relatively short intestine (10-21cm) (pH=6.3). The downward pointing mouth (Fig.1) indicates that this species is a specialised benthic forager. Identifiable stomach contents of *P. maculatus* comprised mostly shrimps and crabs with occasionally some *Cladophora* and small unidentified fish.

DISCUSSION

P. maculatus has a near neutral pH digestive system that would be unlikely to provide the conditions for acid- catalysed spiroisomerisation of CTX-2 (or the putative 52-epi CTX-1 named CTX-4) to CTX-2 and CTX-1, respectively. Thus the CTX-1 and CTX-3 detected in *P. maculatus* flesh (Lewis & Sellin,1992) may arise as an artefact that results from the purification of CTX-2 and -4 on silicic acid supports eluted with acid solvents such as chloroform.

Of the invertebrates inhabiting *Cladophora* beds in Platypus Bay, only shrimps contained detectable levels ciguatoxin-like toxins (Table 1). The toxin levels in shrimps declined to levels below those detectable by mouse bioassay for two subsequent collections. Shrimps (and perhaps crabs) may be a vector in the transfer of gambiertoxins to carnivorous fish. Another possibility is that *P. maculatus* accumulates gambiertoxins from the *G. toxicus* that is ingested along with the small amounts of *Cladophora* ingested incidentally with the invertebrates. This possibility is considered remote since the prominent herbivore in the area (*Siganus spinus*, a species of similar size to *P. maculatus*) consumes almost entirely *Cladophora* and is seldom toxic (unpubl. data). Another possibility is that *P. maculatus* feeds on the dead remains of ciguateric fish.

For shrimps to accumulate gambiertoxins they must be capable of ingesting *G. toxicus*. This appears likely, since brine shrimp have been shown to feed on *G. toxicus* in the laboratory (Kelly et al.,1992). The detection of ciguatoxin-like toxins in shrimps and maitotoxin-like toxins in shrimps and crabs from Platypus Bay indicates that both groups of invertebrates consume *G. toxicus*. Analysis of intestinal content of shrimps (n = 2) revealed a range of detritus similar to or larger than *G. toxicus*, but no *G. toxicus*. This analysis was conducted on shrimps collected at a time when no detectable gambiertoxin could be extracted from these shrimps.

G. toxicus cells have been shown to be toxic to brine shrimps (Kelly et al.,1992). Toxic effects of *G. toxicus* on shrimps may facilitate the selective feeding of fish on shrimps containing the highest toxin levels. Such selective feeding provides a mechanism for funnelling *G. toxicus* toxins, especially gambiertoxins, to *P. maculatus* (Fig.2). It remains to be established if shrimps are capable of biotransforming the gambiertoxins to ciguatoxins or if this capacity is exclusive to fish. The piscivorous fish likely to prey on *P. maculatus* include *Scomberomorus commersoni*, *Sphyraena jello* and *Seriola lalandi* (yellow-tail kingfish).

Environmental and/or genetic factors leading to a proliferation of gambiertoxins and consequent outbreaks of ciguatera remain to be

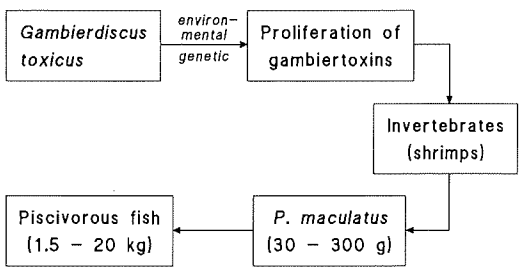

FIG.2. A model for the food chain transfer of ciguatoxins and/or gambiertoxins to *P. maculatus* and piscivorous fish in Platypus Bay. This study has implicated shrimps as a key vector. The size of the fish (kg) involved in this transfer are indicated.

elucidated. Given that *P. maculatus* is at times highly toxic (Lewis & Sellin,1992) and within a year can be non-toxic, it is likely that the gambiertoxins enter the food chain as intense pulses that perhaps last for only several weeks. At these times the shrimps would presumably be highly toxic. Depuration and/or detoxification may account for the apparent rapid loss of gambiertoxins and ciguatoxins from shrimps, crabs and *P. maculatus*.

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CIGUATERA AND HERBIVORES: UPTAKE AND ACCUMULATION OF CIGUATOXINS IN *CTENOCHAETUS STRIATUS* ON THE GREAT BARRIER REEF

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Lewis, R.J., Sellin, M., †Gillespie, N.C., Holmes, M.J., Keys, A., Street, R., Smythe, H., Thaggard, H., and Bryce, S. 1994 08 01: Ciguatera and herbivores: uptake and accumulation of ciguatoxins in *Ctenochaetus striatus* on the Great Barrier Reef. *Memoirs of the Queensland Museum* **34**(3): 565–570. Brisbane. ISSN 0079-8835.

Ctenochaetus striatus is a common detritivorous grazer likely to be a key species transfering ciguatoxin precursors (gambiertoxins) to carnivorous reef fish. Toxins in tissues from *C. striatus* collected in the Great Barrier Reef were characterised by mouse bioassay and chromatography. The biodetritus on which it feeds were collected with an airlift suction apparatus and the toxins present compared with those in *C. striatus*. Toxins resembling gambiertoxins and ciguatoxins predominated in all samples. Lesser amounts of fast acting and unidentified toxins were also detected. Maitotoxin was not detected. Similar concentrations of the ciguatoxins and gambiertoxins were detected in *C. striatus* from John Brewer or Davies Reefs, despite the former having major crown of thorns starfish damage. Toxins detected in *C. striatus* from these reefs were below levels that would result in prey species becoming ciguateric. This assessment is consistent with the historically low risk of contracting ciguatera from carnivorous fish captured at these reefs. We were unable to detect any of the less-polarity gambiertoxins in the liver of *C. striatus*, suggesting that these toxins were biotransformed to the more polar ciguatoxins (ciguatoxin-1, -2 and/or -3) in the liver of herbivorous fish. The concentrations of ciguatoxin in the visceral contents of *C. striatus* were 3- to 6-fold lower than the levels of such toxins in the biodetritus, perhaps as a result of bacterial degradation associated with the active fermentation employed as part of the digestive strategy of this species. Alternatively, the gambiertoxins may have been rapidly assimilated from the intestinal contents of *C. striatus*, a feature that may explain the important role this species apparently plays as a vector for transfer of gambiertoxins (and ciguatoxins) to carnivorous fish.

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Surgeonfish (Acanthuridae) are common benthic herbivores on coral reefs world-wide. Especially common is *Ctenochaetus striatus*, a detritivorous grazer often suggested as a key species involved in the uptake and transfer of toxins involved in ciguatera (Randall,1958; Yasumoto et al., 1971; Banner,1984). *C. striatus* feeds by combing biodetritus from turf algae and coincidentally ingests a variety of toxin producing benthic dinoflagellates in the process. Of these dinoflagellates only *Gambierdiscus toxicus* has been confirmed to produce toxins which accumulate in fish and cause ciguatera (Yasumoto et al., 1977; Murata et al.,1990; Holmes et al., 1991; Lewis et al.,1991; Lewis & Sellin,1992).

Early studies suggested that *G. toxicus* produces one lipid-soluble toxin of similar polarity to ciguatoxin (Yasumoto et al.,1977); however, recent studies (Murata et al.,1990; Holmes et al.,1991; Holmes & Lewis,1992; Legrand et al.,1992) determined that *G. toxicus* produces several less-polar ciguatoxin precursors named gambiertoxins (a class of sodium channel activator toxins). These gambiertoxins apparently undergo oxidative metabolism, at some undefined point(s) in the food chain, giving rise to the various ciguatoxins including CTX-1, CTX-2 and CTX-3, the principal toxins found in the flesh and viscera of ciguateric carnivorous fish (Murata et al.,1990; Lewis et al., 1991; Lewis & Sellin,1992; Lewis et al.,1993). With this new understanding of the toxins involved in ciguatera, we have examined reef biodetritus and the visceral contents, viscera and liver of *C. striatus*. Comparison of the toxicity of *C. striatus* collected from the crown of thorns starfish damaged John Brewer reef and the lightly damaged Davies Reef allowed an assessment of the impact of such damage on toxin levels in fish.

METHODS

Sample Collection

Adult *C. striatus* were collected by spear from back reef areas of John Brewer Reef (18° 38'S, 147° 04'E) and Davies Reef (188 50'S, 147° 39'E). Specimens were collected in 2–5m during 9–10 December 1987 from two sites at John Brewer Reef (n=22 fish at each site) and from one site at Davies Reef (n=30 fish). Viscera and liver were removed soon after capture and visceral contents (including stomach contents) were separated from the viscera by carefully stripping them out. The remaining viscera was subsequently rinsed in seawater to remove any remnants of visceral contents. The visceral contents, viscera and liver were separately pooled for each site, and wet weights determined for each pooled sample. The samples were initially stored (4 days) in an equal volume of methanol (preservative) at 0–4°C. On return to the laboratory samples were stored at –20°C prior to extraction.

Biodetritus samples were taken at approximately 2m from John Brewer (site 2) and Davies Reefs. To mimic the feeding of *C. striatus*, we used an airlift suction apparatus fitted with a plastic bristled brush and powered by compressed air from a SCUBA tank. This allowed removal of biodetritus from the turf algae covering dead coral surfaces by a combination of scrubbing and suction actions. The turf algal areas sampled were typical of areas subject to the major feeding activity of *C. striatus*. Material from 0.8m^2 of turf covered dead coral was collected into a floating plankton mesh sock (50μm mesh). The particulate material remaining in the sock was concentrated to a small volume, diluted 1:1 v/v with methanol and stored as for the *C. striatus* samples.

Isolation of Toxins

Samples were first freed of the methanol preservative before homogenisation in acetone (3x, 3:1 v/w). The dried extracts were then suspended in 90% methanol-water and the hexane- soluble material removed (3x, 1:1 v/v) by liquid-liquid partitioning. The 90% methanol-soluble material remaining was dried, suspended in water and extracted with diethyl ether (3x, 1:3 v/v). The ether-fraction was further separated into cold acetone-soluble and insoluble material following precipitation at –20°C. The acetone-solubles were further fractionated on silicic acid columns (100 mesh, Mallinkrodt using a minimum of 30 g silica/g extract) eluted with chloroform-methanol (c:m) mixtures of increasing polarity as described previously (Lewis et al.,1991). The water-soluble material from each site was extracted with n- butanol and pooled before further fractionation on a silicic acid column (Biosil A, Biorad) eluted with c:m mixtures as described by Holmes et al. (1990).

Mouse Bioassay

Fractions were suspended in a 1% Tween 60/0.9% saline solution and bioassayed by intraperitoneal (i.p.) injection of Quackenbush strain mice (20± 2g, either sex, n=2–5). Signs of intoxication following injection were recorded to allow characterisation of the type of toxin present (ie. ciguatoxin, gambiertoxin, fast acting or undetermined). To avoid non-specific toxic effects ≤30mg of each fraction was injected per mouse. Fractions were designated as containing CTX-1 if bioassay signs of severe laboured respiration and loss of activity as well as at least diarrhoea, hypersalivation or lachrymation. Fractions were designated as containing GTX or less-polar ciguatoxins (e.g. CTX-2, CTX-3) if signs of hindlimb paralysis were observed in addition to the sign for CTX-1. Fast acting toxins were identified by injecting doses varying by 2- to 10-fold and recording time to death. Fast acting toxins typically caused deaths within an hour at doses approximating the minimum lethal dose. This protocol was sufficient to indicate such toxins had dose vs. time to death relationships clearly different from those for ciguatoxins, gambiertoxins or maitotoxins (Holmes et al.,1990,1991; Lewis et al.,1991,1992). Fractions designated as containing ciguatoxin or gambiertoxin on the basis of bioassay signs were quantified from the time to death relationship: log (dose) = 2.3 log (1 + T^{-1}), where dose is in mouse units (MU) and time to death (T) is in hr (Lewis et al.,1992). This approach allowed quantification and toxin characterisation with a minimum number of mice. Animal experiments were conducted in accordance with NHMRC animal ethics guidelines.

RESULTS

Diver Observations on *C. striatus* Feeding

C. striatus is the predominant grazing species at John Brewer and Davies reefs with most *C. striatus* feeding in shallower waters (1–5m). This herbivore was observed to feed throughout the day by combing biodetritus from turf algae covering the exposed dead coral surfaces. *C. striatus* was not observed to remove the turf algae in the

process of removing the biodetritus adhering to these algae. This was confirmed by a visual assessment of the gut contents.

G. TOXICUS COLLECTIONS AND TOXIN ANALYSIS

G. toxicus were found attached to numerous species of macroscopic algae sampled at John Brewer Reef (Table 1). *G. toxicus* were also a conspicuous component of the vacuumed biodetritus but the *G. toxicus* in these samples were not quantified. The biodetritus upon which *C. striatus* feeds and the visceral contents, viscera and liver of *C. striatus* were extracted and bioassayed for the presence of toxins. Table 2 indicates the toxins detected after liquid-liquid partitioning into hexane, ether-(acetone-soluble and -insoluble fractions) and butanol-soluble fractions. The less polar (hexane-soluble) material did not contain detectable toxicity, whereas the more polar fractions were often found to be toxic. The mouse bioassay detected gambiertoxin- and ciguatoxin-like toxins and several fast acting toxins in these more polar fractions.

The toxins in the acetone-soluble ether-fraction were further characterised by mouse bioassay after silica gel chromatography (Table 3). This allowed separation of (i) less polar toxins (GTX-4b-like) which elute with 97:3 c:m, (ii) toxins of similar polarity to CTX-1,-2 or -3 or the more polar gambiertoxins which elute with 9:1 c:m, and (iii) toxins with polarity similar to the maitotoxins which elute with 0:1 c:m (Murata et al.,1990; Holmes et al.,1990,1991; Lewis et al.,

TABLE 1. Population densities of *Gambierdiscus toxicus* on macroalgae on John Brewer Reef (September, 1986)

Macroalgae	*G.toxicus*/100g macroalgae	Depth (m)	n
Spyridia filamentosa	3200	3	1
Padina australis	330-840	1 - 5	3
Halimeda opuntia	40 - 1000	2-10	4
H. incrassata	30	2	1
H. turia	30	3	1
Broadlea sp.	1120	3	1

1991; Holmes & Lewis,1992). Toxins eluting with 97:3 or 9:1 c:m induced signs of toxicity characteristic of the ciguatoxins or gambiertoxins. The most polar toxins induced either CTX-1-like signs, signs of undetermined origin or were fast acting, with the toxicity varying between the sites and the samples investigated. The relative concentration of toxins (MU/g) in the 97:3 and 9:1 c:m fractions at each reef are compared in Fig. 1.

Toxins in the butanol extracts were also characterised after silicic acid chromatography (Table 4). After silica gel chromatography, toxins were found only in the c:m 9:1 and 0:1 eluates. The fast acting toxin from the visceral contents appeared to be less polar than the fast acting toxin in the detritus. Carry-over of ciguatoxins into the butanol fraction may explain the toxicity in the c:m 9:1 eluates. Toxins inducing signs characteristic of the maitotoxins were not detected in the

TABLE 2. Yield (g) and characterisation* of lipid-soluble extracts from *Ctenochaetus striatus* and detritus, Great Barrier Reef.

	John Brewer Reef (1)			John Brewer Reef (2)				Davies Reef			
Fraction	Visceral Content (377g)	Viscera (200g)	Liver (100g)	Detritus (70g)	Visceral Content (434g)	Viscera (410g)	Liver (90g)	Detritus (150g)	Visceral Content (331g)	Viscera (380g)	Liver (130g)
Hexane	5.29	9.59	2.82	0.11	8.14	13.26	4.19	0.18	3.98	20.97	5.89
Acetone-soluble ether	0.62[b]	0.23b	1.11[d]	0.08[b]	0.78[b]	0.42[b]	1.41	0.13[b]	1.32[b]	0.41[b]	1.16[d]
Acetone-insoluble ether	0.05[b]	0.01[d]	0.63	0.01d	0.06[d]	0.03[b]	0.44[d]	0.02[b]	0.12b	0.05	0.84
Butanol	2.30[c]	0.91[d]	0.59[d]	0.23	3.15c	2.60[d]	0.92d	0.17c	2.30[d]	2.98	0.82

* Fractions were separated on the basis of solubility and those lethal to mice at ≤1.0g/kg were characterised as indicated by a-c below (n=2).

[a] Sign(s) in mice typical of ciguatoxin-1 (CTX-1) including lachrymation, hypersalivation and/or diarrhoea.

[b] Signs in mice typical of less polar ciguatoxins (CTX-2, CTX-3, GTX-4b), include those sign(s) induced by CTX-1 plus hind-limb paralysis.

[c] Fast acting toxin.

[d] Sign(s) of CTX-1 in mice for fractions non-lethal at 1.0g/kg.

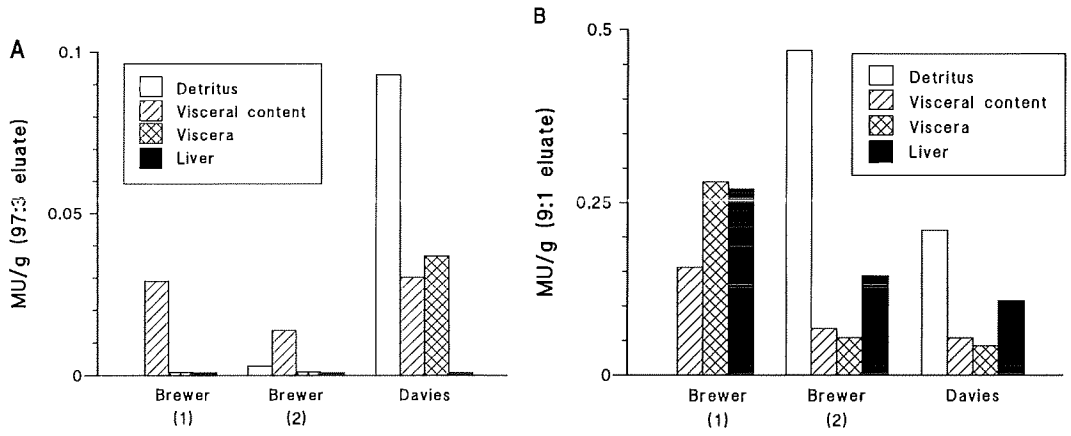

FIG. 1. Concentration of gambiertoxins and ciguatoxins in *Ctenochaetus striatus* and associated reef biodetritus. (A) Toxin levels in the 97:3 chloroform-methanol (c:m) eluates (low polarity gambiertoxins). (B) Toxin levels in the 9:1 c:m eluates (ciguatoxins and high polarity gambiertoxins). Toxin levels are given as total mouse units (MU)/g of sample. Cold acetone-soluble ether extracts were applied to silicic acid columns in each case.

butanol fractions, even after silica gel chromatography.

DISCUSSION

C. striatus feed by combing biodetritus from turf algae covering dead coral surfaces of these reefs (Purcell & Bellwood,1993). The turf algae (and other macroalgae) on these reefs were covered with moderate numbers of *G. toxicus*, indicating that turf algae is a niche in which *G. toxicus* might proliferate.

Several toxins were detected in extracts of the turf biodetritus and *C. striatus* visceral contents, viscera and liver. Ciguatoxin(s) and gambiertoxin(s) predominated in these samples which also contained several fast acting and several unidentified toxins. Interestingly, no maitotoxin was detected. Concentration of ciguatoxins and gambiertoxins in the tissues of *C. striatus* from

TABLE 3. Yield [g (MU)] and bioassay signs* of cold acetone-soluble ether-extracts separated by silicic acid chromatography

	John Brewer Reef (1)			John Brewer Reef (2)				Davies Reef			
Fraction[e] (c:m)	Visceral Content (0.5g)	Viscera (0.19g)	Liver (1.07g)	Detritus (0.06g)	Visceral Content (0.75g)	Viscera (0.38g)	Liver (1.37g)	Detritus (0.11g)	Visceral Content (1.29g)	Viscera (0.38g)	Liver (1.12g)
97:3	0.06 (11)[a]	*0.01 (0)	0.18 (0)	0.002 (0)	0.09 (6)c	0.03 (0)	0.14 (0)	0.01 (14)[b]	0.04 (10)[a]	0.08 (14)[b]	0.07 (0)
95:5	0.04 (3)[b]	0.01 (0)	0.15 (0)	*0.001 (20)[d]	0.07 (37)[b]	0.04 (0)	0.12 (0)	0.02 (0)	0.08 (4)[c]	0.06 (0)	0.12 (0)
9:1	0.37 (59)[b]	0.07 (56)[b]	0.28 (27)[b]	0.02 (33)[d]	0.44 (29)[b,c]	0.21 (22)[b]	0.56 (13)[a]	0.03 (32)[b]	0.53 (18)[b,c]	0.15 (16)[b]	0.22 (14)[a]
8:2	0.04 (2)[b,c]	0.02 (0)	0.002 (0)	0.001 (0)	0.05 (0)	0.03 (0)	0.04 (0)	0.01 (0)	0.18 (0)	0.02 (7)[d]	0.21 (0)
0:1	0.07 (3)[b,c]	0.03 (0)	0.07 (5)[b]	0.01 (0)	0.08 (4)c	0.04 (2)[c]	0.09 (0)	0.02 (20)[d]	0.38 (19)[d]	0.11 (0)	0.11 (0)

* Fractions lethal to mice at ≤1.0g/kg were quantified in mouse units (MU) as described in Methods (n=2-5 mice per fraction). The liver from Brewer (2) induced signs of ciguatoxin intoxication but no deaths (20mg was estimated to contain 0.5MU).

[a-c] Signs in mice as defined in Table 2.

[d] Signs in mice not clear.

[e] Fractions eluted from 100 mesh silicic acid with chloroform-methanol (c:m) mixtures of increasing polarity.

TABLE 4. Yield (g) and bioassay signs* of butanol extracts[e] separated by silicic acid chromatography

Fraction[f] (c:m)	Detritus (0.8g)	Visceral Content (2.5g)	Viscera (2.0g)	Liver (2.2g)
1:0	0.001	0.01	0.006	0.006
97:3	0.002	0.05	0.02	0.001[b]
9:1	0.006[d]	0.74[c]	0.07[b]	0.07[d]
0:1	0.11[c]	1.30	1.00	1.80

* Fractions lethal to mice at ≤1.5g/kg were characterised (n=2).

[a-d] Signs in mice as defined in Table 3.

[e] Butanol extracts (g) were pooled for the three sites, except for the detritus fraction which was from the Davies Reef collection only.

[f] Fractions eluted from Biosil A silica gel with chloroform-methanol (c:m) mixtures of increasing polarity.

John Brewer and Davies Reefs were similar, despite evidence of major damage at the former reef as a result of a crown of thorns starfish infestation. Our sample areas were surveyed in 1985 and had live:dead coral ratios of 1:3 and 2:0 for John Brewer and Davies Reefs, respectively (The Crown-of-Thorns Study,1985). If the area of turf algae is a factor that limits *C. striatus* density in reef areas, increased areas of dead coral would allow larger areas of turf algae which could support higher densities of *C. striatus*. These higher herbivore densities might in turn increase the proportion of such herbivores in the diet of carnivorous fish. This scenario could result in an increase in the rate carnivorous fish accumulate ciguatoxins. Increased areas of turf algae would also reduce the feeding pressure on turf algae which could possibly favour higher densities of *G. toxicus* on turf alae. Environmental factors, as yet unidentified, may also increase the levels of gambiertoxins per unit area of turf algae and such factors are perhaps more important in increasing the rate at which gambiertoxins and ciguatoxins enter the food chain of fish in coral reef areas.

The ciguatoxins in fish are believed to arise through the biotransformation (oxidative metabolism) of gambiertoxins produced by *G. toxicus* (Murata et al.,1990; Holmes et al.,1991; Holmes & Lewis,1992). Since low polarity gambiertoxins were not detected in *C. striatus* liver, we propose that the liver is a site, perhaps the major site, for biotransformation of the gambiertoxins in this species. Concentrations of ciguatoxin-like toxins in the biodetritus were considerably less than (3- to 7-fold) the concentrations found in the visceral contents of *C. striatus*. *C. striatus* does not employ acid digestion (gut was found to have a pH = 6.4) but fermentation is an important step in the digestion and assimilation of biodetritus by *C. striatus* (H. Choat pers. comm.). Such a reduction in toxin levels between the biodetritus and the visceral contents may stem from microbial degradation of the gambiertoxins to less potent forms. Alternatively, the uptake of gambiertoxins from the visceral contents of *C. striatus* may be rapid. A rapid uptake of gambiertoxins by fish (and man) may be a feature common to this class of polyether toxins.

This study found that levels of gambiertoxins entering *C. striatus* were typically higher than levels in the liver of this species. Consequently the gambiertoxins and their biotransformed products (ciguatoxins) do not appear to be accumulated in a simple, additive manner, suggesting that depuration of ciguatoxins and/or gambiertoxins may be significant in *C. striatus*. Such depuration by herbivores could, at least in part, contribute to the rapid decline in the ciguatoxin levels in a population of moray eels (Lewis et al.,1992) and the rapid decline in ciguatera incidence in some Pacific Island countries (Lewis,1992). A similar conclusion can be drawn from the study of Bagnis et al. (1985) who showed that a decline in *G. toxicus* numbers paralleled the decline in *C. striatus* toxicity.

Fish sampled in this study had relatively low levels of ciguatoxins compared with *C. striatus* from French Polynesia (Yasumoto et al.,1971; Bagnis et al.,1985). To initiate a ciguatera outbreak at these locations on John Brewer and Davies Reefs, we suggest that orders of magnitude higher gambiertoxin production per unit area of turf algae are required. Assays with higher sensitivity and specificity for toxins involved (e.g. antibody-based assays selective for the different gambiertoxins and their metabolites) or sites harbouring more toxic fish are likely prerequisites to the further study of toxins in *C. striatus*.

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CELL BIOASSAY FOR THE DETECTION OF CIGUATOXINS, BREVETOXINS, AND SAXITOXINS

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Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M. & Wekell, M.M. 1994 08 01: Cell bioassay for the detection of ciguatoxins, brevetoxins, and saxitoxins. *Memoirs of the Queensland Museum* **34**(3): 571–575. Brisbane. ISSN 0079-8835.

We have developed an assay using neuroblastoma cells for detection of sodium channel-specific marine toxins based on an end point determination of mitochondrial dehydrogenase activity in the presence of veratridine and ouabain. This cell bioassay allows detection of either sodium channel blocking agents, such as saxitoxins, or sodium channel enhancers such as brevetoxins and ciguatoxins. The assay responds in a dose dependent manner, differentiates the toxic activity as either sodium channel blocking or enhancing, and is highly sensitive. Assay response to brevetoxins and to ciguatoxic extracts is rapid, allowing dose dependent detection within 4-6hr. The method is simple, utilizes readily available reagents, uses substantially less sample than required for mouse bioassay, and is well within the scope of even modest tissue culture facilities. This cell-based protocol has the potential to serve as an alternate and complementary method to the standard mouse bioassay.

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Monitoring programs for marine toxins have depended in large part upon mouse bioassays. Although mouse assays have for many years provided a fairly reliable assessment of risk, there is mounting pressure to develop alternative methods to reduce the reliance on animal testing. Kogure et al. (1988) and Jellett et al. (1992) developed tissue culture assays in which mouse neuroblastoma cells are treated with veratridine and ouabain resulting in altered cell morphology and decrease in viability. Toxins which block sodium channels antagonize this effect, rescuing the cells in a dose dependent manner. Evaluation is either through the visual scoring of 200 or more cells per sample or well, a potentially time consuming and operator dependent task, or is dependent upon the physical removal of affected cells through the cumulative steps of rinsing, fixing, and staining.

We have developed a cell bioassay for detection and quantitation of sodium channel activating toxins such as the brevetoxins and ciguatoxins. We have modified and simplified the above assays for determination of sodium channel blockade. Assessment of cytotoxicity in the present method uses a colorimetric index of metabolic activity. Those cells which are metabolically active reduce a tetrazolium compound, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium), to a blue-colored formazan product (Mosmann, 1983)). This method requires minimal processing and the results can be read on a standard multiwell scanning spectrophotometer (ELISA plate reader).

MATERIALS AND METHODS

TOXINS

Purified saxitoxin (Calbiochem) was diluted to the appropriate concentration with complete tissue culture medium prior to cell assay. Brevetoxins PbTx-1 and PbTx-3 (Calbiochem) were dissolved in methanol and diluted 1:100 in complete tissue culture medium, from which serial dilutions in complete medium were made. A ciguatoxic fish extract (methanol fraction), prepared from wrasse (*Cheilinus rhodochrous*) and mouse bioassay data were generously provided by Dr. Yoshitsugi Hokama, University of Hawaii. A stock solution of this material was prepared in the same manner as for the brevetoxins. Extracts of crab viscera and mouse bioassay data were generously made available by Cheryl Eklund and James Bryant, FDA, Bothell, WA.

MTT BIOASSAY

Cultures were prepared for bioassay as described by Jellett et al. (1992) with modifications described below. Neuro-2a cells (ATCC, CCL131) were grown in RPMI 1640 (Sigma) containing 10% fetal bovine serum (Gibco),

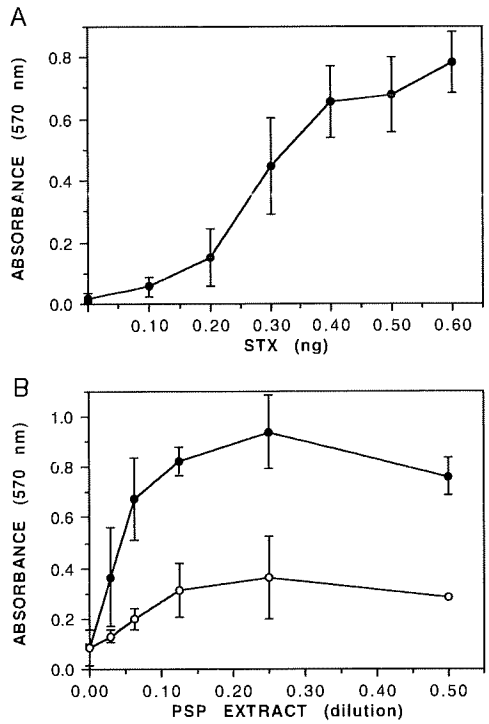

FIG.1.Effect of increasing concentration of pure saxitoxin upon MTT development in the neuroblastoma assay (1a). Standard MTT development time of approximately 15min allowed detection in the range of 0.1ng/10µl to 0.6ng/10µl saxitoxin addition per culture well. Aqueous extracts of Dungeness crab viscera examined for PSP activity in the MTT neuroblastoma assay (1b). Extracts that had tested at 122µg/100g (●) and no detectable activity (○) using the mouse bioassay were tested at various dilutions in the cell bioassay. Circle without error bar is the average of 2 replicates. The error bar indicates ±SD.Values represent mean of 3-4 replicates.

glutamine (2mM) (Sigma), sodium pyruvate (1mM) (Sigma), streptomycin 50µg/ml (Sigma), and penicillin 50units/ml (Sigma) (complete growth medium). Cells were seeded into 96-well plates at a density of 5 x 10^5 cells/ml in 200µl complete growth medium per well. Cultures were incubated at 37°C/5% CO_2 for approximately 24hr.

Culture wells received 10µl of sample and 10µl additions of aqueous stocks of 10mM ouabain (Sigma) and 1mM veratridine (Sigma) pH 2. Each sample concentration was tested in replicate (3 to 5 wells). A minimum of 15 wells per plate were processed as ouabain/veratridine treated controls (no sample addition), and a minimum of 5 wells served as untreated controls (without ouabain/veratridine and without sample). In the case of sodium channel activators, such as the brevetoxins and ciguatoxic extract, 10µl samples were added to replicate culture wells in both the presence and absence of ouabain and veratridine. Control wells received added culture medium to make up for volume differences.

Following incubation with samples, the overlaying medium was removed from cultures, and without a wash step, 60µl of a 1:6 dilution of MTT stock (5mg/ml in PBS, pH 7.4) in complete growth medium was added to each well. Cultures were incubated for approximately 15min at 37°C, medium was then removed, and 100µl of DMSO was added to each well. The plates were immediately read with an automated multiwell scanning spectrophotometer using a test wavelength of 570nm and a reference wavelength of 630nm.

RESULTS

Saxitoxin dependent inhibition of the ouabain/veratridine induced cytotoxicity was measured directly by alterations in MTT metabolism (Fig.1a). Purified saxitoxin was detected at a level of 0.1ng/10µl addition using an approximate MTT development time of 15min. Assay sensitivity could occasionally be enhanced by increasing MTT development time to c.45min, with a resultant detection limit of c.0.02ng/10µl addition (data not shown). Assay sensitivities were comparable to that reported by Jellett et al. (1992). In the absence of ouabain/veratridine treatment saxitoxin at the concentrations tested had no measurable effect. For the purpose of comparison, 0.1ng/10µl and 0.02ng/10µl saxitoxin are equivalent to shellfish extracts of 2µg/100g tissue and 0.4µg/100g respectively.

As a preliminary test of detection of naturally incurred PSP in samples, acid extracts of Dungeness crab viscera exhibiting positive and negative PSP activity by the AOAC mouse bioassay (122µg/100g and none detected/100g respectively) were examined by the MTT neuroblastoma assay (Fig.1b). The cell bioassay detected mean values (with standard deviations) of 124±44µg/100g in the positive extract (mouse bioassay) and 33±2µg/100g in the extract negative by mouse bioassay. The dose response curves tended to plateau with increasing concentrations of extract (dilutions of <1:4), suggesting a competing or potentially interfering cytotoxic component.

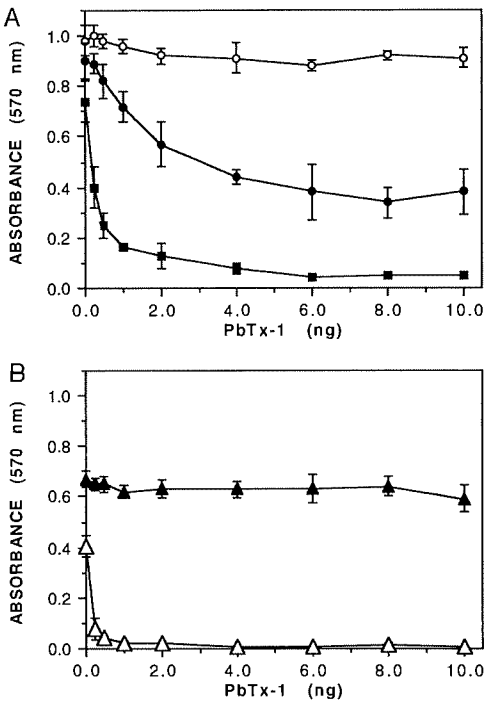

FIG.2.Brevetoxin PbTx-1 cytotoxicity as measured by the MTT neuroblastoma assay. (a, b) PbTx-1 cytotoxicity was assayed at 2hr (○), 4hr (●), 6hr (■), and 18hr (Δ). (b) Cytotoxicity was insignificant in the absence of ouabain and veratridine at the maximum incubation time of 18hr (▲). Values represent mean of 4 replicates. The error bar indicates ±SD.

Brevetoxins and ciguatoxins significantly enhance veratridine induced sodium influx in neuroblastoma cells (Catterall & Risk,1980; Bidard et al.,1984). We reasoned that this effect would accelerate the rate of ouabain/veratridine induced cytotoxicity and could therefore be the basis of a detection method for sodium channel activators such as brevetoxins and ciguatoxins. In the dose range explored, titratable cytotoxicity was observed as early as 4hr (Fig.2a) and was total at 18hr. Brevetoxin in the absence of ouabain/veratridine was not cytotoxic even at the highest concentration and incubation time tested (10ng/10μl, 18hr exposure) (Fig.2b). PbTx-3 produced similar results as observed for PbTx-1 in the cell bioassay (data not shown).

A ciguatoxic extract was examined with the same MTT cell assay format as utilized for brevetoxins. The extract was diluted and applied to neuroblastoma cells in the presence or absence of ouabain/veratridine. Within 6hr the sample produced significant dose dependent cytotoxicity only in cells treated with ouabain/veratridine (Fig.3). Even after prolonged exposures of up to 22hr the ciguatoxic extract was not cytotoxic in the MTT cell bioassay in the absence of ouabain/veratridine treatment (data not shown).

DISCUSSION

In the present study we sought to develop a diagnostic cell-based assay for determining either sodium channel blocking or enhancing activity. Furthermore, we explored the possibilities of improving previous methods by simplifying the end-point assessment of cells treated with sodium channel blocking agents in the presence of ouabain and veratridine.

Simplifying the assay was met by incorporating a sensitive colorimetric test of cellular metabolism, MTT, originally described by Mossman (1983). The method is a rapid, versatile, quantitative, and simple technique based upon the metabolism of a tetrazolium salt, MTT, by mitochondrial dehydrogenase activity in viable cells. This assay does not require washing or fixation steps. MTT metabolism has established itself as an accepted *in vitro* method in such diverse areas as the assessment of growth factor activity (Kotnik & Fleischmann,1990), radiosensitivity of cultured cells (Wasserman & Twentyman,1988), and the evaluation of chemotherapeutic agents upon target cell lines in culture (Carmichael et al.,

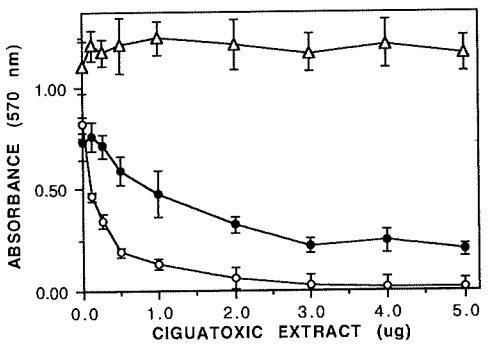

FIG.3. Ciguatoxic extract from wrasse analyzed by the MTT neuroblastoma assay. Ciguatoxic extract was diluted and applied to cells in the presence of ouabain and veratridine, 6hr (●) and 22hr (o), or without ouabain and veratridine, 22hr (Δ). Values represent mean of 4 replicates. The error bars indicate ±SD.

1987; Alley et al.,1988; Manger et al.,1989). Due to its many advantages and wide acceptance it seemed reasonable to explore the utility of MTT in a modified cell bioassay for the detection of marine toxins active at the sodium channel, and had been earlier suggested by us as a potentially useful approach (Manger,1993).

An additional goal of our studies was to incorporate modifications in the cell-bioassay to allow detection and quantitation of marine toxins that activate sodium channels. Insight as to how this might be accomplished came from Catterall & Risk (1980) and Bidard et al. (1984). Their research demonstrated that these toxins enhanced the ^{22}Na influx effect produced by veratridine treatment in neuroblastoma cells. We reasoned that this brevetoxin or ciguatoxin activity would also relate directly to an observable enhancement of ouabain/veratridine induced cytotoxicity in our assay. This was observed in our modified MTT neuroblastoma assay as exhibited by a dose dependent enhancement of cytotoxicity following treatment with either of these toxins. The lack of noticeable cytotoxicity in the absence of ouabain/veratridine is in agreement with specific toxin activity via interaction with sodium channels.

The MTT cell bioassay was significantly more sensitive than the mouse bioassay. The animal assay can detect saxitoxin to a lower limit of 40µg/100g tissue (Hungerford & Wekell,1992). In contrast, the cell bioassay can routinely detect purified saxitoxin at a level of 0.1ng/10ul, which is the equivalent of 2µg/100g tissue. Occasionally, with extended MTT development time, the observed limit of detection was 0.02µg/10µl (0.4µg/100g).

Examination of crab viscera samples with the MTT cell bioassay demonstrated good correlation with mouse bioassay results. A sample determined to have 122µg/100g tissue of PSP by mouse bioassay resulted in a mean value of 124±44µg/100g tissue using the MTT cell bioassay. Interestingly, a crab viscera sample that was PSP negative by mouse bioassay had a mean value of 33±2µg/100g tissue in the cell bioassay, however, this level of saxitoxin is below the standard detection limit of the animal test (40µg/100g). Examination of additional crab viscera extracts by MTT cell bioassay have produced results in agreement with the mouse bioassay (data not shown).

The modified MTT cell bioassay is also more sensitive to the brevetoxins than the mouse bioassay. The LD_{50} for mice is 0.01mg/20g animal, i.p. injection (Hungerford & Wekell,1992). This would correlate to 0.1mg/100g tissue extract and would be the equivalent of a 1ng/10µl sample in the MTT cell bioassay. In the present study the MTT cell bioassay detected brevetoxins at levels of 0.25ng/10µl.

The ciguatoxic extract tested in our studies produced death in 20g mice following injection of 50mg in 1ml within 2.5hr (Amra et al.,1990). This represents about 1.8 mouse units or the equivalent of 15ng CTX-1 as estimated by the method of Legrand et al. (1989). Thus, the sodium channel activity of this extract was readily detected in the MTT cell bioassay at levels of less than 10^{-4} mouse units, corresponding to low or sub pg concentrations of CTX-1.

Mouse bioassays for brevetoxins and ciguatoxins involve long observation periods, ranging from several hours to 48hr (Hungerford,1992), whereas, the MTT cell bioassay can be processed within 4-6hr. The MTT cell bioassay is also well suited to automation, providing a convenient biological assay that can accommodate a large number of samples and which can be accomplished within one day. Although *in vitro* methods cannot presently substitute entirely for the data derived from animal studies, these methods do offer the potential to reduce the reliance upon animal testing and to facilitate the rapid screening of test samples. In the event that mouse bioassays are prohibited or limited by law, cell bioassays for these marine toxins may provide an alternative screening method.

Subsequent to these preliminary studies we have confirmed the estimated assay sensitivity to ciguatoxins using purified CTX-1 and CTX3C (Manger et al., in press).

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DETECTION OF CIGUATOXIC FISH BY USING THE BINDING PROPERTY OF CIGUATOXINS TO VOLTAGE-DEPENDANT SODIUM CHANNELS. *Memoirs of the Queensland Museum 34(3) 576. 1994*:- Binding studies indicate that CTX (coded -1B), the principal toxin isolated from moray eel viscera and CTX-4B (or GT-4B) isolated from wild dinoflagellate, *Gambierdiscua toxicus*, competitively inhibit binding of the brevetoxin (3H)-PbTx-3 to rat brain membranes. Affinity of CTX-1B is around 30 times higher than that of PbTx-3 while CTX-4B has around the same affinity as the brevetoxin. Results confirm that the two toxins act at the voltage dependant sodium channel of rat brain membranes. Experiments on minor toxins isolated from ciguatoxic material are underway. Preliminary results indicate a common property of the compounds to inhibit the binding of PbTx- 3. This property is used to evaluate the ciguatoxicity of hazardous fish. A rapid extraction procedure and a routine binding assay have been established.

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EVALUATION OF INTRAVENOUS MANNITOL FOR TREATMENT OF ACUTE CIGUATERA FISH POISONING. *Memoirs of the Queensland Museum 34(3): 576. 1994*:– The Ciguatera Double Blind Study is an investigator-initiated, grant supported, multicenter, randomized, controlled trial which is designed to: 1) investigate the efficacy of intravenous 20% mannitol in comparison to a placebo (intravenous 5% dextrose in water) for treatment of acute ciguatera fish poisoning; 2) determine the response time to treatment; and 3) determine relapse rate 48hrs post treatment. Mannitol and the 5% dextrose were randomly assigned to patients who presented with ciguatera fish poisoning to 1 of 4 hospitals. Medical treatment was provided through a protocol. Patients response was monitored at 10min, 30min and 2.5hr after therapy was begun. Patient followup was done for 48hr after the treatment was given.

Neal Palafox, Box 686, John Hopkins School of Public Health, Maryland U.S.A.; 12 April, 1993.

CONFOCAL LASER SCANNING MICROSCOPY: A NEW TOOL FOR STUDYING THE EFFECTS OF CIGUATOXIN (CTX-1B) AND D-MANNITOL AT MOTOR NERVE TERMINALS OF THE NEUROMUSCULAR JUNCTION IN SITU

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Molgó, J., Juzans, P. & Legrand, A.M. 1994 08 01: Confocal laser scanning microscopy: a new tool for studying the effects of ciguatoxin (CTX-1B) and D-mannitol at motor nerve terminals of the neuromuscular junction in situ. *Memoirs of the Queensland Museum* **34**(3): 577–585. Brisbane. ISSN 0079-8835.

The confocal laser scanning microscope was used in conjunction with the fluorescent probe FM1-43 to study the effects of ciguatoxin (CTX-1B) and D-mannitol at motor nerve terminals of the neuromuscular junction in situ. CTX-1B caused time-dependent changes in the surface area of motor nerve terminals and perisynaptic Schwann cell at living neuromuscular junctions.These changes were completely prevented by tetrodotoxin indicating that they are related to both entry of Na^+ and increased quantal acetylcholine release. D-mannitol at concentrations reported to exert an effective hydroxyl radical scavenger action neither prevented the action of CTX-1B nor antagonized its effects. However, at higher concentrations D-mannitol exerted osmotic effects that caused shrinkage of both motor nerve terminals and Schwann cell somata previously swollen by the action of CTX-1B probably by shifting water from the intracellular to the extracellular compartment.

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Ciguatoxins (CTX) are a family of potent lipid-soluble cyclic polyethers (Scheuer et al., 1967; Tachibana et al.,1987; Legrand et al., 1989; Murata et al.,1989,1990; Lewis et al., 1992; Lewis & Sellin,1991) involved in ciguatera fish poisoning (Anderson & Lobel, 1987; Russell & Egen,1991; Swift & Swift, 1993). The poisoning is characterized by severe gastrointestinal and neurological disturbances (Bagnis et al.,1979; Withers,1982; Gillespie et al.,1986) which develop after consumption of coral reef fish.

The chemical structures (Murata et al., 1989, 1990; Lewis et al.,1991) of structurally related ciguatoxins (CTX-1B or CTX-1 or CTX, which is probably the major toxin involved in ciguatera, CTX-2 and CTX-3) are reminiscent of brevetoxins, (Baden,1989; Murata et al., 1989, 1990; Lewis et al.,1991; Gawley et al., 1992) and they share a common binding site with the brevetoxins on the neuronal voltage-sensitive sodium channel proteins (Lombet et al.,1987; Lewis et al.,1991).

CTX selectively acts on Na^+ channels in the node of Ranvier of single myelinated nerve fibers in such a way that voltage-clamped Na^+ channel currents are activated at potentials about 30mV more negative than unmodified channels and fail to inactivate during long-lasting depolarizations (Benoit et al.,1986). It is likely that a persistent activation of Na^+ channels by CTX at the resting membrane potential leads to a membrane depolarization and the spontaneous action potentials reported on neuronal, axonal and muscle membranes (Benoit et al.,1986; Bidard et al., 1984; Molgó et al.,1990). Tetrodotoxin (TTX) which blocks voltage-gated sodium channels in those membranes, prevents such actions (Benoit et al.,1986; Bidard et al., 1984; Molgó et al., 1990). CTX has also been reported to increase intracellular Ca^{2+} concentration in NG108-15 hybrid cells bathed in standard medium or in a Ca^{2+}-free medium supplemented with EGTA (Molgó et al.,1992a,1993b). CTX-induced Ca^{2+} mobilization prevents further effect of bradykinin (1μM) suggesting that CTX also stimulates the inositol 1,4,5-trisphosphate-releasable Ca^{2+} store (Molgó et al.,1993b). Since TTX prevents the CTX-induced increase in intracellular Ca^{2+} concentration it would appear that Na^+ influx through voltage-gated Na^+ channels somehow leads to release of intracellular Ca^{2+}. Such a direct relationship of Na^+- dependent Ca^{2+} mobilization in neuronal cells is unknown.

CTX increases the rate of release of [^{3}H]-aminobutyric acid and [^{3}H] dopamine from rat brain synaptosomes. These actions are sensitive to blockade by TTX but are unaffected by Ca^{2+} channel antagonists like nitrendipine and D-600 (Bidard et al.,1984). Since CTX has no action on

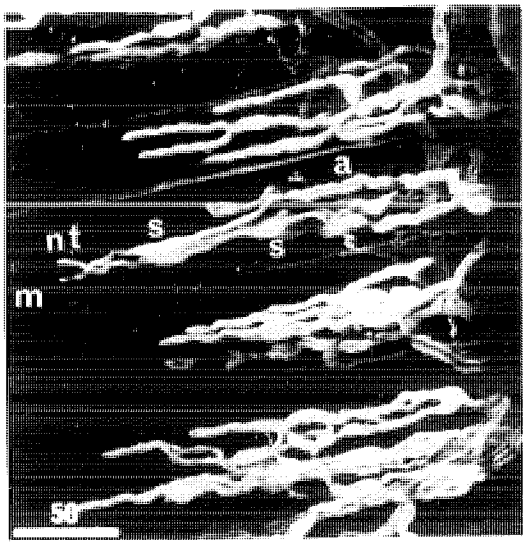

FIG.1. Low magnification view of motor nerve terminals (NT), Schwann cells (SC) and intramuscular axons (a) in a living frog cutaneous pectoris neuromuscular preparation. Notice the nonmyelinating Schwann cells covering the branches of the nerve terminals. Tridimensional reconstitution by a projection of 30 horizontal section series. The structures have been stained with the fluorescent membrane dye FM1-43 for 60min and then washed free of FM1-43.

Na^+-K^+-ATPase activity, it was suggested that the enhancement of neurotransmitter release may be due to a depolarization-induced Ca^{2+} influx (Bidard et al.,1984). CTX was reported also to enhance Ca^{2+}-dependent ACh release from pure cholinergic synaptosomes (Molgó et al.,1992b). If CTX depolarized synaptosomal membranes to levels above that needed to activate voltage-gated Ca^{2+} channels, then it would be expected that membrane depolarization, via Ca^{2+} influx, would contribute to this Ca^{2+}-dependent ACh release caused by CTX. However, blockade of Ca^{2+} channel subtypes in Torpedo synaptosomes (Moulian et al.,1993) by simultaneous application of FTX, a toxin purified from *Agelenopsis aperta* venom, synthetic omega-conotoxin and Gd^{3+} (Molgó et al.,1991b) did not prevent ACh release caused by CTX in the presence of Ca^{2+} (Molgó et al.,1993a). These results may suggest that CTX exerts its effects on ACh release from Torpedo synaptosomes by increasing synaptosomal Na^+ levels sufficiently to reverse the Na^+/Ca^{2+} exchange system which normally uses the Na^+ gradient to extrude Ca^{2+}. In the reversed mode the exchanger will import Ca^{2+}.

CTX also increases spontaneous quantal acetylcholine release at frog neuromuscular junctions even in a nominally Ca^{2+}-free medium supplemented with EGTA (Molgó et al.,1990). TTX completely prevented activation of the release process by CTX suggesting that the CTX effect depends on Na^+ entry into the terminal (Molgó et al.,1991a). Furthermore, ultrastructural studies performed at neuromuscular junctions in which quantal transmitter was exhausted irreversibly by CTX, after 3–4hr of toxin action, revealed a marked depletion of synaptic vesicles per nerve terminal cross-section. The depletion of synaptic vesicles was accompanied by enlargement of the presynaptic membrane coupled to the swelling of the terminal (Molgó et al.,1991a; Comella, Molgó & Legrand unpubl. results) suggesting that CTX impairs the recycling process that, under normal conditions, maintains the synaptic vesicle population during quantal release.

Experiments described here aim to characterize some of the basic changes occurring at the neuromuscular junction in situ during the action of CTX. For this purpose we have used a lipophilic dye, that becomes fluorescent only after incorporation into the outer leaflet of surface membranes, in conjunction with the recently evolved confocal laser scanning microscope which allows optical sectioning of the neuromuscular junction at a desired thickness and a subsequent 3-dimensional reconstitution of the imaged motor nerve terminals.

Confocal laser microscopy appears as one of the most exciting and valuable techniques for optical sectioning, high resolution three dimensional imaging and reconstitution of fluorescence-labelled or reflecting cellular structures. This kind of analysis can be done on living nerve-muscle preparations without the need of physical sectioning and enables the investigation of processes, like the time course of action of CTX, which is not readily studied in fixed preparations.

MATERIAL AND METHODS

Experiments were performed using isolated cutaneous pectoris nerve-muscle preparations from adult male frogs, *Rana esculenta* (20–25g) between October and April. The excised nerve-muscle preparation was pinned to the bottom of a rhodorsil-lined plexiglass chamber (2ml), exposed for 5–60min to the dye (FM1-43,

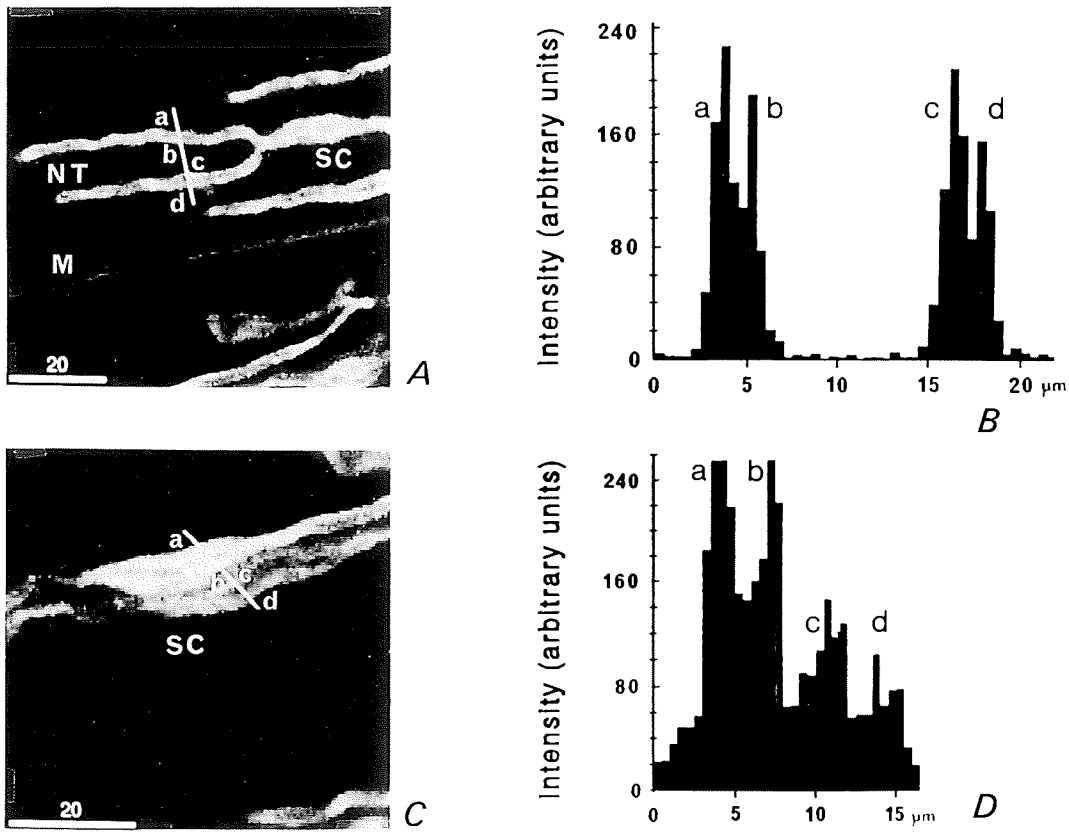

FIG.2. Images of a neuromuscular junction (A) and of a perisynaptic Schwann cell (C) stained with the dye FM1-43. In B and D, the intensity of the fluorescence between the lines shown in A and C is indicated. The peaks of the histograms in B and D (a,b,c,d) correspond to the zones labelled in the images A and C. The images A and C represent the 3-D reconstitution by a projection of 30 serial sections (0.5µm steps).

Molecular Probes, Eugene, Or., U.S.A) [N-(3-triethyl ammonium) propyl]-4-(dibutylamino-styryl pyridinium, dibromide (2µM) dissolved in standard physiological solution of the following composition (mM): NaCl, 115.0; KCl, 2.1; $CaCl_2$, 1.8; and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 5 (pH=7.25); and then washed with the standard physiological solution. The experiments were carried out at 20°C. Only neuromuscular junctions of surface fibers were studied. In some experiments excitation contraction of cutaneous pectoris muscles was uncoupled by treating the preparations with 2M formamide (Sigma, St. Louis, U.S.A) as previously described (del Castillo & Escalona de Motta,1978). In other experiments, D-mannitol (Sigma, St. Louis, U.S.A) was added to the standard solution and osmolality was determined using a Knauer (Berlin, Germany) freezing-point osmometer. Ciguatoxin (CTX-1B) was extracted from *Gymnothorax javanicus* (moray eel) liver and viscera (Legrand et al.,1989; Murata et al., 1990). Tetrodotoxin was from Sigma (St. Louis, U.S.A.).

Neuromuscular junctions were imaged with a Sarastro-2000 confocal laser scanning microscope (Molecular Dynamics, California, U.S.A.) composed of an upwright NIKON optiphot-2 microscope equipped with a single argon-ion laser beam emmitting light at 488nm (high power, maximum output 25mW), with a 3% neutral density transmission filter to prevent dye bleaching. A 510nm dichroic mirror and a 510nm long pass emission filter were used. The aperture setting was 50µm. The photomultiplier was set at a constant level in a given experiment (between

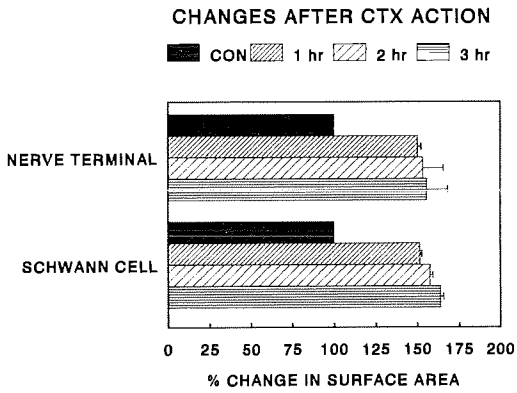

FIG.3. Relative changes caused by 10nM CTX-1b on the surface area of motor nerve terminals and Schwann cell somata with respect to controls at different times of toxin action. The black columns denote respective controls.

600–900V). Images were acquired with single scans or after averaging. Neuromuscular junctions were routinely visualized with a 40x water immersion objective (0.55 numerical aperture).

Control of the scanner module and image analysis of the data files was achieved with a Silicon Graphics workstation (Mountain View, Ca, U.S.A) integrated into the Sarastro system. Images were analyzed with a Silicon Graphics Personal Iris 4D/35G workstation using a UNIX™ operating system and the software Image Space from Molecular Dynamics. A series of optical sections were taken at 0.13–0.5μmsteps. Images from each experiment were processed identically and stored on rewritable magneto-optical disks. In all experiments neuromuscular junctions were imaged before and after the various treatments.

RESULTS AND DISCUSSION.

STAINING OF THE NEUROMUSCULAR JUNCTION IN SITU

The fluorescent staining appears on motor nerve terminals, on myelinated nerve fibres, and in perisynaptic Schwann cells somata (Fig.1). This staining is difficult to wash out after such a long exposure (60min) to the dye. The mechanism of staining seems to be due to the high affinity of the dye for lipid membranes coupled with an inability to penetrate, so that the dye seems to partition only into the outer leaflet of surface membranes (Betz et al.,1992). In contrast to previous work by Betz et al. (1992), we have found that the FM1-43 dye also stains living motor nerve terminals in an activity-independent fashion. Staining in the various membrane structures was detected on resting preparations exposed for only 5min to the dye and then washed out, with dye-free medium. This staining lasted for more than 12hrs.

When the nerve terminal and the Schwann cell somata were imaged at higher magnification by a stack of horizontal scans, the image of the 3-D volume described by the section series (look through projection) revealed both surface and internal structures (Fig.2). The intensity of fluorescence was more marked at the contours and edges than in the interior of both structures. Pixel intensity plots of line scans (Fig.2b,d) showed peaks corresponding to the limits of the nerve terminal membrane and Schwann cell somata membrane. The axoplasma of the terminal and the cytoplasma of the Schwann cell exhibited lower intensity. Having characterized the dye staining in motor terminals and Schwann cells, we performed further experiments in order to determine whether CTX-1B was still active in enhancing quantal transmitter release after application and washout of the dye. Under these conditions, as in control junctions (see Molgó et al.,1990), CTX-1B (2.5nM) increased the frequency of miniature endplate potentials (data not shown). These results indicated first that the FM1-43 dye did not perturb the effect of CTX-1B and second that the dye was suitable for following eventual changes in the nerve terminal surface area during the action of CTX-1B.

EFFECT OF CTX-1b ON MOTOR NERVE TERMINALS IN SITU

In junctions in which muscle contraction was prevented by prior treatment with formamide, stained with the FM1-43 dye and then washed out, one of the nerve terminals was selected and, imaged before and after different times of CTX-1B (10nM) addition to the standard medium. Usually 10 horizontal section images (0.5μm step) were made for complete reconstructed view of the nerve terminals at each time period investigated. Increases in the nerve terminal surface area were evident within 15–17min of CTX-1B (10nM) addition to the medium, this increase in surface area continued for 3hrs. Relative changes in surface area at 1, 2 and 3hrs of CTX-1B action (Fig.3) were greatest during the first hour (50±2.0%; n=3) compared with the second and third hour of CTX-1B exposure. After the second and third hour nerve terminals only increased

3.4±0.15 and 5.7±0.29% respectively with respect to the first hour (Fig.3). When junctions were pretreated with TTX (1μM) no such changes were observed during 3hrs of CTX-1B action. Thus, the increase in nerve terminal surface area of motor nerve terminals might be related to both increase of intraterminal Na^+ concentration and to the enhanced quantal release. None of the 6 nerve terminals imaged during 3hrs with CTX-1B showed fluorescent spots inside the terminals, as observed with high K^+ medium (Betz et al., 1992). This result supports the previous view that CTX-1B blocks the recycling of clear synaptic vesicles (Molgó et al.,1991a).

Effect of CTX-1B on Perisynaptic Schwann Cells In Situ

Satellite cells of the nervous system, oligodendrocytes and astrocytes in the central nervous system and Schwann cells in the peripheral nervous system, have a regulatory role in synaptic transmission. Thus, glial cells can be depolarized by K^+ accumulation near active neurons in situ (Orkand et al.,1966), can respond to many chemical transmitters in vitro (Orkand,1982; Dave et al., 1991) and express a diversity of ion channels (Ritchie,1992; Sontheimer,1992). At the frog neuromuscular junction non-myelinating Schwann cells cover the motor nerve terminal and send fine processes around it (Birks et al., 1960; Dreyer & Peper,1974). The Schwann cell processes are generally located between the active zones at irregular intervals (Couteaux & Pécot Dechavassine,1970). We tested whether perisynaptic non-myelinating Schwann cells are affected, like motor nerve terminals, by CTX-1B (10nM). Schwann cells also markedly increased their surface area during 1, 2 and 3hrs of CTX-1B action (Fig.3). After 3 hours of CTX-1B action the increase was more marked (64±2.9%) than in motor nerve terminals. The changes of the Schwann cells lying directly over the nerve terminal were similar to the changes observed in cells located lateral to the nerve terminals. TTX (1μM) completely prevented such changes when applied before CTX-1B. Therefore, in addition to acting on motor nerve terminals CTX-1B also acts on Schwann cells. Is this related to the action of the CTX-1B on sodium channels of Schwann cells or is it the result of the changes in quantal acetylcholine release caused by CTX-1B? The role of the nonmyelinating perisynaptic Schwann cells at the frog neuromuscular junction during synaptic activity and particularly during transmitter release remains unknown. Recent studies have shown that motor nerve stimulation induces an increase in intracellular Ca^{2+} concentration in Schwann cells (Jahromi et al.,1992). Since Schwann cells undergo profound changes during the action of CTX-1B it is likely that they may play a role in the maintenance of the neuromuscular junction.

Effect of CTX-1B on Muscle Fibres In Situ

When skeletal muscle fibres in which muscle contraction was prevented (by formamide treatment) were imaged at junctional sites before and after 3hr of CTX-1Baction, the changes observed in muscle fibre surface area were of the order of 1−1.5% (n=4). Attempts to investigate the effects of CTX-1B in muscle fibres with functional excitation-contraction coupling failed due to the spontaneous contractions induced by the toxin which prevented imaging during the first hr of toxin action.

Effects of D-Mannitol after CTX-1B Action

Mannitol was reported to markedly improve neurologic and muscular dysfunction in patients with acute ciguatera (Palafox et al.,1988). Although these observations were uncontrolled, the dramatic clinical improvement suggested that mannitol may have a valuable therapeutic effect on this intoxication. The mechanism of action of mannitol is obscure (Russell & Egen,1991). One possibility that was suggested is that D-mannitol may neutralize the toxin by some covalent coupling or complexation. Another possibility is that mannitol may exert osmotic effects by increasing extracellular osmolality. Finally, one should take into account that mannitol reacts with free radicals and is considered as an effective hydroxyl radical scavenger (Halliwell & Gutteridge,1985). The possibility that hyperosmotic D-mannitol may exert its action on ciguatera due to its hydroxyl radical-scavenging properties or its water-draining effect has been suggested by Pearn et al., (1989).

A free radical is an atom or molecule that contains one or more unpaired electrons so that to attain stability either donates its electron to other molecules or acquires an extra electron from adjacent molecules. Indeed, free radicals are highly reactive and, because of their instability, damage cells and tissues. D-mannitol reacts with the very reactive and short-lived hydroxyl radical in a way that its concentration can be limited. Hydroxyl radicals also stimulate

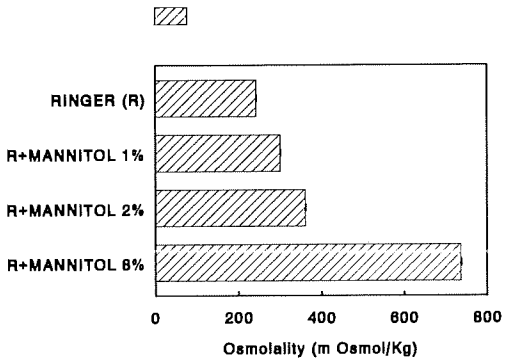

FIG.4. Osmolality of the standard Ringer's solution (R) and of the various standard solutions (R) to which D-mannitol was added.

phospholipase A_2 leading to release of arachidonic acid. One interesting property of hydroxyl radicals is their ability to initiate lipid peroxidation by extracting a hydrogen atom from polyunsaturated fatty acids such as arachidonic acid. This leads to cell membrane damage and frequently to cell death.

We performed experiments on isolated frog neuromuscular junctions in order to determine whether D-mannitol could modify the actions of CTX-1B. For this purpose, we used a dose of D-mannitol which has no osmotic effects per se but which protects kainate-induced death of cerebellar neurons in culture by scavenging hydroxyl radicals (Dykens et al.,1987). When preparations were pre-treated with 20μM D-mannitol, subsequent addition of CTX-1B (2.5nM) caused similar effects to those observed in the absence of D-mannitol i.e. there was an increase of spontaneous quantal release and spontaneous asynchronous contractions (data not shown) and depolarization of the muscle membrane (Molgó et al.,1990). Thus, it appears that 20μM D-mannitol does not prevent the actions of CTX-1B at the neuromuscular junction. When nerve terminals and Schwann cells were imaged during 3hrs of CTX-1B action with the confocal laser scanning microscope the typical changes above reported, i.e. changes in surface area of nerve terminals and Schwann cells, were observed.

We conclude that D-mannitol concentrations which has been reported to exert an effective hydroxyl radical-scavenger action neither prevented binding of CTX-1B nor antagonized effects of the toxin.

Further experiments were performed with higher concentrations of D-mannitol (54.9mM =1%; 109.8mM=2% and 439.1mM =8%) added to the standard Ringer's solution to determine osmotic effects of this agent. Since solutions containing 1, 2 and 8% D-mannitol added to the standard Ringer solution (Fig.4) had osmolalities that are 24% (1% D-mannitol), 48.8% (2% D-mannitol) and 203.7% (8% D-mannitol) higher than the standard Ringer's solution and, increases in osmotic pressure causes dramatic increases in spontaneous quantal release. At the frog neuromuscular junction a 50% increase in osmotic pressure by addition of sucrose causes a reversible 45-fold increase in miniature endplate potential frequency, as previously reported (Fatt & Katz,1952). We did not attempt to study the effects of CTX-1B in the presence of such high concentrations of mannitol. Instead we tried D-mannitol after CTX-1B action at the neuromuscular junction to determine whether this agent at different osmolaties could modify the changes in the nerve terminal and Schwann cells previously described with the toxin. D-mannitol effectively caused a shrinkage of nerve terminals and Schwann cells previously swollen by the action of CTX-1B (Fig.5). When the effects of mannitol were quantified in nerve terminals it was evident that 2% mannitol applied for 30min decreased the nerve terminal surface area by 21±1.0% and the Schwann cell surface area by 15.2%, while the muscle fibre was decreased by 11.4±O.2% (n=4). In control junctions D-mannitol reduced the nerve terminal surface area by only 13±1.2%, the Schwann cell surface area by 10±0.5% and the muscle surface area by 11±0.6% indicating that mannitol was more effective in reducing nerve terminal surface area in nerve terminals treated with CTX-1B than in control nerve terminals. However, muscle changes caused by mannitol were no different in CTX-1B treated junctions as compared to controls. D-mannitol, at concentrations that increased the osmolality of the standard solution by c.50%, reversed swelling of motor nerve terminals and Schwann cells observed during long-term effects of CTX-1B.

These findings are important since the clinical improvement observed in acute ciguatera after mannitol treatment may be ascribed to the osmotic action exerted by this agent in the peripheral nervous system and skeletal muscle fibres, which would result in a shift of water from the intracellular to the extracellular compartment.

DISCUSSION

Cell swelling of motor nerve terminals and perisynaptic Schwann cells was a common

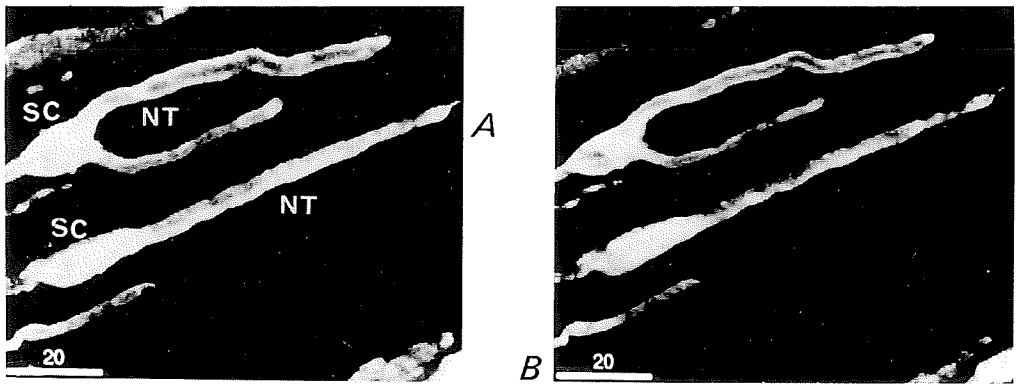

FIG.5. Nerve terminal (NT) and perisynaptic Schwann cell somata (SC) from a junction treated for 3hr with 2.5nM CTX- 1B (a) and after 30min of D-mannitol (2%) added to the standard solution (b). Note the shrinkage of structures after mannitol action.

response to CTX-1B application at living neuromuscular junctions. Imaging methods are the only way in which the shape changes accompanying cell volume changes can be determined. However, determinations of cell volume are not easy, even in a relatively simple synapse as the neuromuscular junction. The term cell volume is a complex concept because neither motor endings nor perisynaptic Schwann cells have simple individual geometric shapes and relationships. Furthermore, the mechanisms of volume homeostasis in motor endings have not been explored.

Changes in nerve terminal volume caused by CTX-1B may result from the fusion of synaptic vesicles to the presynaptic membrane and the influx of Na^+ across the presynaptic membrane. Previous electron microscopic studies of motor endings in fixed specimens revealed time-dependent increase in the nerve terminal perimeter, alterations in nerve terminal mitochondria and profound depletion of synaptic vesicles after CTX-1B action (Molgó et al., 1991; Molgó, Comella & Legrand, unpubl.).

The Na^+ content of the nerve terminals is expected to be increased by CTX-1B. Under normal conditions, water is in thermodynamic equilibrium across the terminal membrane. However, any change in the intracellular Na^+ concentration will result in a rapid water flow from the extracellular to the intracellular compartment. Because the nerve terminal is readily distensible, transmembrane water movements will result in nerve terminal swelling. Schwann cell somata swelling in situ is probably also related to the increase in Na^+ concentration through activation of sodium channels sensitive to the action of both CTX-1B and TTX. The contribution of enhanced quantal transmitter release to the swelling of Schwann cells remains to be established.

D-Mannitol at concentrations reported to exert an effective hydroxyl radical scavenger action neither prevented the action of CTX-1B nor antagonized its effects. However, at higher concentrations mannitol exerted osmotic effects that caused shrinkage of both motor nerve terminals and Schwann cell somata previously swollen by the action of CTX-1B probably by shifting water from the intracellular to the extracellular compartment.

This report demonstrates that CTX-1B causes time dependent changes in the surface area of motor nerve terminals and perisynaptic Schwann cells in living neuromuscular junctions. We have shown that confocal laser microscopy is a new tool for research on the effects of ciguatoxins on living tissues. While the extent of its future applications in the field of the ciguatoxins is hard to predict, its potential for neurobiological research appears enormous.

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MODIFICATION OF NERVE CONDUCTION IN THE RAT BY BREVETOXIN (PBTX-3). *Memoirs of the Queensland Museum 34(3): 586. 1994*:– Brevetoxins are lipid-soluble polycyclic ether toxins isolated from the marine dinoflagellate *Ptychodiscus brevis*. The toxins PbTx-2 and PbTx-3 bind to a specific receptor site (site 5) on the voltage-dependent sodium channel, a site shared with ciguatoxin. This study set out to examine the effects of PbTx-3 and a possible antagonist on the parameters of nerve conduction.

Electrophysiological studies were carried out on the ventral coccygeal nerve of male Wistar rats. Prior to experimentation each animal was anaesthetised with intramuscular Leptan (420μl/kg). A Medelec MS92a electromyography unit was used for recordings. PbTx-3 (15μg/kg) was administered intravenously over 15 minutes. In antagonist experiments lignocaine (500μg/kg) was delivered intravenously, over 30 minutes.

PbTx-3 produced a significant increase in both the magnitude and duration of supernormality to that of control nerves. This toxin also increased the absolute and relative refractory periods and decreased the conduction velocity. Lignocaine returned these parameters towards control values.

These results demonstrate that PbTx-3 alters nerve conduction parameters of rats in a similar way to ciguatoxin. It is suggested that brevetoxin may provide a suitable model in further studies pertaining to possible therapeutic agents for ciguatera poisoning.

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CIGUATERA RESEARCH - AN HISTORICAL PERSPECTIVE. *Memoirs of the Queensland Museum 34(3): 586. 1994*:– Ciguatera research at the University of Hawaii was initiated in the mid-1950's by the late Professor A.H. Banner, who formulated four principal objectives: 1, What is the molecular structure of the toxin? 2, What is the origin of the toxin? 3, Can a diagnostic test be devised that distinguishes toxic from nontoxic fish? 4, Can an effective human therapy be found?

Elucidation of the molecular structure was of central concern since success with the other three goals would be greatly enhanced, or depend on, a knowledge of structural features.

Inadequate supplies of toxic fish, establishment of a suitable bioassay, and technology of the 60's made for slow progress. Even after the discovery of a dinoflagellate as the primary toxin producer in 1977, moray eels had to remain the sole source of toxin, since *G. toxicus* cultures yielded only the water-soluble maitotoxin, which was distinctly different from the lipid-soluble ciguatoxin extracted from carnivorous fish.

While an extensive search for an algal food source or a toxin precursor produced no useful leads, it gave rise to significant discoveries, most prominent among them of palytoxin, which in time became a benchmark in marine natural product chemistry, and indeed, in all of organic chemistry.

The first clue that ciguatoxin belonged to the structural type of polyethers did not come from sophisticated instrumentation, but from its behaviour in chromatography which paralleled that of okadaic acid, a compound first isolated and characterized as a constituent of a sponge and subsequently identified as a metabolite of the dinoflagellate *Prorocentrum lima*. Interestingly, okadaic acid has low mammalian toxicity and has become an important probe in the study of cellular regulation.

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REEF MANAGEMENT AND SEAFOOD MONITORING PROGRAMS FOR CIGUATERA

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Park, D.L. 1994 08 01: Reef management and seafood monitoring programs for ciguatera. *Memoirs of the Queensland Museum* **34**(3): 587–594. Brisbane. ISSN 0079-8835.

Ciguatera (CTX) toxins in fishery products are odorless, tasteless, and generally undetectable by simple chemical test; bioassays traditionally monitor suspect fish. Assurance that susceptible foods are safe to eat will come from marketplace screening, separation of adulterated product to less risk uses, and, where feasible, prediction of potentially hazardous food production/harvesting areas. An effective screening method for use in the marketplace must be: (a) easy to use and interpret; (b) able to test a large number of samples in a short period; (c) accurately differentiate between toxic and non-toxic product; (d) low cost; (e) available in sufficient quantity to meet private, industrial, and regulatory agency demands; and (f) where possible identify toxins involved. The solid-phase immunobead assay (S-PIA, Ciguatect™) has the highest potential for this purpose. The kit can be used on fishing vessels, at receiving docks, processing plants, distribution organizations, retail outlets, consumers, and regulatory agencies and is designed for non-laboratory use by untrained personnel.

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Ciguatera fish poisoning is a centuries old illness, endemic to tropical and subtropical areas (WHO,1984), sometimes shipped to nontropical population centres (North America). Humans are exposed through consumption of fish which have accumulated toxins produced by dinoflagellates. An estimated 50,000–500,000 cases of ciguatera occur each year (Ragelis, 1984). Symptoms are gastrointestinal, neurological and cardiovascular and can persist for weeks and even years (Juranovic & Park,1991). U.S. public health agencies (Food and Drug Administration and National Marine Fisheries Service) have been striving to implement a combative seafood safety program for years.

Public health research on ciguatera has focused on protection of human health and enhancement of commerce in subtropical reef fish. To set up an effective seafood monitoring program, it is necessary to understand how products become toxic and so develop an analytical technique for detection. Historically, methods of analysis for ciguatoxins (CTX) have been labor-intensive, time-consuming, and not able to identify individual toxins (Juranovic & Park,1991).

TOXIN PRODUCING ALGAE AND PRINCIPAL TOXINS INVOLVED IN CIGUATERA

CTX accumulates in benthic feeding herbivorous fish and then up the food chain to man. Benthic toxigenic dinoflagellates suspected in ciguatera poisoning include *Gambierdiscus toxicus, Prorocentrum lima, P. concavum, P. emarginatum, P. mexicanum, P. rathynum, Amphidinium carterae, Ostreopis ovata, O. siamensis, O. lenticula, Coolia monotis, Scrippsiella subsalsa,* and *Thecadinium* sp.

Of several toxins which may be responsible for ciguatera, ciguatoxin has been isolated as the major toxin from large carnivores while smaller amounts have been detected in herbivores. An explanation for this could be that CTX accumulates preferentially in large carnivores due to its greater lipid solubility. Murata et al. (1990) reported the structures of ciguatoxin from the moray eel (*Gymnothoraxjavanicus*) and its likely precursor from *G. toxicus*. The congener was shown to be a less oxygenated analog of ciguatoxin. However, it has not been demonstrated that the toxin produced by the dinoflagellate is the precursor to ciguatoxin(s) accumulating in fish. Until sufficient quantities of individual toxins become available and suitable detection methods for these toxins are developed, it will be difficult to determine toxin properties. At least five toxins are implicated in ciguatera; they are ciguatoxin (CTX), maitotoxin (MTX), scaritoxin (STX), okadaic acid (OA), and a recently named toxin, prorocentrolid (Bagnis et al.,1974; Chungue et al.,1977; Maulin et al., 1992; Tachibana, 1980; Tindall et al.,1984; Yasumoto et al.,1971; Yasumoto et al.,1984; Yasumoto & Murata,1988a; Yasumoto & Murata,1988b; Yasumoto & Scheuer,1969). Recent studies suggest that in ex-

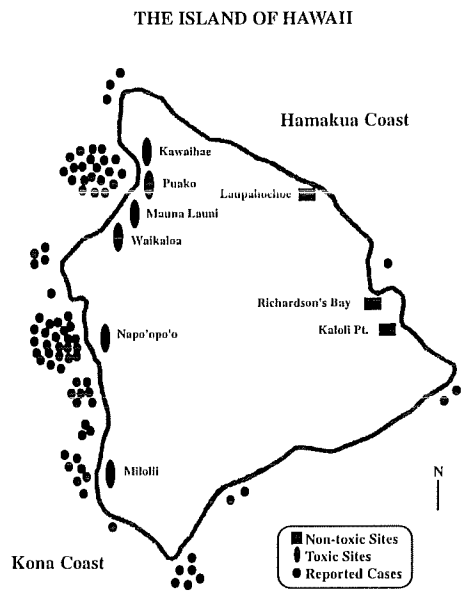

FIG. 1. Reports of cases of ciguatera fish poisoning outbreaks recorded by Hawaii State Department of Health (1981-1990). Toxic (Kona Coast) and non-toxic (Hamakua Coast) sites for collecting biomarker model are noted.

cess of 20 toxins may be involved in the ciguatera phenomenon (Juranovic et al.,in press; Legrand,1991; Lewis et al.,1991; Lewis & Sellin,1992; Lewis,1992). Relative concentrations and toxin profiles for each toxic fish vary greatly and are unknown due to the lack of individual toxin reference standards and specific analytical methods.

Okadaic acid is available commercially as a standard reference material. For CTX, however, ciguatoxin and possibly maitotoxin and their analogs are the toxins with the highest toxic potentials. The toxic potential of okadaic acid is several orders of magnitude lower than ciguatoxin.

ANALYTICAL METHODOLOGY

Analytical methods for phycotoxins vary according to the application, i.e., screening, identifying, etc. (Park,1994). For ciguatera biossays have been used in the laboratory but are unsuitable as a marketplace test. Most earlier methods were based on biological endpoints which had major limitations on levels of detection and specificity. Many native tests for fish toxicity have been examined, including discoloration of silver coins, or copper wire, the repulsion of flies or ants, and rubbing the liver on the gums to ascertain if it causes a tingling feeling (Juranovic & Park,1991). With the possible exception of rubbing the liver on the sensitive tissues of the mouth, all have proven invalid. As more reference material and standards became available, chemical and immunochemical methods have emerged.

Bioassays have one common disadvantage: the lack of specificity for individual toxins. Alternative methods based on immunochemistry (Hokama et al.,1977) are applicable to screening fish in the marketplace. The original assay, a radioimmunoassay (RIA) for ciguatoxin, was developed using antibodies produced against a conjugate of human serum albumin and ciguatoxin (isolated from toxic moray eel) injected into sheep and rabbit. This assay was used successfully to test ciguatera and to screen for toxic amberjacks (*Seriola dumerili*) where 15% of the fish were rejected during a 2-yr study on the Hawaiian market (Kimura et al.,1982). Despite this success, the assay was not suitable for routine use due to high cost, instrumentation requirements, and time involvement.

In 1983, a competitive enzyme immunoassay (EIA) commonly called the 'sticktest' was developed using the polyclonal antibody used in the RIA, and evaluated on Hawaiian reef fishes (Hokama et al.,1983; Hokama et al.,1984; Hokama,1985). As with its predecessor, this antibody demonstrated close structural similarity of CTX, MTX, brevetoxin, and OA. EIA used liquid-paper applied to bamboo sticks to isolate and bind the toxins (Hokama,1985). This assay was able to distinguish between toxic and nontoxic fish. Test results revealed a high number of false-positives, although no false-negatives were observed (Hokama et al.,1987; Hokama & Miyahara,1986).

The stick test was modified further using monoclonal antibodies specific for CTX, OA, and a synthetic fragment of OA, that are more specific than the sheep antibody (Hokama et al.,1990; Hokama et al., 1992; Hokama et al.,1986). This antibody gave peak titers of 1.5ng, 10ng and 50ng, respectively, for CTX, the fragment of OA, and OA (Hokama et al.,1992). Competitive inhibition analyses showed that 4ng purified CTX blocked completely the antibody reaction with crude CTX, OA and the fragment of OA at similar concentrations (approximately 50ng). This assay was used to test fish specimens

from documented cases of ciguatera (Hawaii Department of Health) with 98% agreement (Hokama et al.,1989). A preliminary collaborative evaluation study of the rapid enzyme immunoassay stick test was conducted (Ragelis, 1987,1988). Eight of the nine laboratories involved obtained results within acceptable limits for each of 3 fish cake samples homogenized with ciguatoxin. The relative standard deviation of reproducibility (RSD_R) was 23–30%. Due to the lack of a chemically identifiable standard, the full collaborative study was not conducted.

This assay was modified to a solid-phase immunobead assay format (Hokama,1990) commonly known as the 'paddle test', using bamboo paddles coated with liquid paper. This format was used to test 26 cases of ciguatera with 100% agreement. In a study comparing the stick and paddle tests, 436 specimens with varied levels of toxicity showed 80% agreement (Hokama,1990).

Patents covering the stick and paddle tests were purchased by HawaiiChemtect International. The original format was modified to an innovative rapid solid-phase immunobead assay (S-PIA, Ciguatect™) for ciguateric toxins (CTX) and diarrheic shellfish poisoning (DSP) outbreaks (Park & Goldsmith,1991; Park et al.,1992b). Toxins are determined by binding them to a membrane attached to a plastic strip and exposing the toxin-ladened membrane to a monoclonal antibody-colored latex bead complex which has a high specificity for the toxins of interest. The intensity of the color on the membrane denotes the toxins. CTX toxicity potential can be determined directly on edible tissue or following specific extraction procedures. The method has been used to evaluate CTX potential in fish obtained from Hawaii, Australia, and the Caribbean (Park et al.,1992b). The Ciguatect™ test kit has been compared to the mouse assay for the detection of toxic fish, i.e. fish of tropical origin for sale in Canada (Todd *et al.*,1992) and fish collected from St. Thomas, U.S. Virgin Islands (Dickey et al.,this memoir). Both studies reported a high percentage of fish toxic to the mouse and positive for CTX-related toxins by the Ciguatect™ test kit or following a rapid extraction and purification procedure. CTX-related toxins are present in a significant number of fish; however, the toxicological or public health significance is unknown, i.e., would the toxin(s) present (profile, potency and concentration) pose a significant risk for acute poisoning and/or chronic toxicity? Todd and co-workers used 135–250g equivalent fish flesh for injection into the mouse where 85% of the mice died within 24hours. Dickey et al. (this memoir) used 45–180g equivalent fish flesh and 67% of the mice died within 48 hours. Interpretation of mouse assay results must be made with caution, however. Mouse toxicity results have been useful in confirming toxins in ciguatera outbreaks, although the mouse is relatively resistant to CTX. Because of the lack of specificity, the mouse bioassay should not be used to predict ciguatoxicity. This was particularly apparent with the Dickey et al. study where for 22% of the specimens one animal died within a short time frame and the duplicate animal survived 48 hours. 43%, of the animals that died, died within 30min. Short death times (<30min) with the mouse are not considered ciguatoxic. Additionally, the statistical procedure used by the authors can only be applied when the method used for comparison by definition is 100% accurate for sensitivity and specificity (Riegelman & Hirsch,1989). The mouse assay is unlikely to be 100% accurate in determining ciguatera toxicity. As was pointed out by Hoffman et al. (1983) and Vernoux (1993), the mouse can have utility when symptomatology is used as well (symptomology was used as a criteria in the Dickey et al. study). A preferred procedure for the evaluation of a test method is outlined below.

For those products where additional testing is desired, possibly for samples testing positive, the University of Arizona and HawaiiChemtect International have developed a rapid extraction method (REM™) capable of extraction and partial purification of ciguateric toxins in <30 mins (Park et al.,1992a). The REM procedure isolates and purifies CTX-related toxins on the same chemical basis as used in more exhaustive CTX extraction procedures. For the REM™, toxins are extracted with a chloroform:water:methanol mixture and partitioned into selected phases by varying polarity. When the REM™ is used in combination with the Ciguatect™ test kit, the limit of detection for ciguatoxin, okadaic acid and related toxins is <0.05ng/g fish flesh. Also, at this point chemical methods based on thin layer (TLC) or high performance liquid chromatography (HPLC) technology can be used to confirm individual toxins.

Methods based on thin layer (TLC) and high performance liquid chromatography (HPLC) have been developed for selected individual toxins associated with CTX (Lee *et al.*, 1987; Legrand,1991; Dickey *et al.*, 1990). These methods can be applied as a regulatory tool where

TABLE 1. Precision parameters of collaborative data for solid-phase immunobead assay (Ciguatect™) determination of ciguatoxins and related polyether compounds in parrot fish, surgeon fish and amberjacks from the Hawaii Island.

	mean	S_r	S_R	RSD_r (%)	RSD_R (%)
	Fish Fillets				
Parrot Fish (*Scarus* sp.)	1.2	0.16	0.53	13.5	44.4
Surgeon Fish (*Ctenochaetus* sp.)	1.7	0.15	0.50	9.0	29.7
Amberjack (*Caranx* sp.)	3.6	0.15	0.51	4.3	14.3
	REM Extracts				
Parrot Fish (*Scarus* sp.)	3.1	0.18	0.37	5.8	11.9
Surgeon Fish (*Ctenochaetus* sp.)	3.8	0.18	0.38	4.8	9.9
Amberjack (*Caranx* sp.)	4.9	0.18	0.37	3.7	7.6

S_r= Standard deviation of repeatability
S_R = Standard deviation of reproducibility
RSD_r= Relative standard deviation of repeatability
RSD_R = Relative standard deviation of reproducibility

sophisticated laboratory facilities are available. HPLC techniques have been applied to analysis of okadaic acid in fish and shellfish (Gamboa *et al.*, 1992; Yasumoto,1985; Lee et al.,1989; Dickey et al.,1990). Park and co-workers (unpubl. data) have developed a TLC method for okadaic acid in fish tissue and dinoflagellate cultures. Specificity of this methodology is enhanced by exhaustive purification of toxins extracted from fish tissue. Unfortunately, CTX and okadaic acid develop similarly on TLC. These methods, although not suitable for routine screening programs, could play an important role in confirming the presence of individual toxins in fish products.

HPLC methodology have been reported for ciguatoxin and several analogues (Murata et al.,1990; Lewis et al.,1991; Lewis & Sellin,1992). These studies reported four major ciguatoxins. Legrand (1991) and co-workers (Legrand et al.,1990) used HPLC methodology to isolate multiple ciguatera toxins from wild *Gambierdiscus toxicus* and toxic herbivorous and carnivorous fish.

METHOD VALIDATION

Any method intended to be used in a seafood safety monitoring program must pass stringent in-house evaluation and be validated through an inter-laboratory study to determine precision (reproducibility, repeatability) and accuracy (recovery) parameters of the method. Method validation programs are administered by AOAC International (AOAC) and International Union for Pure and Applied Chemistry (IUPAC). These validation programs include two phases. The first phase is a ruggedness test or feasibility (mini-collaborative) study. Acceptable results in this phase lead to a collaborative study (Phase II). These studies (mini- and full-collaborative) involve the distribution of coded samples (in duplicate) of fish, preferably authentic ciguatera fish poisoning specimens, to participating laboratories. Known amounts of specific standards are also added to some of the samples. The samples are analyzed following exact method protocols and results returned to the study organizer. Phase I of the validation process, i.e., mini-collaborative study, was carried out for the S-PIA using fish fillets and REM™ extracts (Park et al.,1992a,b). The AOAC/IUPAC inter-laboratory mechanism was used. The precision of the S-PIA (Ciguatect™) to detect CTX has been evaluated through analysis of toxic and non-toxic fish fillets (amberjack, surgeon, and parrot fish) and REM™ extracts of the same fish obtained from fishing areas around the Hawaiian Islands. Toxicity potentials of purified extracts were determined using the mouse and brine shrimp (*Artemia* sp.) assays. The analysis showed acceptable repeatability and reproducibility parameters (Table 1). University of Arizona, FDA and NMFS laboratories participated in the study. The study confirmed excellent performance and interpretation of results, and demonstrated acceptable precision parameters (Park et al.,1992a,b). A full-collaborative study of the test kit is recommended. The validation study will include test portions of naturally and artificially contaminated fish with ciguatoxin and okadaic acid. Toxicity potentials will be determined using the mouse and brine shrimp assays.

SEAFOOD SAFETY MONITORING PROGRAMS

An effective food safety monitoring program comprises: 1, monitoring fish harvesting areas for CTX; 2, establishment of regulatory limits, and; 3, screening commercial fish products. Unacceptable product can be further tested to identify the toxin(s).

MONITORING FISH HARVESTING AREAS

Since seafoods commonly associated with

ciguatera poisoning outbreaks are associated with highly mobile fish, collecting and testing such fish alone could provide misleading information so testing less mobile species (e.g. invertebrates) should be included.

The Ciguatect™ S-PIA screened 36 species of nearshore invertebrates off the Island of Hawaii for ciguatoxin and related polyethers (Fig.1) (R.G. Kvitek, Moss Landing Research Laboratories, and D.L. Park, University of Arizona, unpubl. data). Specimens included snails, sea urchins, sea cucumbers, crabs, brittle stars, bivalves, and zoanthids. Invertebrates were collected at 6 'toxic' locations along the Kona coast, and at 3 'non-toxic' sites along the Hamakua coast where there had been only one reported case of ciguatera since 1980.

A significant positive correlation between assay results and site-specific ciguatera history was found for the cowry *Cypraea maculifera* (Fig.2). While assay results for most other species indicated low or no ciguatoxin, cone snails (*Conus*), ophiuroids (*Ophiocoma*) and sea cucumbers (*Holothuria*) tested positive frequently. There was no correlation, however, for these three genera between assay results and site history. These results suggest that invertebrates, particularly grazers and deposit feeders, and especially cowries, accumulate ciguatoxins and related polyether compounds at sites known for ciguatera fish poisoning outbreaks and have the potential utility of being bio-indicators of reef toxicity. This marine specimen, or other invertebrates native to other areas, could be an integral part of the ciguatera monitoring program.

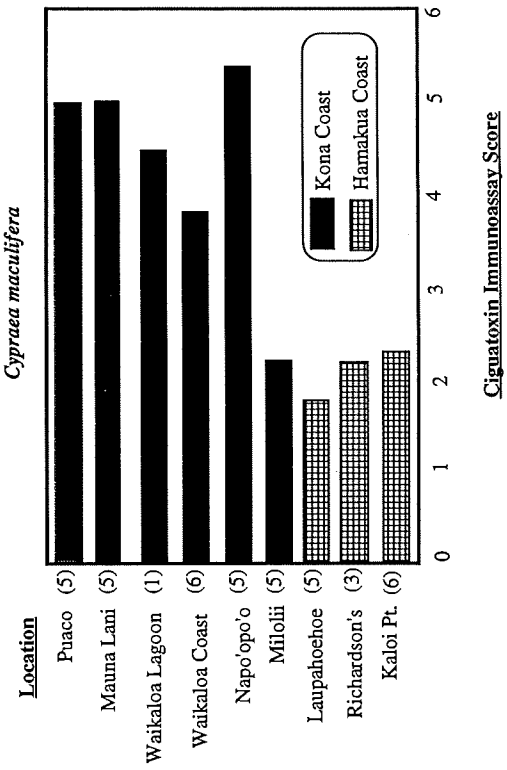

FIG. 2. Ciguatoxin immunoassay scores for *Cypraea maculifera* from Kona and Hamakua Coasts of Hawaii. Color intensity on the test strip assigned a value between 0-6 where 0 = nondetectable and 5 = color intensity equal to 5ng okadaic acid. Numbers in () indicate number of individual specimens tested and values pooled.

Establishment of Regulatory Limits

Based on mouse and mosquito bioassay data, several levels of concern have been proposed. Since multiple toxins of varied toxin potential are involved with CTX poisoning, it is not practical to use a single compound for this regulatory limit. Historically, a seafood safety monitoring program for ciguatera has been hampered by the lack of reference standards, particularly ciguatoxin. Okadaic acid is the only toxin associated with CTX poisoning in sufficient quantities to serve as a reference standard. Toxicity of okadaic acid, however, is significantly lower than ciguatoxin where acute mouse toxicity potentials for ciguatoxins and okadaic acid are 0.45 and 210μg/kg, respectively. The term okadaic acid equivalents (OAE) could be used, however, to *standardize analytical methods* and in the *establishment of regulatory limits*, provided the relative potencies of all toxins involved with the CTX phenomenon are used in calculation of action levels. For this to be feasible, the test employed must recognize all toxins involved in the poisoning in a similar manner and the action level focus on the toxin of highest potency. Again, the term OAE would be used because multiple toxins are involved in the poisoning.

Screen Fish in the Marketplace/Commercial Channels

Any method for screening marketplace seafoods must: a, be easy to use and interpret; b, be rapid, i.e., able to test a large number of samples in a short time; c, accurately differentiate between toxic and non-toxic samples; d, have low cost; e, be available in quantities to meet private, industrial, and regulatory agency testing

demands; and f, where feasible, confirm toxin identity.

The S-PIA method (Ciguatect™) has high potential for screening market place fish. When fully validated, the kit may be used at the harvesting, processing, distribution, retail or other point through the marketing route. Testing fish early after capture is recommended, since this will minimize cost expended for the product and potential economic loss to the industry. The kit can be used on-board fishing vessels, at receiving docks, processing plants, distributing organizations, retail outlets, consumers, and regulatory agencies. The self-contained assay is available as a single analysis kit designed for non-laboratory use by untrained personnel. Organizations conducting large numbers of analyses would be more inclined to use the laboratory kit which contains sufficient material for >50 tests.

The Seafood Safety Monitoring Program would involve large-scale testing of fish according to an acceptable sampling plan. Fish or lots testing negative to the screening procedure would be allowed to proceed normally in commercial channels. Each point identified above would be a quality control point. Product testing positive for toxic potential would be diverted to lower risk uses or retested to confirm toxic potential. This can be done by using the REM procedure which isolates, purifies and concentrates the toxins before retesting or by using alternative test methods for specific toxins.

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CIGUATERA POISONING: CURRENT ISSUES IN LAW

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The current situation with regard to liability under Queensland law relevant to ciguatera poisoning is reviewed. It is argued that all sectors of the fishing industry should be acquainted with their responsibilities under common law and under the statutes of Workplace Health & Safety Act, Trade Practices Act, and Sale of Goods Act to prevent litigation in the event of an incident.

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Areas of Queensland law relevant to ciguatera poisoning are (i) Liability pursuant to Common Law and (ii) Statutes (Workplace Health & Safety Act, Trade Practices Act, Sale of Goods Act).

LIABILITY AT COMMON LAW

Liability at common law can be based on breach of contract, Tort or Statute. Breach of Statute will be dealt with below. Breach of contract, usually in the form of breaching implied duties of care, gives rise, to the extent that privity of contract allows, to similar duties to that which arise in tort liability. Tort, or civil wrong, is based on a concept of a duty of care. For an action to lie in tort, three elements are required to be proven: 1) damage, 2) a relationship of proximity, and 3) want of reasonable care, in circumstances of foreseeable risk.

The starting point is the case of *Donaghue* v. *Stevens*[1], which involved purchase by Donaghue of a bottle of ginger beer, in circumstances where, due to the bottle being opaque, the contents of the bottle could not be seen. The bottle in fact contained the remains of a decomposed snail which fact was not ascertained by Ms Donaghue until after she had consumed the contents of the bottle. She was not the original purchaser of the bottle, which had been bought by a friend and arguably no contractual relationship existed as between her and the maker. In the leading decision Lord Atkin held

'*you must take reasonable care to avoid acts or omissions which you can reasonably foresee would be likely to injure your neighbour - who, then, in law is my neighbour. The answer seems to be - persons who are so closely and directly affected by my act that I ought reasonably have them in contemplation as being so affected when I am directing my mind to the acts or omissions which are called in question.*'

In applying this principle to ciguatera poisoning, the first element to consider is the question of proximity or to whom is the duty owed. Based on the 'neighbour' principle of Lord Atkin, it would be any person whom the provider of fish ought reasonably have in contemplation as likely to be affected. This would include the eventual consumer, whether or not that person be the purchaser of the fish. The relationship vis-a-vis the consumer would be: commercial catcher, marketer, vendor (fresh), and provider (prepared). Each of these (individual or corporate) would owe a duty of care to the consumer of the fish.

The duty is to protect from foreseeable risk of harm. To determine whether or not that duty has been breached, consider:(i) whether there was a foreseeable risk of injury; (ii) whether the foreseeable risk gave rise to the injury - causation; (iii) whether the foreseeable risk could be prevented; and (iv) whether the foreseeable risk should, in all the circumstances, be reasonably prevented.

The standard by which the test of breach is measured is that of the reasonably prudent person[2], which in respect of ciguatera would be 'the reasonably prudent commercial catcher, marketer, vendor or provider'.

Whether a risk of injury is, or is not, foreseeable, depends on the circumstances of an incident. In the Wagon Mound No. 2[3], it was held '*....a person must be regarded as negligent if he does not take steps to eliminate a risk which he knows and ought to know is a real risk and not a mere possibility which would never influence the mind of a reasonable man.*'

In respect of causation, it must be the foreseeable risk which gives rise to the injury. This does not mean that the 'precise' injury must be

foreseen but rather the general nature or category of the injury, i.e. strain, break, poisoning[4].

The case of *McLean* v. *Tedman*[5] deals with the issue of prevention. That case dealt with the system of work adopted by garbage collectors and provided that once the collector had raised an alternative system of work (which could have been adopted and so avoided risk of injury), that it was up to the employer to establish that such system would not work in the circumstances of the case.

Finally, the Court will need to decide whether or not, in all the circumstances of a matter and where the three elements of forseeability, causation and prevention have been made out, as to whether or not there has been a failure to provide reasonable care. In reaching its conclusion the Court will, inter alia, consider matters such as: seriousness of the risk, i.e. its potential to harm; effect on the person upon whom the duty is cast, i.e. whether or not it will unwarrantedly impede the process of industry; and cost of implementation or effect of implementation or alternatives[6].

Further, in reaching its decision and considering the elements of breach, the Court will have regard to practical matters such as: prior complaint, state of general knowledge and/or specialised knowledge on the issue of risk, what steps have been taken to investigate and eliminate risk, whether risk of a similar nature or magnitude has been removed or otherwise dealt with, whether or not subsequent to injury an alteration has been made, and custom and practice within the industry.

From the layman's perspective of ciguatera, the following elements are discernible: species of fish, location of breeding ground, range of symptoms - from mild to serious, prohibition on species/breeding ground, incubation period for onset of symptoms, sensitisation to the ciguatera toxin, increased toxicity in respect of certain parts of the fish, size of fish, treatment, inability to detect.

These factors need to be considered in the light of an individual experience to determine whether or not liability will be incurred. For example, if a commercial catcher of fish sold Red Bass, caught anywhere in Queensland, or narrow barred Spanish mackerel, caught off Platypus Bay, then there is no doubt he would be liable in Tort to any person who consumed the fish and became symptomatic. Equally, it may be the case that any provider of prepared fish who provided as part of a seafood restaurant menu, barracuda liver taken only from large barracuda, would be liable. This case would, of course, be dependent on the state of knowledge. However, it should be remembered that it is not the individual's state of knowledge that is relevant, but rather the state of knowledge of the reasonably prudent provider etc. It is arguable that a seafood provider in Queensland should be aware of the existence of ciguatera poisoning and its likely causation. This is especially so given the regular press coverage given to the subject and the existence of appropriate Departmental information.

At the other end of the scale, it is probably not arguable that liability would accrue to a provider who sold barramundi which in turn lead to symptoms of ciguatera poisoning. This is particularly so, noting the low incidence of ciguatera poisoning linked to the species where only one case is ascribed during the period 1965–1984[7].

Somewhere between these extremes will, of course, be the grey area of concern to the industry. For example, the selling of narrow barred Spanish mackerel which has been linked with 226 cases in Queensland between 1965–1984[8]. This is of more concern when Gillespie, Lewis et al. (1986) statements are considered: '*a large number of cases of ciguatera are not reported to health authorities, so the true incidence of ciguatera is difficult to assess*' and '*Whether these figures reflect a trend towards an increasing incidence of ciguatera or increased public awareness is not known, but it is certain that the abovementioned Reports represent only a proportion of the outbreaks that have occurred*'.

Therefore given what appears to be a relatively high incidence of ciguatera cases/outbreaks associated with narrow barred Spanish mackerel, given the potential of ciguatera poisoning to cause severe health problems and given that species such as Red Bass which are known to cause risk in other Pacific countries (but which have been involved in few reported cases locally)[10] are prohibited, then it is arguable that if the consumption of commercially caught narrow barred Spanish mackerel gave rise to ciguatera poisoning, that liability would accrue. This may not be that clear as, for example, it may be the case that a professional fisherman could argue that narrow barred Spanish mackerel were only of concern if caught, for example, off Fraser Island. This is a matter which depends on its own facts and will be clarified as research continues.

There may well be arguments as to why it is not reasonable to remove narrow barred Spanish mackerel from the catch in areas other than the zones of concern, i.e. Cairns/Townsville, Rock-

hampton and Fraser, or to remove certain sizes of catch. Such arguments are plausible, but it is emphasised are dependent on the relevant facts and level of knowledge.

In summary, to assess whether or not liability accrues in any given circumstance, it is necessary to: (i) show that a relationship of proximity exists, (ii) show that there is a failure or want of reasonable care, by demonstrating that there was a foreseeable risk of injury, which gave rise to the type of damage which was foreseen, which could reasonably have been prevented, and (iii) the consideration of whether or not such breach has occurred will be dependent on the facts and circumstances of the poisoning and the events that precede it.

The author considers it inevitable that there will be successful litigation in respect of ciguatera poisoning. It is simply a matter of time.

WORKPLACE HEALTH & SAFETY ACT - DUTIES OF CARE

The most important changes to Occupational Health & Safety in Australia, are the introduction of Robens-style legislation. Robens' legislation is based on the self regulation of Occupational Health and Safety in workplaces as opposed to regulation by way of sanction imposed from outside the workplace.

The Queensland Workplace Health and Safety Act, which is the embodiment of the Robens model, was assented to on 12th May, 1989, with Section 6, 36 and 57 commencing on 10th June, 1989 and the remaining provisions commencing on 31st July, 1989[11]. Regulations were enacted and commenced on 31st July, 1989, excepting regulations dealing with diving, which commenced on 30th October, 1989[12]. This Act amended, or repealed the Construction Safety Act, the Inspection of Machinery Act, the Health Act and the Shops and Factories Act. It is now the Act dealing with Occupational Health & Safety for the great majority of Queensland workers.

Since enactment there have been substantial amendments to both the Act and the Regulations. The most significant amendment being the inclusion of the rural industry within the parameters of the Act by amendment in 1990.

Central to the Robens' model are: duties of care, internal workplace assessment, and broad based prescriptive alternatives, i.e. codes of practice[13].

The duty of care is expressed as a legislative formula in the Act: '*an employer who fails to ensure the health and safety at work of all the employers, employees, save where it is not practicable for the employer to do so, commits an offence against this Act.*'[14].

With the definition of practicable in the Act being, *practicable, means practicable having regard to:-*

(a) the nature of the employment or, as the case may be, the particular aspect of the employment concerned; and

(b) the severity of any potential injury or harm to health or safety that may be involved, and the degree of risk that exists in relation to such potential injury or harm; and

(c) the state of knowledge about the injury or harm to health or safety that may be involved, about the risk of that injury or harm to health or safety occurring and about any ways of preventing, removing or mitigating that injury, harm or risk; and

(d) the availability and suitability of ways to prevent, remove or mitigate that injury or harm to health or safety or risk; and

(e) whether the cost of preventing, removing or mitigating that injury or harm to health or safety or that risk is prohibitive in the circumstances.[15].

This duty reflects broadly the common law principle of the duty of care which has evolved through personal injuries case law and was enunciated by Lord Atkin in the case of *Donaghue* v. *Stevens.*

Section 9 provides for the duty of care and imposes the relationship in respect of employers and employees. The Act, however, does not solely relate to workplace health and safety, but extends well beyond what is perceived to be the employment relationship. This is the result of the origins of the Workplace Health & Safety Act[16].

Of particular relevance to the commercial fishing industry is Section 10 of the Workplace Health & Safety Act: '*(1) An employer who fails to conduct his or her undertaking in such a manner as to ensure that his or her own health and safety and the health and safety of persons not in the employer's employment and members of the public who may be affected are not exposed to risks arising from the conduct of the employer's undertaking, except where it is not practicable for the employer to do so, commits an offence against this Act.*'

It is clear that this would include commercial catcher, marketers, vendors (fresh), and the provider (prepared).

The definition of practicability applies to Section 10 and the terms of the definition should be

considered. Basically, practicability provides for a similar test as is used for breach of duty of care under tort. The principal difference is that there is no requirement for causation. That is, no injury needs to occur for there to be a breach of the Workplace Health & Safety Act. That means that if a risk exists which could be reasonably removed and ought to be reasonably removed and is not so removed, then an offence occurs.

Matters discussed above in respect of the breach of duty of care under tort, i.e. forseeability, preventability and reasonableness are equally applicable to a consideration of practicability. There are however, in the writer's view, some essential differences between the duty owed pursuant to tort and the duty under the Workplace Health and Safety Act. These duties arise from the fact that the Workplace Health and Safety Act is a quasi criminal act[17], which means its provisions must be strictly construed to the benefit of the individual against whom the sanction is imposed. This is of particular relevance to the last element of practicability which, as noted above, is: '*(e) Whether the cost of preventing, removing or mitigating that injury or harm to health or safety of that risk is prohibitive in the circumstances.*

This on a strict construction should be considered in the light of the abilities of the individual commercial catcher, marketer, vendor and provider to meet that cost. In other words, rather than an application of the test of the reasonable person, the individual should be considered.

Nonetheless, given the industry's knowledge of ciguatera poisoning and potential risk of harm to members of the public the Act may well have been breached.

Breach of the Workplace Health & Safety Act will also support an action at common law for damages. Further, the penalties range from a fine of $3,000 or 6 months imprisonment for a person (other than a body corporate) where the Act is contravened to a fine of $30,000 or 6 months imprisonment where a death or serious bodily injury occurs (again this is for a person other than a body corporate). Offences for bodies corporate range from $12,000 to $120,000[18]. By Section 124 of the Act, a person who is a managing director or other governing officer or who at any time acts or takes part in the management, administration or government of the business in Queensland of a body corporate can be liable to punishment by imprisonment.

In my view the industry must comply with provisions of the Workplace Health & Safety Act and should consider its position vis-a-vis whether or not it is practicable for the risk to be removed.

TRADE PRACTICES ACT & SALE OF GOODS ACT

When a consumer purchases an item from a retailer there is an oral contract and into this oral contract certain terms are implied by law. These implied terms are measured in law to balance the relationship between retailer and consumer to protect the consumer from the 'caveat emptor' (i.e. buyer beware) principle.

The implied terms of the contract are either conditions or warranties. Basically, a condition is a vital or fundamental term whereas a warranty is a collateral or subsidiary term.

Where there is a breach of a condition the consumer can return the goods, get a refund and sue for compensation for any loss suffered as a consequence of the breach. Where there is a breach of warranty on the other hand the consumer cannot return the goods and get a refund, but the consumer can sue for compensation for any loss suffered as a consequence of the breach.

Therefore, if a retailer breaches a condition or a warranty, he/she is exposing himself/herself to an action by the consumer for compensation for any loss suffered as a consequence of the breach.

Certain conditions and warranties are implied by the Commonwealth's Trade Practices Act and the States' Sale of Goods Act. Most significant in the current context is the implied condition that the goods be of merchantable quality.

Under Section 66(2) of the Trade Practices Act, goods are of merchantable quality '*...if they are fit for the purpose or purposes for which goods of that kind are commonly bought as it is reasonable to expect, having regard to any description applied to them, the price (if relevant) in all other circumstances.*' A similar definition is provided by s17(2) of the Queensland Sale of Goods Act.

Basically, to be of merchantable quality the goods must:-(a) pass without objection in the trade and description given to them in the contract, and

(b) be of fair and average quality within the description, and

(c) be fit for the usual purpose for which such goods are used, and

(d) one with variations allowed by the agreement of even kind, quality or quantity within each unit and among all units, and

(e) be adequately contained, packaged and labelled, and

(f) conform to the promises or affirmations of fact made on the label or container.

The implied condition or merchantable quality does not apply where the defects are brought to the consumer's attention prior to sale or where reasonable examination of the product occurs and the defects ought to have been revealed by this examination. This situation is of course unlikely to occur in relation to ciguatera affected fish.

It could be argued that where ciguatera affected fish is sold it may not be of merchantable quality as it would not be of fair and average quality within the description and would not be fit for its usual purpose, i.e. human consumption. A person would then arguably sue for the damage that has been suffered.

Where the goods are not of merchantable quality, the retailer may be exposing him/herself to a suit by the consumer for compensation for loss suffered as a consequence of such breach. These rights of redress are of course only available to the purchasers of the affected fish.

CONCLUSION

It is arguable that action could be taken against industry members in relation to ciguatera poisoning, either by suit under common law, by prosecution under the Workplace Health & Safety Act, or action pursuant to the Trade Practices or Sale of Goods Act. I suggest the industry should be pro-active and consider what steps can be taken to address potential liability.

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2.*Glasgow Corporation* v. *Muir* [1943] 1 AC 447.

3.[1967] AC 617.

4.See generally *Hamilton* v. *Nuroof* (1956) 56 Commonwealth Law Reports 18.

5.[1984] 155 CLR 306.

6.See generally *General Cleaning Contractors* v. *Christmas* [1953] AC 180, *Read* v. *J. Lyons & Co Limited* [1947] AC 156.

7.Table 3, Ciguatera in Australia, Vol. 145 *Medical Journal of Australia*, December, 1986, p. 584, Gillespie, Lewis et al.

8.*ibid.*

9.*ibid*, p. 586.

10.*ibid*, p. 587.

11.Ss 1 & 2 commenced on the day of Assent.

12.Regulations 1 & 2 commenced on 29th July, 1989.

13.See Section 34 of the Workplace Health & Safety Act.

14.See Section 9 of the Workplace Health & Safety Act.

15.See Section 6 of the Workplace Health & Safety Act.

16.The Queensland Workplace Health and Safety Act, M. Quinlan, T. Farr, J. Payne, *Journal of Occupational Health & Safety* - Australia, New Zealand, 1989, 5(3), p. 265 - 274.

17.See Section 118, 119 and 124.

18.See Section 118 & 119 and for definition of serious bodily injury see Section 6.

STRUCTURES OF MAITOTOXIN AND CIGUATOXIN CONGENERS ISOLATED FROM CULTURED *GAMBIERDISCUS TOXICUS*. *Memoirs of the Queensland Museum 34(3): 600. 1994*:– Maitotoxin (MTX) was isolated from cultured cells of *Gambierdiscus toxicus* from the Gambier Islands (GII1 strain). To determine the structure, the toxin was cleaved into 3 fragments (A, B, C) by sodium periodate oxidation, followed by sodium borohydride reduction. Structures of fragments A and B were determined by 2D NMR experiments. The structure of fragment B, the largest fragment of 2306 Dalton, was negative FAB MS/MS experiments. Comparison of the spectra between the fragments and intact MTX allowed us to assemble the whole structure of MTX. MTX has molecular weight 3422 (nominal, as disodium salt) and is constructed from 142 carbon chain, comprising 32 ether rings, 21 methyls, one exomethylene, 28 hydroxyl groups, and two sulfate esters.

Two ciguatoxin (CTX) congeners, CTX3C and CTX4A, and a new polyether toxin named gambierol were isolated from the culture of *G. toxicus* from Rangiroa Atoll (GRI1 strain). CTX4A is 52-epiCTX4B and CTX3C is 1,2,3,4-nor-E-homo-CTX4B. The ladder-shaped polyether skeleton of gambierol differs from the other two. Production of CTX4A and CTX3C, by cultured *G. toxicus* unambiguously confirmed the generic origin of ciguatera toxins.

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CIGUATERA: DILEMMAS IN CLINICAL RECOGNITION, PRESENTATION AND MANAGEMENT

JOHN PEARN

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Both the clinician and consulting scientist are confronted with several key problems in the recognition and management of the ciguatoxic victim. Failure to consider the possibility of ciguatera in a patient presenting with any one or more of the pleomorphic constellation of symptoms and signs which are the hallmark of the disease, remains the most important ongoing dilemma of management. A differential diagnosis involves 'formulation of a list of diseases, commensurate with the elicited history and the observed signs, arranged in decreasing order of likelihood'. In mild single cases the difficulty of raising a differential diagnosis is compounded by lack of some symptoms. Another dilemma is interpretation of the chronicity of symptoms and this remains a clinical research challenge. A further dilemma is use of Mannitol and timing its introduction. Clinical research shows that Mannitol is not effective if administered more than 48 hrs after symptoms appear.

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The pleomorphic nature of ciguatera, the subjectivity of many of its symptoms and the absence of any definitive laboratory diagnosis for clinical cases make this condition one of the most challenging in clinical medicine.

The research dilemmas of ciguatoxin classification, source, assay and lesion pathogenesis are paralleled by clinical dilemmas of diagnosis, symptom interpretation and management. In the evolution of the understanding of any human disease there exists a 'window of time' in which one has to make the best of all available clinical experience, however anecdotal and however imperfect, in the practical management of an individual victim. In the case of ciguatera we are hopefully nearing the end of this era of clinical empiricism. Recent identification of the molecular structure of several of the ciguatoxins (Murata et al,1990), advances in understanding of sodium channel pathophysiology (Benoit et al.,1986; Lombet et al.,1987) and unequivocal histological evidence of nerve and muscle changes all contribute to the better interpretation of the miscellany of symptoms and signs which is the hallmark of this 'treacherous and increasingly-occurring marine fish public health hazard' (Russel & Egan,1991).

Many clinical dilemmas remain. These uncertainties are perplexing for the physician but like all dilemmas their resolution will advance the understanding of this enigmatic, common and important disease.

DIFFERENTIAL DIAGNOSIS

Diagnosis of ciguatera is essentially clinical. Currently, it is the failure to consider the possibility of ciguatera, in a patient presenting with any one or more of the pleomorphic constellation of symptoms and signs which are the hallmark of this disease, which remains the most important ongoing dilemma of management. This fact, the overlooking of the possibility of ciguatera rather than any omission of documenting the symptoms and signs remains the major problem in the management particularly of sporadic cases.

One dilemma is that there is no published work on the proportion of sporadic versus multiple cases in any published case series. Although the clinical syndrome is now very well defined (Gillespie et al.,1986) the syndrome boundaries for subacute and chronic cases still remains uncertain. The 'gold standard' of the chronic ciguatera syndrome must include case studies of multiplex (or epidemic) cases, followed prospectively.

The concept of differential diagnosis is 'the formulation of a list of diseases, consistent with the elicited history and the observed signs, arranged in decreasing order of likelihood'. All familiar with ciguatera are aware of the multiplicity of other different diagnoses which are included in the list of possibilities generated by the perplexed victim and his or her family, by the attending first aider, or by the admitting doctor in the emergency room of the referral hospital.

Differential diagnosis, in sporadic cases, includes such conditions as viral and bacterial enterocolitis, viraemias of diverse types, some types of hypersensitivity reaction, poisoning with other organic and inorganic agents and various types of neuroses.

Bacterial and viral gastroenteritis can be accompanied by prostration, rash, arthralgia and myalgia and bradycardia. Viral infections can cause puzzling constellations of symptoms and signs including rashes, arthralgia and myalgia, gastro-intestinal disturbances and neurogenic paraesthesiae. In the past, patients with undoubted ciguatera have been labelled as suffering from chronic viral diseases, auto-immune disease, possible insecticide and heavy metal poisoning, psychosis and neurosis, hysteria and malingering.

Subacute and chronic cases, or cases presenting for the first time after several days of symptoms, are always difficult to diagnose. A particular difficulty is the fact that loss of energy, loss of appetite and subjective feelings of weakness are very common indeed in the general population.

The differential diagnosis of ciguatera is always a two-stage process. The first stage is to deduce that one of the icthyosarcotoxaemias is present; the second is to run through the other possibilities of puffer fish poisoning (fugu), maitotoxaemia, clupeotoxism and histamine poisoning. This latter condition, due to histamine poisoning, occurs especially after the ingestion of spoiled *Pomatomus*, or common 'tailor' fish of eastern Australia; and occasionally after the ingestion of *Arripis* or 'Western Australian salmon' (Smart,1992). The differential diagnosis of the icthyosarcotoxaemias also includes the various forms of diarrhoeal and paralytic shellfish poisoning, especially after the ingestion of mixed seafood meals which include both potentially toxic species such as coral trout (*Plectropomus maculata*) and mackerel (*Scomberomorus commersoni*) together with oysters and scallops.

The author has encountered several cases presenting first following rechallenge with ciguatoxic food one case involving the ingestion of battery-fed chicken, in which the poultry was probably fed on fish meal. In this type of case the diagnosis of the putative original ciguatoxic intoxication can only be made in retrospect.

CHRONICITY OF CIGUATERA

One of the main clinical dilemmas is interpreting the true significant of chronic symptoms. How long can ciguatera last? Most experienced workers have followed cases prospectively and know that objective signs of poisoning usually persist for a few days or several weeks only; yet all know that the subjective, often distressing symptoms such as prostration, arthralgia and myalgia and disordered cutaneous sensation can persist in an unbroken continuum of such subjective symptoms for many months. Can ciguatera produce symptoms, say, after two or three years? At this stage of scientific knowledge there are numerous anecdotal case reports, but doubt persists about the true persistence of symptoms for more than one year or so. At this point of scientific endeavour, no cumulative frequency histograms have been generated, by symptoms, for proven cases followed prospectively. Thus, the chronicity of ciguatera remains an important clinical research issue for the future.

Recent neurophysiological experiments have indicated that the toxin is acting at its affector sites, in organ-bath preparations in fractions of nanomolar concentrations. This fact, combined with its fastness in some neurophysiological experiments lends plausible support to the concept that true symptoms may persist for years rather than months. The principal target of ciguatoxin is on unmyelinated fibres. It is not implausible that one of the most toxic substances known to science (ciguatoxin), and one of such demonstrated strong attachment to its receptor site in the sodium channel, might produce bizarre autonomic-related symptoms for very long periods after the initial insult. Permanent damage to nerves, or residual binding of the toxin to its target receptors, may help explain the often observed phenomenon of recrudescence of symptoms, even in the face of an otherwise subclinical dose of toxin.

INDIVIDUAL SUSCEPTIBILITY

There is considerable individual clinical susceptibility to ciguatoxin. Not infrequently, different family members eating the same toxic fish, and often apparently in similar amounts, are affected to different degrees. The mass of toxic fish eaten is obviously important; and in the case of *very* toxic fish even small differences in plate portions may be reflected in large differences in the mass of toxin which is ingested. Experience in Japan with fugu fish poisoning is that the eating of very large portions of otherwise relatively safe fish has resulted in fatalities (Matsubara,1981).

Such cases highlight particularly the importance of portion size - and conversely, the need for prudence in the face of potentially risky meals.

Personal clinical experience with managing multiple affected victims who have eaten from the one ciguatoxic fish suggests that individual clinical variation is the rule, rather than the exception. All experienced workers have encountered situations where some members may be totally unaffected following the ingestion of a ciguatoxic fish meal, whilst others eating portions of similar size may be severely affected. Research biologists undertaking the mouse assay for ciguatoxin, also encountered this in a situation where pairs of mice were being used in the biological assay. Not infrequently one member of the pair will be dead within 1-3 hours and the other (although usually affected) will survive. These 'mouse-splits' so often parallel the clinical discordance one sees among the human victims of mini-epidemics.

The basis for this variable susceptibility remains unknown. A significant genetic component is likely although, even within affected families (in family outbreaks), there is not infrequently widespread variation in the severity of symptoms and objective signs. Different species react differently to the toxin, both in terms of quantitative response as crude evidence of poisoning on the one hand, and in qualitative syndromic variation on the other. The 'straub tail' seen in poisoned mice is quite different from the syndrome seen in the (more sensitive) afflicted cat, often used as the practical test animal in real life domestic situations where a family is wishing to consume a risky species of fish.

Some believe that children are particularly susceptible and certainly in various LD_{50} assays for other toxins, neonatal mice are more sensitive than the standard 19–21 gram adults which are more traditionally used in the specific ciguatoxic mouse assay. I have encountered clusters of family poisonings where children appear to be more severely affected. The dilemma remains however that children so often ingest more of the fish, and in a particularly toxic fish meal a relatively small increase in ingested mass (in relation to a child's body weight) may result in a supra-threshold level of ingested toxin. Similarly, sex differences in responses to the toxin are often hinted at, anecdotally in the case of women whom it is thought may be particularly susceptible to the long term effects. No formal attempts at initial dose quantification, with long term follow up by sex, have been reported.

GEOGRAPHIC VARIATION SYMPTOMATOLOGY

Confusion exists about the relative incidence of different symptoms in different parts of the world. Whilst all case series report such things as circum-oral tingling, diarrhoea and vomiting, other symptoms such as dysuria (Gillespie et al., 1986), dental pain, pruritus and piloerection are reported much more frequently in certain geographic regions than in others. Some differences are undoubtedly due to sampling errors, differences in case descriptions and different standards of history taking and of reporting. However, there are obviously different toxins and different toxin subtypes in different areas. Indeed, it seems inescapable that the human clinical syndrome of ciguatera is the result of ingestion of a cocktail of different ciguatoxins. *A priori*, it would be unrealistic not to expect different clinical syndromes under these circumstances, in different parts of the world. There is some evidence that antibody profiles to toxins from fish taken from different parts of the world differ in their cross-reactivity. This gives further credence to the belief that there are subtle differences in ciguatera syndromes in different parts of the world. Nevertheless, in all reported series, a profile of core symptoms is seen and includes gastrointestinal symptoms, neurological complications such as paraesthesiae and temperature dysaesthesiae, myalgia and arthralgia. This also reflects different case definitions which are used.

The role of mannitol therapy (Palafox et al., 1988; Pearn et al.,1989) remains indeterminate, although the necessary double-blind study (from the Marshall Islands) is in progress. In the writer's experience, administration of intravenous mannitol in a dose of 1g/kg body weight, given as an oedema-reducing regimen over a maximum administration time of 45 minutes, produces dramatic alleviation of symptoms within 2–3 hours in some patients. The role of mannitol therapy in cases presenting to medical attention after this time remains controversial and this dilemma will not be resolved until treated cases are followed prospectively. I give mannitol, in cases presenting acutely even although the symptoms may be milder, in the anticipated belief that the risk of long term sequelae will be reduced. What has been established is that mannitol given to the ciguatera patients is safe, and that no synergism between toxin and mannitol has been observed.

To the clinician practising in high-risk endemic regions of the tropical littoral, multiple-case out-

breaks pose no problem in diagnosis and with the advent of mannitol therapy management is much more straightforward. The major problem in the clinical management of ciguatera remains in the need for more widespread awareness of the possibility of the disease, and earlier diagnosis. To the first aider, nurse or physician encountering (particularly sporadic) cases, often distant in place and sometimes distant in time from the fish source, missed diagnosis still remains the biggest challenge in the management of this important disease.

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CIGUATERA: RISK PERCEPTION AND FISH INGESTION

JOHN PEARN AND RICHARD LEWIS

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A survey of 37 attendees at the Clinical Ciguatera session of the Ciguatera Management Workshop, Bribie Island, April 1993, completed a questionaire to assess risk perception relative to fish ingestion among a group acutely aware of the ciguatera threat. The perceived risk difference between ingestion of fish personally purchased in the marketplace and fish served in a restaurant was assessed with responses from different groups (males/females, clinicians/biologists) within the sample being compared. No one would accept a risk of 10% in purchasing fish personally but one clinician would accept a risk of 20% in a seafood restaurant and 25% of respondees would accept a higher risk in a restaurant than in their purchasing unprepared fish.

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One of the most practical questions relating to human ciguatera poisoning is the question "What risk will I accept before eating a fish meal?". The answer to this question governs fishing policy, marketing regulations, species selection for gourmet dining and individual choice of menu.

Many factors influence the statistical risk of contracting ciguatera. Fish species, size of an individual fish, fish habitat (Lee,1980), season of catch and size of portion all modify the intrinsic risk of developing ciguatera (Bagnis et al,1979; Lawrence et al,1980; Russell & Egan,1991).

The statistical risk of contracting ciguatera varies with country and species (Hokama et al, 1993). The risk of contracting ciguatera on Niutao Island in Tuvalu is 1 in 10 (Dalzell,this memoir). In Micronesia, the risk of ciguatera from eating Moral eel viscera may be > 1 in 20. With Hawaiian jackfish (*Caranx* sp., or 'papio') the risk is 1 in 100 (Hokama et al,1993). In Queensland, the risk of ciguatera is <1 in 3,000 (Gillespie et al,1986) and from a meal of coral trout (*Plectropomus maculata*) it is <1 in 5,000.

Individuals modify their behaviour not on the basis of these objective figures, but on the perceived subjective risk (Pearn,1973,1977). Subjective risk is determined by such factors as sex (women usually being more conservative in the face of a gambling situation), personality (optimists being less conservative), past experience, concepts of probability and the perceived outcome including fatality (Pearn,1973). In the specific risk of ciguatera following fish consumption, it is known that the objective risk of fatality is <1 in 1,000 of clinical cases in Australian (Tonge et al,1967) and French Polynesian reports (Bagnis et al,1979).

An objective risk of contracting ciguatera of <1 in 1,000, with a risk of fatality of 1 in 1,000,000 for a random fish meal, is for many a low or trivial risk. On the other hand, many deny themselves the pleasures of gourmet fish meals because of the subjective (or perceived) risk which is seen to be more threatening than these low figures imply. We were interested to obtain data on this phenomenon of perceived or subjective risk in the context of human fish-consumption behaviour. Besides perceptions of subjective risk, risk-taking behaviour is known to be influenced by the social setting in which fish is consumed, and of course by peer influence. This has significant implications for consumption of risk species in seafood restaurants and in other social settings.

During a Workshop on Ciguatera Management a gathering of world experts on ciguatera participated in a risk-assessment study of professed personal decision-making in the face of a hypothetical ciguatera risk. We report here the results of this study of professed risk-taking behaviour in the context of a ciguatera threat.

METHODS

SUBJECTS

The subjects included all 37 individuals (31 males and 6 females) who attended the 'Clinical Ciguatera' Session of the International Workshop on Ciguatera Management, held at Bribie Island, Queensland, on 15th April 1993, under the auspices of the (Australian) Fisheries Research and Development Corporation and the

TABLE 1. Maximum acceptance risks for buying a marketplace fish which might be ciguatoxic. Thirty-seven world experts in ciguatera, at the International Ciguatera Management Conference, Queensland, Australia, 1993.

GROUP	RANGE OF MAXIMUM ACCEPTANCE RISKS	MEDIAN ACCEPTANCE RISK
All female subjects	0 - 10%	0.1%
All male subjects	0 - 10%	0.2%
Clinicians	0.01-10%	0.01%
Research scientists	0 - 10%	1.0%
All subjects	0 - 10%	0.1%

Queensland Department of Primary Industries. No subject refused to take part in this study. All were university graduates and (comprising as they did the world leaders in this subject) all were fully informed of the implications of ciguatera poisoning. Of the 37 subjects, nine were practising clinicians and 25 were biological research scientists working in this field. Subjects came from Australia, USA, France, Japan and the United Kingdom. All were proficient in English.

QUESTIONNAIRE

Each subject completed a personal questionnaire, anonymously, giving details of sex, discipline (research scientist, clinician etc) and professed acceptance risks in the instance of two separate question-scenarios. A brief (5 min) verbal exposition about the nature of the study was presented by one of us (JP) prior to the completion of the questionnaire.

Each subject was asked to respond to two specific questions:-

1.Imagine you are staying in a country where ciguatera occurs. You are buying fish in a shop or market, to take home for yourself or your family. What (maximum) level of risk of ciguatera would you accept, before buying the fish?

2.Imagine you are in a seafood restaurant, in a country where ciguatera occurs. Fish is served. It is a species known occasionally to be ciguatoxic. At what (maximum) risk level for ciguatera, would you eat the fish meal?

RISK SCALES

Subjects recorded their personal (subjective) acceptable risks in 3 ways: a) by a linear (Likert) scale marked from 0 to 100%, on which the subject draws a line at their personal risk acceptance level, b) by a vulgar fraction, and c) by a percentage figure. In each of these systems, a risk of '0' means that an individual will not accept any risk whatsoever. In the context of the specific questions we asked this implies that the individual would not eat a risk-species of fish under any circumstances. A risk of 100% or 1.0 implies that an individual would go ahead and consume fish even if it was certain that the subject would contract ciguatera from such ingestion. The specific risk of 50% (with the appellation '1 in 2' risk) was marked on the Likert scale.

RESULTS

Of the 37 subjects, all but one recorded their professed risk-acceptance levels in each of the 3 modalities. For Question 1, about acceptance risks for ciguatera when buying fish in the market place (Table 1), 7 subjects (19%) said they would not accept *any* risk, and would not buy risk species of fish for which there was any chance whatsoever of contracting ciguatera; 13 (35%) said they would accept a risk of 1% or greater, that is a risk of 1 in 100 or greater; 4 (3 research scientists and 1 male clinician) said they would accept a risk of 10% (1 in 10) of contracting ciguatera.

For question 2, concerning restaurant consumption of potentially toxic fish (Table 2), 8 subjects (22%) said they would not eat any fish species in a seafood restaurant, where there was any risk whatsoever of contracting ciguatera; 8 (22%) said they would accept risks of 10% (1 in 10) or higher. This widespread difference in professed behaviour, in the face of a medical risk, parallels the widespread attitude to risk seen in other medical situations (Pearn,1973).

Analysing the individual responses to each of the two questions revealed that 21 subjects (58%) did not change their professed risk-acceptance level when confronted by the different social and peer pressures inherent in eating in a seafood restaurant. Nine subjects (25%) professed to accept higher risks in the seafood restaurant scenario, with a median increase in risk, in this group, by a factor of five. In the open-ended section of the questionnaire marked 'comments',

TABLE 2.Maximum acceptance risks for eating a potentially ciguatoxic fish meal in a seafood restaurant. Thirty-seven world experts on ciguatera, the International Ciguatera Management Conference, Queensland, Australia,1993.

GROUP	RANGE OF MAXIMUM ACCEPTANCE RISKS	MEDIAN ACCEPTANCE RISKS
All female subjects	0 - 5%	0.2%
All male subjects	0.01 - 20%	0.1%
Clinicians	0.01 - 5%	0.1%
Research scientists	0 - 20%	0.1%
All subjects	0 - 20%	0.1%

6 wrote that they would accept higher potential risks in a seafood restaurant scenario, because of social and peer pressures, and because of such themes as 'being an honoured guest', or 'good manners in a group situation'.

DISCUSSION

This study shows that the majority of subjects, themselves expert in ciguatera, accepted risks for contracting the disease which were greater than the real life objective risks around the Pacific rim and in the Caribbean. No worker professed to accept a planned fish-buying risk greater than 10% (1 in 10), although 1 individual would be prepared to accept risks of 20% (1 in 5) of contracting ciguatera from eating in a seafood restaurant. It is the objective (mathematical) risk of ciguatera which concerns questions about fishing industry policy, species prohibition and the funding for management, monitoring and research. By contrast, subjective risk determines the choice of fish for personal and family consumption, menu selection and such diverse themes as legal and compensation issues. Decision-making in the face of a threat always involves a balance between perceived or subjective risk on the one hand, and the outcome (or utility) of a won gamble on the other. In the study reported here, the 'utility' - the joy of enjoying a gourmet fish meal together with the risk of escaping clinical ciguatera - this 'utility' is as consistent for a within-group pattern as it is possible to imagine. The collective 'utility' - good health after risk-fish ingestion, or its inverse, clinical ciguatera - was fully understood by all participants, all of whom were giving papers on the subject at an international conference.

Many subjects think of personal risk in quite specific and individual ways. Optimists tend to regard themselves as invulnerable and will take quite high (objective) risks. Pessimists on the other hand and those with obsessive traits will reject risks and not enter a gambling situation where the risks are mathematically very low (e.g. <1 in 1,000 or even <1 in 10,000). Almost everyone behaves inconsistently in their life's behaviour when it comes to risks. Some will accept quite high risks in some areas of human activity (speeding in the car, for example; or driving after drinking) but will not accept very low risks in other areas. For example almost all home owners will not leave their home uninsured against fire, even though the objective risks are <1 in 60,000 and the outcome often not as severe as the consequences of a motor vehicle accident.

The relationship between ciguatera and public and commercial liability is a topical theme. There is an undoubted duty of care to reduce the risk of ciguatera to individual subjects. This applies both to legal liability in common law and to statutes in various Workplace Safety Acts and in Fair Trading Acts. The courts of various countries try to set what is a 'reasonable' or 'practicable' risk, with penalties potentially imposed on those who expose individuals to risks greater than these arbitrary levels. The current study reported here shows that experienced, informed ciguatera scientists and clinicians collectively take greater risks than are currently accepted as 'safe' in the fishing industry and in restaurant commerce. What the implications of this are, in the evolution of regulations and for case law, is for the future to determine. Certainly, the law always demands public health regulations and commercial 'duty of care' to be set at much safer levels (that is lower risks of exposure) than that pragmatically accepted by individuals functioning in their own personal lives. This research confirms this general observation in the specific context of ciguatera. Large individual differences exist in risk-taking behaviour generally (Pearn,1973; Pearn,1977), differences which are shown here to apply to ciguatera specifically.

Health regulations and case-law practice (the latter set by precedent) strive to protect all in society - not only those whose personal behaviour tends to be risky. In the developed world, the perspective of the fishing industry is thus to see a majority of informed individuals who will accept a risk of upto 1 in 1,000 (0.1%) of contracting ciguatera from dining in a seafood restaurant. Of the world's ciguatera experts 69% professed that they would be happy to accept such a risk under these circumstances. Whether or not this is 'risky' behaviour is also a subjective judgement. Current regulations and some legal opinion indicates that even those who profess to accept such risks must be protected. Health regulations and local custom (such as the banning from sale of the red bass, *Lutjanus bohar*) operate to ensure that the objective risk to diners is significantly less than the subjectively- acceptable risk level.

The phenomenon of subjective risk is culture-dependent. Many individuals and indeed many communities in the developing Pacific countries accept the risk of ciguatera as a fact of life. In some such communities individuals accept risks >1 in 10. Where to set public health risk acceptance levels is thus difficult. If one is too conservative, education and local community policies will tend to reduce the impact of a highly nutritious, high quality delicious food source with consequent greater dependence on tinned fish and tinned meat - the so-called dietary colonialism. In Western countries of the Caribbean and the Pacific rim, objective risk rates also vary from society to society.

The fact that a significant proportion (25%) of subjects recorded that they would, in a restaurant setting, accept a higher risk than their own personal food-buying 'baseline' risk, imposes special responsibilities and duties of care on commercial restaurateurs. This implies that the special vulnerability of patrons, a proportion of whom are caught against their will and feel that they have to take higher risks than they would in other circumstances, need special protection. At the very least, it implies that the objective mathematical risk of a random fish meal producing ciguatera should be reduced as much as possible, and suggests that restaurateurs should be aware of the geographical source of risk-species which they serve.

Attitudes to subjective risk are never static. They change as scientific knowledge of ciguatera increases; and will change further as practical test systems for detecting individual ciguatoxic fish become available. When they do, risk-acceptance habits of the fish-eating public will change again, as new community baselines are set for the risk of ciguatera.

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CLINICAL ASPECTS OF CIGUATERA: AN OVERVIEW

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Ciguatera is a polymorphous disease posing important health, nutritional, economic and social problems for inhabitants of endemic areas, and occasionally for those in non-endemic areas. Limited progress has been made in understanding the pathophysiology of the disease and in developing effective treatment.

Clinical features of the disease are reviewed, and incidence, morbidity and mortality data are outlined. Methods to prevent ciguatera and progress in treatment of ciguatera are discussed, and key issues and needs for future research are described. These include: 1, consistent epidemiologic data, using a consistent case definition; 2, the human immune response to ciguatoxins; 3, the pathophysiological mechanisms underlying human disease, potentiation of disease by alcohol, and the phenomenon of sensitisation; 4, better tests for ciguatoxins; and 5, effective and safe treatment for affected patients.

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Ciguatera is the disease caused by the consumption of fishes contaminated with ciguatoxins, which originate from *Gambierdiscus toxicus* (Adachi & Fukuyo), a unicellular dinoflagellate alga associated with coral reefs (Adachi & Fukuyo,1979). Most toxic fish are captured during inshore fishing near coral reefs. Ciguatera is a circumtropical disease, likely to affect >25,000 persons annually. Its greatest impact is in Pacific island countries (Lewis,1992a). Although rarely fatal, possibly because fish succumb before concentrations lethal for humans can be accumulated (Lewis,1992b), its morbidity is considerable. Ciguatera has been reviewed several times (Gillespie et al.,1986; Lewis,1986; Hokama,1988; Vernoux,1988; Hokama,1991; Juranovic & Park,1991; Russell & Egan,1991; Lewis,1992a; Lewis & Ruff 1993).

Often regarded as an interesting tropical medical curiosity rather than a subject for serious medical study, a good deal of the clinical literature on ciguatera is rather repetitive and anecdotal and does relatively little to advance our understanding of the disease and its management.

Clinical manifestations of ciguatera are protean. In areas where the disease is not endemic, the diagnosis is often not considered by physicians unfamiliar with ciguatera, and a wide variety of erroneous diagnoses may be made, including neurosis. In many parts of the world, increasing international travel, and increasingly widespread transport and consumption of warm water fish, especially coral reef fish, make it more likely that cases will be seen outside endemic areas. The possible severity, chronicity and possibility of effective treatment make it important to consider the diagnosis in those presenting with a compatible illness soon after eating fish.

CLINICAL FEATURES

CLINICAL MANIFESTATIONS

Ciguatera results in variable combinations of gastrointestinal, neurological, general and cardiovascular manifestations. Symptoms usually develop 1-6 hours after ingestion of toxic fish - in about 90% of cases within 12 hours (Gillespie et al,1986, Gillespie,1987), but in a few after more than 24 hours (Bagnis et al.,1979; Bagnis & Legrand,1983; Narayan,1980). Gut involvement usually consists of an acute self-limiting syndrome akin to gastroenteritis, which may be severe, but generally lasts less than 24-36 hours (Gillespie et al 1986, Gillespie,1987; Frenette et al.,1988; Engleberg et al.,1983). Symptoms may include abdominal pain, nausea, vomiting, diarrhea and tenesmus (rectal pain). Resulting intravascular volume depletion ('dehydration') and electrolyte disturbances may be severe, particularly in young children. Volume depletion and hypotension may be compounded by myocardial depression and disturbed vasomotor regulation (including deranged blood pressure control). Neuromuscular disturbances are most commonly sensory, but may also be motor. Although neurological dysfunction is typically sug-

gestive of predominant involvement of peripheral nerves, effects may occur at any level of the nervous system from cerebral cortex to muscle. Neurological manifestations are usually bilateral, but may be asymmetrical (Hamburger,1986) or unilateral (Hashmi et al.,1989). Manifestations may include coma, seizures, ataxia (disordered co-ordination and balance), cranial neuropathies including ophthalmoplegia (paralysis of eye movement), myelopathy (spinal cord dysfunction), peripheral sensory, motor and autonomic neuropathy and myositis (muscle inflammation).

Typical sensory symptoms are distal limb, perioral and lingual paraesthesia and dysesthesia (disordered sensation) - with prominent numbness and tingling - and often a very unpleasant form of hyperesthesia (abnormal, heightened sensation) particularly associated with cold objects producing a distressing burning sensation (Gillespie et al.,1986). Sometimes a reversal of temperature sensation occurs, such that cold objects feel hot and vice versa. Reduced distal sensation and reduced or absent tendon jerks are the commonest neurological signs. A sensation of fizzy, metallic taste may occur. Muscle weakness - most commonly distal or generalised, occasionally asymmetrical - sometimes involves bulbar and respiratory muscle groups. Airway protection and ventilatory support may be required in severe cases. Diffuse muscle pain is common, and may be associated with elevated blood levels of muscle enzymes and biopsy evidence of myositis (Nakano,1983).

General (non-localising) symptoms include malaise, lassitude, irritability, depressed mood, pruritus (itching), sleep disturbance and unusually vivid dreams. Headaches, arthralgia (joint pain, particularly involving shoulders, elbows, knees and ankles), pruritis (localised or generalised), dental pain, a sensation of looseness of the teeth and dysuria (painful urination) may also occur. A variety of skin rashes, most commonly maculopapular, are sometimes present and may be associated with desquamation (peeling) during the healing phase.

Bradydysrhythmias (slow cardiac rhythm disturbances), atrio-ventricular heart block, myocardial depression and loss of vasomotor regulation with hypotension, often postural, may occur during the early phase and tend to resolve more quickly than general and neurological symptoms. Autonomic dysfunction may be manifested by sweating, lacrimation (excessive tears), salivation and internal ophthalmoplegia (paralysis of ocular accommodation and pupillary responses).

Symptoms often fluctuate from day to day and at different times of day. The time course is generally one of improvement over days to weeks, but symptoms not uncommonly persist for months, or rarely years. Consumption of alcohol commonly exacerbates symptoms (Gillespie et al.,1986; Gillespie,1987). Death is rare (0.1% of recorded cases) (Gillespie et al.,1986; Juravnovic & Park,1991; Gillespie,1987; Bagnis et al.,1979; Bagnis & Legrand,1987). Clinical manifestations and severity may vary considerably, even among individuals poisoned by the same fish. In the absence of a specific human diagnostic test for ciguatera, this wide variation in clinical manifestations and the clinical nature of the diagnosis make reliability difficult. Diagnosis is especially difficult when only one person presents with less than a full hand of symptoms. Nerve conduction studies may be helpful, and demonstration of toxin in any remaining fish samples, while very useful, is often not possible. Commonly used clinical criteria for diagnosis of ciguatera are gastrointestinal and neurological symptoms following ingestion of potentially toxic fish. This combination, however, occurred in only 25/53 (55%) of patients in one common source outbreak (Engleberg,1983), and 52/57 (91%) of patients in another (Frenette,1988).

Person-to-person Transmission

Although the vast majority of ciguatera cases are caused by ingestion of toxic fish, various forms of person-to-person transmission have been described, and are indicative of the potent, persistent and lipid-soluble nature of ciguatoxins. These include: transmission via milk to breastfed infants (Bagnis & Legrand,1987; Thoman,1989; Blythe & De Sylva,1990), though hyperaesthesia of the nipples of a lactating mother may interfere with breast-feeding (Pearn et al.,1982); transplacental transmission, resulting in transient neurological manifestations in the newborn following maternal illness near term (Pearn et al.,1982); and apparent sexual transmission from female to male (penile pain after intercourse in the male partner of an affected woman) (Geller et al.,1991) and vice versa (pelvic and vaginal pain after intercourse in the female partners of affected men)(Lange et al.,1989).

Sensitisation and Recurrent Attacks

These are two of the most enigmatic aspects of ciguatera, and increase its morbidity as well as its social and economic consequences. Not only does immunity not follow an attack of ciguatera,

but there is evidence from a variety of locations that second and subsequent attacks tend to be more severe than first attacks (Bagnis et al., 1979). Also well documented is the phenomenon of sensitisation. Persons who have previously had ciguatera may suffer a recurrence of typical ciguatera symptoms after eating fish which do not cause symptoms in other persons (Narayan, 1980). Consumption of alcohol or chicken may have the same effect (Gillespie et al.,1986; Gillespie,1987). Such sensitisation can occur many months or even years after an attack of ciguatera. Both these factors are most troublesome in areas where people depend heavily on fish as their major dietary source of protein.

The basis for sensitisation and recurrent attacks tending to increase in severity is not known, but has been generally presumed to be immunological, although the symptoms are not typically allergic. A serum bank is being established at CSL Limited in Melbourne, Australia, as a basis for exploring the nature of sensitisation following ciguatera (Sutherland & Lewis,1992).

PATHOLOGY AND PATHOPHYSIOLOGY

Human pathological studies of ciguatera are few. Nakano (1983) reported high blood levels of creatine phosphokinase (CPK, a muscle enzyme) in 7 men affected with ciguatera on Midway Island, Central Pacific. The CPK level, initially >1000 IU/L (normal <200 IU/L) in each, returned to normal within 10 days. While their motor and sensory nerve conduction velocities remained normal, electromyography revealed changes consistent with an acute myopathic process. Insertional and spontaneous activity were normal. Mild recruitment (minimal effort) produced small motor units of short duration; maximal recruitment (maximal effort) revealed enhanced motor units of low amplitude. Repetitive nerve stimulation suggested possible neuromuscular junction fatigue in 2 patients. Muscle biopsies from 3 patients showed muscle fibre splitting, degeneration and necrosis, with subsarcolemmal tubular aggregates and small lipid vacuoles.

A near-fatal case in Hawaii was associated with prominent generalised muscle spasms and high blood levels of CPK (41,000 IU/L, reference range 45–35) and other muscle enzymes (Kodama et al.,1989). Palytoxin present in smoked mackerel from the Philippines was thought to be responsible. Similar cases have also been described following parrot fish ingestion in Japan (Noguchi et al.,1987). A possible association between polymyositis (a chronic inflammatory disease of muscle) and ciguatera occurring some years previously has been suggested (Stommel et al.,1991) but remains speculative.

The major morbidity of ciguatera, however, is probably attributable to its effects on peripheral nerves. Ayyar & Mullaly (1978) reported slowed sensory conduction velocities without decrease in sensory nerve action potential amplitude in affected patients. Other studies (Allsop et al.,1986; Cameron et al.,1991; Cameron & Capra,1991) documented increased distal motor and sensory latencies, reduced motor and sensory conduction velocities, prolongation of the absolute refractory, relative refractory and supernormal periods, reduced sensory amplitudes and F wave latencies. These findings are consistent with a neuropathic process which in traditional neurological terms is predominantly demyelinating rather than axonal in type (primarily damaging the myelin sheaths of nerves, which are part of Schwann cells, rather than the nerve fibres themselves).

The report of human nerve biopsy in ciguatera (Allsop et al.,1986) found striking edema of vacuoles in Schwann cell cytoplasm adaxonally (immediately abutting axons), with axonal compression and vesicular degeneration of myelin. Nakano (1983) described diffuse slowing of brain electrical activity, elevated cerebrospinal fluid pressures and abnormal brainstem auditory-evoked responses in ciguatera patients, although these are not common findings.

One interesting finding by Cameron & Capra (1991), in the rat tail nerve *in vivo,* is that a blood ethanol (alcohol) level of 0.05% was found to significantly increase the magnitude and duration of the abnormal supernormal response observed in ciguatoxin-treated rats. The mechanism of this potentiation, which is consistent with common clinical experience in humans, is yet to be elucidated. The nature of the human immune response to ciguatera is essentially unknown.

TREATMENT

Despite advances in understanding the nature and pharmacology of ciguatoxins, this has yet to translate into major specific therapeutic advances. No specific antidote is known for any of the many marine dinoflagellate toxins, including those causing ciguatera. Therapy remains primarily symptomatic and supportive. Many types of treatment have been tried and although some important uncontrolled observations have been reported, particularly in relation to man-

nitol, no double-blind controlled clinical trial results are available for any treatment modality.

Supportive and symptomatic therapy may include bed rest, analgesia, fluid and electrolyte replacement, airway protection and ventilatory support, circulatory support (including positive inotropic agents), management of dysrhythmias (most commonly bradycardias and atrioventricular block, occasionally necessitating temporary cardiac pacing), general care of the unconscious patient, antihistamines and cool showers for pruritis, hypnotics, etc. In French Polynesia standard (Bagnis et al.,1992) but unproven (Calvert,1991), therapy for hospitalised patients has consisted of intravenous infusions of vitamins C and B6 (pyridoxine) and calcium gluconate. A wide variety of traditional remedies, including a considerable number of plants, are used in various areas (Cooper,1964; Narayan, 1980; Amade & Laurent,1992; Dufva et al.,1976; Bourdy et al.,1992) Screening of traditional plant remedies with a novel mouse bioassay has found that an extract from leaves of *Argusia argenta* can reduce the effects of ciguatoxin (Amade & Laurent, 1992). Efficacy or safety in humans of traditional remedies are unknown.

Occasional success has been reported with low dose amitriptyline, a tricyclic antidepressant, particularly for chronic paraesthesia and other neurological symptoms (Bowman,1987; Davis & Villar,1986; Calvert et al.,1987). Fluoxetine (an antidepressant drug which is a relatively specific serotonin-uptake inhibitor) was reported to reduce chronic fatigue in two patients with ciguatera in whom symptoms had persisted for over nine months (Berlin et al.,1992). Nifedipine (a calcium channel blocker) (Calvert et al.,1987) and tocainide (a lignocaine-like local anaesthetic agent) (Lange et al.,1988; Lange & Kreider,1988) have some theoretical appeal but experience with their use is very limited.

The most dramatic reported experience of successful treatment of ciguatera has been that of Palafox et al. (1988) in the Marshall Islands, who treated 24 patients with acute ciguatera with intravenous infusions of mannitol, an osmotic diuretic agent most commonly used in the treatment of cerebral edema. Mannitol is inexpensive and readily available, but must be given by intravenous infusion and accompanied by careful patient monitoring. Two patients in coma and one in shock are reported to have responded within minutes, with full and rapid recovery, hitherto virtually unknown in severe ciguatera (recovery typically takes at least one, and more usually two weeks). Neurological and muscular manifestations improved dramatically; gastrointestinal symptoms resolved more slowly. A variety of case reports and uncontrolled observations involving small numbers of patients (Pearn et al., 1989; Williamson,1990; Stewart,1991) documented a clear clinical impression that in some patients (including young children) (Williams & Palafox,1990), mannitol is dramatically efficacious, notwithstanding the highly variable natural history of the disease. Patients at the more severe end of the disease spectrum and who are treated early (within 24 hours of symptom onset) would appear most likely to benefit from mannitol. The mechanism of action of mannitol in ciguatera is unclear - possibilities suggested (Pearn et al., 1989) include a direct anti-ciguatoxin effect via a scavenger mechanism, or an osmotic effect reducing Schwann cell edema, thereby ameliorating neurological dysfunction. Experimental studies on interactions between ciguatoxin and mannitol indicate that mannitol does not act to reduce the affinity of the sodium channel for ciguatoxin, nor does mannitol act as a scavenger for ciguatoxin (Lewis unpubl. data), suggesting that the osmotic effect is the most likely mode of action.

In the first controlled trial of mannitol (Bagnis et al.,1992) 34 patients were treated, compared with 29 patients treated with vitamins B6 and C and calcium. Patients were well matched, and a clinical score based predominantly on the number and severity of subjective symptoms showed significant benefit 1 and 24 hours after onset of treatment, particularly for paraesthesiae and gastrointestinal symptoms. The study suffers from a number of weaknesses: it is unclear whether the patients or the observers were blinded, the clinical score was based excessively on subjective criteria, no follow-up beyond 24 hours is reported, and the differences between treatment groups, while statistically significant, would appear not to be of major clinical significance. The clinical condition of some patients deteriorated in the first 24 hours despite mannitol infusion. Further studies of mannitol treatment are underway, at least in the Marshall Islands, Kiribati, Fiji and Florida. A rigorously conducted, double-blind controlled clinical trial, including as many objectively determined parameters as possible and with adequate follow-up is needed. At present, given the safety of mannitol and the rapidity with which benefit may be evident, the administration of mannitol would seem justified in patients whose illness is

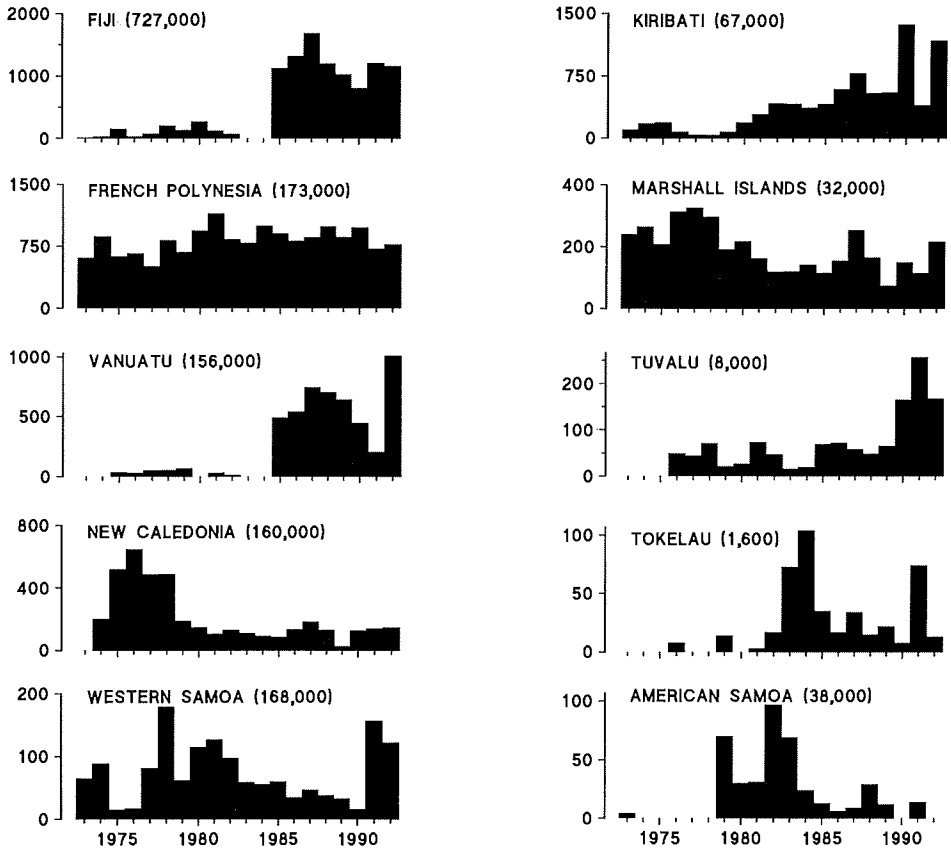

FIG. 1. Annual cases of ciguatera for selected Pacific countries,1973-1992. Data from SPEHIS,1973-1992. The 1988 population estimates for each country are indicated in parenthesis (FAO,1990). Prior to 1982, data for the Marshall Islands also included data from the Federated States of Micronesia, the Northern Marianas and Palau.

moderate or severe, and particularly those who present during the acute phase of the illness, typically within 24 hours of the onset of symptoms. A dose of 1g mannitol per kg body weight, as a 20% solution, infused over about 30 minutes, has been most commonly used (Palafox et al.,1988; Pearn et al.,1989). The clinical impression is that half this dose, infused over 60 minutes, appears to be less effective (Pearn et al., 1989). No adverse experiences have been reported with use of mannitol in patients with ciguatera, but it is prudent to ensure that patients are replete in intravascular volume prior to commencement of mannitol infusion.

The remoteness of small and widely scattered island communities from health care services, particularly in the Pacific, imposes limitations on availability of medical treatments, particularly one requiring careful supervision and intravenous infusion. A safe orally-active therapy requiring minimal supervision is desirable.

All patients suffering from ciguatera should be advised to avoid fish and alcohol for at least 3 months, and to reintroduce them into their diet cautiously, recognising that ingestion of either may precipitate a relapse. Many sufferers of ciguatera, particularly in Western cultures and where fish are not a crucial foodstuff, lose all inclination to again eat reef fish.

INCIDENCE OF CIGUATERA

The most comprehensive regional database on ciguatera (SPEHIS,1973-1992) also includes other forms of marine food poisoning (scombroid poisoning, clupeotoxism, mullet poisoning, puf-

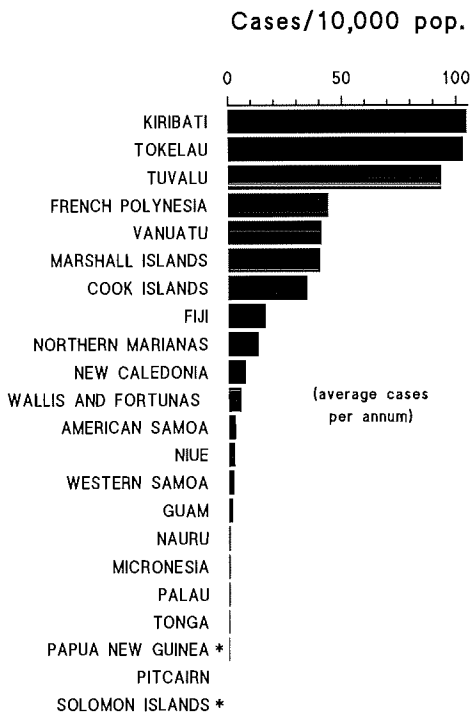

FIG. 2. Incidence of ciguatera in Pacific Island countries. Cases per 10,000 population are indicated Data are given per annum (p.a.) and were averaged from SPEHIS data (1973-1991) covering the period 1985-1990. Asterisks indicate incomplete reporting to SPEHIS from these countries.

fer fish poisoning and invertebrate intoxications). However, ciguatera typically dominates as a cause of fish poisoning in the Pacific region (Lewis,1992a). Ciguatera is reportedly prevalent throughout Pacific island countries with the exception of the Solomon Islands and Pitcairn Island (Fig.1). Ciguatera is invariably substantially underreported. In Australia it is estimated that as few as 20% of cases are reported and <10% of ciguatera cases in Western Samoa are reported to SPEHIS (Lewis, 1992a). Similar levels of underreporting are likely in other countries. Underreporting may vary within and between countries, and over time.

For countries of the South Pacific, the highest average incidence of reported ciguatera for the period 1985-1990 was c.100/10,000 population per annum(p.a.) in Kiribati, Tokelau and Tuvalu (Fig.2). The average reported incidence of ciguatera was less than half these levels in French Polynesia, Vanuatu, the Marshall Islands and the Cook Islands. The remaining 13 countries reported <15 cases/10,000 people p.a. Over the same period, the average reported incidence of ciguatera in Queensland (population 2.9 million) was 0.16 cases per 10,000 p.a., a level similar to that reported for Tonga. By way of comparison, in the Iles Saintes, Guadeloupe, in the Caribbean, annual ciguatera incidence has been estimated to be 30 (Czernichow et al.,1987), in the US Virgin Islands (Caribbean) to be 73 (Morris et al.,1982) and in Miami to be 5/10,000 population (Lawrence et al.,1980).

INDIRECT EFFECTS OF CIGUATERA

Throughout Pacific island countries there is a heavy dependence on the inshore fishery resource of reefs for dietary protein and animal fats. Johannes (1990) suggested that the inshore fisheries resource is of greater importance per capita to Pacific island countries than in any other region of the world. Nowhere is the impact of ciguatera greater than in atoll countries of the Pacific where intake of reef fish is often above 100g/per person per day (Lewis,1992a). Ciguatera is also important in relative terms, being one of the more commonly reported diseases (SPEHIS,1973–1991).

Ciguatera may have indirect effects on health by predisposing victims to poor nutrition and other diseases, and via its social and economic effects. The ability of subsistence communities to provide food, especially difficult on the poorer Pacific atolls, may be impaired due to the necessity of reducing fish consumption to reduce the risk of ciguatera (Lewis,1986). Ciguatera may have direct economic effects, reducing trade opportunities in potentially ciguateric fishes and damaging tourism (Lewis,1986). The effect of ciguatera on fish consumption is likely to be least in countries where alternative dietary protein sources to locally caught fish are costly and few, and where a system of traditional beliefs acts to reduce perceptions of the adverse effects of ciguatera (Lewis,1992a). People in larger and developed countries (e.g. Australia) with more diverse food sources and a less traditional orientation to the sea may be less accepting of ciguatera than are people in many Pacific Island countries.

The need to avoid fish after an outbreak of ciguatera may exacerbate undernutrition, especially among children (Eason & Harding,1987).

Fear of poisoning may accentuate dependence on imported food. In many Pacific locations, as much as 90% of fish eaten comes out of a can (Lewis,1986). Increased intake of imported food is often associated with a higher salt, fat and refined carbohydrate diet that contributes to an increase in chronic degenerative diseases such as diabetes (Zimmet et al.,1981), gout (Prior et al., 1987), hypertension (Zimmet et al., 1980) and atherosclerotic vascular disease (Taylor & Thoma,1985) in indigenous Pacific populations.

PREVENTION

INDIVIDUAL LEVEL

Individuals can reduce their risk of contracting ciguatera by: 1, avoidance of warm water reef fish, particularly those with a known propensity to be toxic, and avoidance of certain pelagic fish which feed on them (e.g. barracuda and mackerel), especially in areas with a history of ciguatera; 2, avoidance of all fish at locations which are a known recent or current source of toxic fish. 3, complete avoidance of moray eels, which are commonly highly toxic (Murata et al.,1990; Lewis et al.,1991; Lewis et al.,1992), except when captured in areas with no history of ciguatera; 4, avoidance of carnivorous fish may reduce, but does not eliminate, the risk of contracting ciguatera. Ciguatoxins tend to be concentrated as they pass up the food chain, and larger fish (particularly 2.5kg) are more likely to be toxic (Hessel et al.,1960); 5, avoidance of the head, roe and viscera of potentially toxic fish. Concentrations of ciguatoxins in fish liver may be up to 50 times higher than in muscle (Banner, 1976); 6, eating a small portion (20–100g) from any one fish at the first sitting (Lewis,1992a); 7, feeding a large fish flesh meal to a cat which is observed for at least 6 hours prior to human consumption of portions of the same fish (Lewis,1992a; Cooper,1964); 8, washing the flesh of herbivorous fish (such as parrot and surgeon fish), in several changes of water prior to consumption has been recommended on the basis that this may remove some of the water-soluble maitotoxin (Juranovic & Park,1991). This has not, however, been demonstrated to be useful

PUBLIC HEALTH MEASURES

These include: 1, education of fisherpeople and the public in affected areas about the risk of ciguatera and how this risk can be reduced (Ahmed,1991); 2, closure of known highly toxic areas to fishing (Ahmed,1991); 3, bans on the sale of high risk fish from known toxic locations. Such bans have been employed in American Samoa (Dawson,1977), Queensland (Lewis et al.,1988), French Polynesia (Lewis,1986), Fiji (Sorokin, 1975), Hawaii (Ahmed,1991; Gallop & Pon, 1992) and Miami (Craig,1980); apparently with some success, but with attendant economic loss; 4, detection of ciguatoxic fish prior to consumption. Such tests should be specific and sensitive for the toxins implicated in human disease. They should be sufficiently sensitive to detect 0.1 nM ciguatoxin-1 per kg of fish flesh (Lewis,1992b). To be used effectively at the community level, they should be robust, temperature-insensitive, reliable, inexpensive and simple to use.

Hokama pioneered development of such a test to detect ciguateric fish (Hokama,1991). A radioimmunoassay (RIA), subsequently modified to a simpler enzyme immunoassay (EIA) (Hokama, 1985) has been further simplified to a 'stick test' which has been used to screen fish caught in Hawaii (Hokama et al.,1990). All of 57 fish implicated in cases of ciguatera, and provided by the Hawaiian Department of Health in 1987-89 tested positive on a stick enzyme immunoassay (S-EIA) using a monoclonal antibody against ciguatoxin (MAb-CTX) (Hokama et al.,1990). All 86 *Caranx* sp (jack) and *Seriola dumerili* (amberjack) provided by sports fisherpersons and found to be negative on the S-EIA test, were consumed without incident (Hokama et al.,1990). However a high proportion, 1195/2190 (55%), of randomly tested fish of 19 different, potentially ciguatoxic species tested borderline or positive (Hokama et al.,1990), suggesting a high rate of false positive tests. The false negative rate however, which is of greater importance, would appear to be acceptably low.

Although the test has problems of specificity, cross-reacting with a variety of polyether toxins, such as okadaic acid, which play an uncertain role in ciguatera, and is not sufficiently robust to be used in the field (Hokama et al.,1990), it holds promise as a practical measure in ciguatera control, particularly for large fish processed commercially. Several groups are in the process of developing such antibody-based tests, including Hawaii Chemtec Inc, which plans to commercialize a modified version of the Hokama test. Research on detection of ciguatoxins using fluorescence high pressure liquid chromatography (HPLC) is also in progress. HPLC-based assays, perhaps linked to fluorescence or mass spectral detectors, have the potential to confirm ciguatoxins in small samples of fish flesh.

A rapid inexpensive test may eventually supplant the riskier process in use in some Pacific island areas, whereby an adult human eats or a cat is fed fish from an area, several times a year, to reassess the toxicity present in locally- caught reef fish. Such testing may be used particularly to protect children from ciguatera (Cooper,1964).

Long-term monitoring of populations of dinoflagellate(s) associated with ciguatera, their toxicity and toxicity of fish at various levels of the food chain at a range of sentinel sites may be of benefit in predicting ciguatera in an area. This may enable timely action, such as closing an area to fishing, or restricting types or sizes of fish caught, before an outbreak occurs (Ahmed, 1991). Such monitoring, particularly in areas of human impact on coral reefs (particularly through construction activities, other forms of coral damage, terrestrial and marine pollution, including sewage and agricultural runoff), could also make an important contribution to our understanding of the genesis of ciguatera. Such monitoring should be initiated with baseline studies prior to major developments likely to damage or alter a coral reef. There is widespread concern, particularly in the Pacific, that coral reef damage and pollution associated with population increase and economic development may increase the incidence of ciguatera (Lewis,1992a, Lewis,1986). The possible effects of global warming, stratospheric ozone depletion and other global environmental changes on ciguatera are unknown and provide additional justification for long-term environmental monitoring.

Restriction of human activities likely to be associated with coral reef damage. In some Pacific islands, such as the Line islands (Ross,1947), Gilbert Islands (Cooper,1964), and Hao, Moruroa and Mangareva in French Polynesia (Ruff,1989a,b) military dumping of material on reefs, construction activities and nuclear test explosions have been associated with outbreaks of ciguatera. Similarly, outbreaks have followed shipwrecks, shore modification and other construction activities in the Marquesas (Lewis, 1984a) and Hawaii (Gallop & Pon,1992; Lewis, 1984b). Although not supported by firm data, local Aboriginal people in East Arnhem Land ascribe the occurrence of ciguatera near the Gove peninsula to the construction of a township and alumina plant at Nhulunbuy in the early 1970s.

FUTURE DEVELOPMENTS

Key issues and areas for research include: 1, the need for a consistent case definition of ciguatera, a crucial basis for comparable epidemiologic and clinical data; 2, better tests for ciguatoxins, including ones which can be applied to human clinical samples. Antibodies which are more selective and have higher affinity for the ciguatoxins than those currently available are needed; 3, understanding of the pathophysiological mechanisms underlying human disease, potentiation of the disease by alcohol, and the phenomenon of sensitisation; 4, understanding of the human immune response to ciguatera may provide a basis for more effective control, particularly through immunisation; and 5, treatment for ciguatera which is simple to administer (preferably orally), inexpensive, and which is demon-strated to be effective and safe. In the short term, a well-conducted randomised controlled double-blind trial of mannitol therapy is needed.

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PATHOLOGICAL CHANGES IN MURINE HEARTS INDUCED BY INTERMITTENT ADMINISTRATION OF CIGUATOXIN

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Terao, K., Ito, E., Ohkusu, M. & Yasumoto, T. 1994 08 01: Pathological changes in murine hearts induced by intermittent administration of ciguatoxin. *Memoirs of the Queensland Memoirs* **34**(3): 621–623. Brisbane. ISSN 0079-8835.

Ciguatoxin (CTX) at doses of 0.1 or 0.05μg/kg were given orally by intubation into male ICR mice once a week for 25 weeks (0.1μg/kg group) or 40 weeks (0.05μg/kg group). Until about 10 weeks after the beginning of the experiments the mice in both groups showed no abnormal clinical signs. After about 18 weeks, mice treated with 0.1μg/kg showed marked hypertrophy of the hearts; no pathological changes were seen in the hearts of the other mice. There was swelling or rupture of the endothelium of the capillaries and widening caused by exudation or collagen fibers in the interstitial space. Occasionally, degenerated or swollen mitochondria were prominent in the myocardium. Accumulations of platelets in the capillaries were frequently observed. Mice treated with low CTX dose showed no pathological changes even at the ultrastructural level until 40 weeks. tHUS CTX has a potent cumulative effect on the cardiac tissue.

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Ciguatera is one of the most serious tropical food poisonings of fish in the vicinity of coral reefs; its symptoms are neurological, gastrointestinal and cardiac (Baden,1983). Several toxins were isolated from contaminated fishes or cultured dinoflagellates and the chemical structures determined (Murata et al.,1989). Among them, CTX is the most potent.A feature of ciguatera is its obstinacy and recurrence of attacks (Bagnis,1968, Bagnis et al., 1979). Our study of short-term, successive administration of CTX at a low dose, confirmed that CTX has a cumulative effect on the cardiac tissue. In experiments reported here we re-examined the potency of the cumulative effects of the toxin on the mouse heart tissue.

MATERIALS AND METHODS

TOXIN

CTX used herein was isolated from reef snappers (*Lutjanus bohlar*) from Micronesia and Okinawa. Phycotoxin was purified as described by Legrand et al. (1989). One mouse unit is the minimal lethal dose of CTX 24hrs after i.p. administration into 20g mouse and is equivalent to 0.35μg/kg body weight (Yasumoto pers. comm.). Oral LD_{50} was not determined because the available dose of phycotoxin was limited.

EXPERIMENTAL ANIMALS

Male ICR mice (4 weeks of age weighing 20-23g) were obtained from Charles River Japan Inc., Tokyo. 40 mice were divided into 3 groups. Group 1: Five mice were given physiological saline with a stomach tube once a week for 40 weeks and served as control. Group 2: Twenty mice were given CTX (0.1μg/kg of body weight). Group 3: Fifteen mice were given low CTX (0.05μg/kg). Mice in groups 2 and 3 were given the phycotoxin in a similar manner to group 1. Two mice each from group 2 were sacrificed by cervical dislocation 5 hours after the intubation at the 12th, 14th, 18th and 23rd week from the beginning. After the treatment all surviving mice were fed a standard diet (CE-2, Nihon Clea Inc., Tokyo). Two mice from the group 3 were also killed and controlled in a similar manner to mice in group 2 at the 18th and 40th week.

MORPHOLOGICAL EXAMINATION

After necropsy all internal organs were fixed in 10% neutral formalin and embedded in paraffin. The slides for light microscopy were stained HE and PAS. For TEM, pieces of the heart, kidney, and liver were fixed by cold paraformaldehyde-glutaraldehyde solution (final concentration: 2%) and immersed in buffered 1% OsO_4 at room temperature. Then dehydrated in a series of graded ethanol, embedded in Epon 812, and cut with a diamond knife on a Porter II ultratome. The ultrathin sections were stained with uranyl

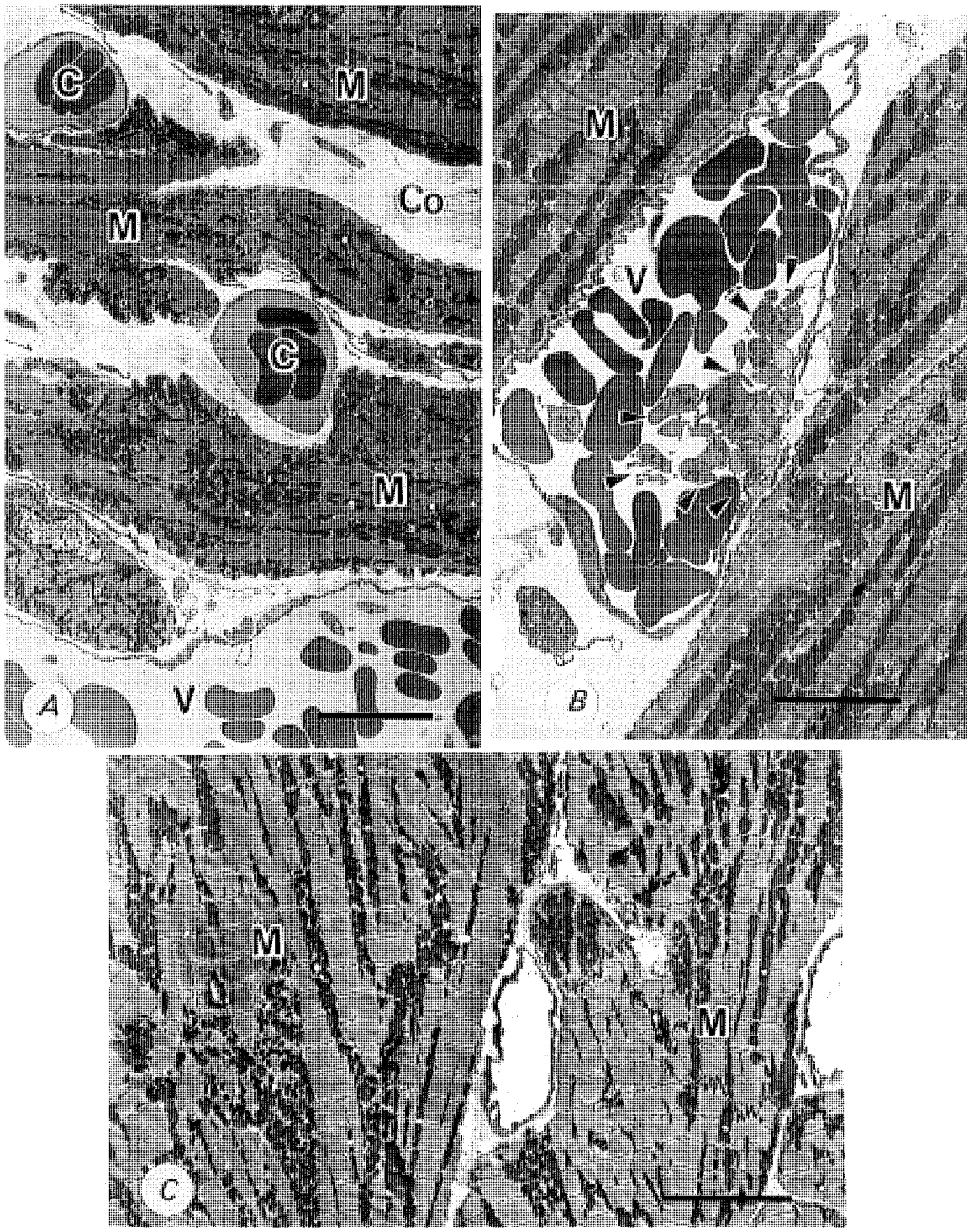

FIG. 1. A, Electron micrograph of heart from mouse given 0.1 μg/kg CTX orally by intubations once a week for 25 weeks. Between cardiac muscles (M) are thick bundles of collagen (Co). Blood capillaries (C) are embedded in the collagen fibers. V: vein. Bar: 10μm. B, Electron micrograph of the heart from mouse given 0.1μg/kg CTX orally by intubations once a week for 25 weeks. In a small vein (V) a thrombus (arrow heads) is developing. M: cardiac muscle. Bar: 10μm. C, Electron micrograph of the heart from a mouse given 0.05 μg/kg CTX orally by intubation for 40 weeks. No discernible changes are seen. M: cardiac muscles. Bar: 10μm.

acetate and lead citrate and examined with a Hitachi H700H TEM.

RESULTS

Oral administration of CTX at a dose of 0.1μg/kg resulted in no abnormality, whereas i.p. injection of the same dose caused severe watery diarrhoea within 10 minutes. In contrast, mice injected i.p. with the phycotoxin at a dose of 0.05μg/kg induced no diarrhoea at all.

Even at the ultrastructural level a single oral dose of 0.1μg/kg of CTX produced no abnormal changes in the heart muscle. Long-term, intermittent administration of CTX at a dose of 0.05μg/kg induced no pathological changes in the heart tissue until 40 weeks. In contrast, intermittent administration of 0.1μg/kg CTX once a week for over 12 weeks resulted in severe morphological changes in heart tissue.

Mice given 0.05μg/kg CTX showed no abnormal behaviour through the experiment. Mice treated with CTX at an intermittent oral dose of 0.1μg/kg also showed no abnormality during the first 12 weeks. After that, however, shock often occurred shortly after administration of CTX. Usually the animals recovered spontaneously within 10 minutes. After 18 doses or at the 18th week two mice were sacrificed. Both ventricles of the animals were dilated at necropsy. Histopathologically, multiple single cell necroses were often seen in the mural or papillary muscles of the left ventricle. TEM examination showed swelling of myocardiac cells and edema between bundles of muscle fibres. Mitochondria in these cells became rounded and the matrix was electron-dense. Occasional dissociation of intercalated discs was noted. Blood capillaries were embedded by bundles of collagen fibers and electron-dense flocculent materials (Fig.1A). Capillaries and small veins were often occluded by accumulation of blood platelets (Fig.1B).

Mice given 0.05μg/kg CTX produced no changes in heart tissue until 40 weeks (Fig.1C).

DISCUSSION

The most prominent morphological changes after administration of CTX occurred in the heart muscles (Terao et al.,1990,1991,1992). Almost all cardiac muscle cells and the endothelium of blood capillaries in the cardiac interstitium were markedly swollen and ruptured with severity of change dependent on dose. These edema may be caused by the increased influx of Na^+ channels of cardiac muscle cells (Ohizumi,1990). In our previous report, short-term successive administration of CTX at low dose resulted in a cumulative effect of CTX on the mouse heart (Terao et al., 1992). In the present study, an intermittent dose resulted in similar severe morphological changes in the heart tissue. CTX may bind very tightly to Na^+ channels on the cardiac muscle cells or to those on the endothelium of the capillaries in the interstitium.

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THE MOUSE CIGUATOXIN BIOASSAY: DIRECTIONS FOR USE TO CONTROL FISH FOR CONSUMPTION

JEAN-PAUL VERNOUX

Vernoux, J. P. 1994 08 01: The mouse ciguatoxin bioassay: directions for use to control fish for consumption. *Memoirs of the Queensland Museum* **34**(3): 625–629. Brisbane. ISSN 0079-8835.

Ciguatera fish poisoning causes serious health problems around the world but the diversity and heterogeneity of ciguatoxins are delaying chemical and immunological remedies. Realistic methods for ciguatoxin screening of fish are needed for public health studies. The mouse bioassay may prove useful since it is simple and relatively cheap. Qualitative and semi-quantitative analyses for ciguatoxins from 50, 100 or 200g of fish tissue using respectively 2, 6 or 12 animals are precisely described.

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Ciguatera is a human illness, sporadic in nature, caused by the ingestion of a wide variety of fish typically associated with coral reefs. These fish are transvectors for multiple ciguatera toxins (mainly ciguatoxins) acquired through their diet (Vernoux & Abbad el Andaloussi,1986; Legrand et al.,1990; Lewis & Sellin,1992). Fish poisoning is present in many coral reef areas of the world (Bagnis,1981) with 10,000–50,000 persons affected each year. Its incidence has been estimated at 1–4/000 population in the Pacific area (Bagnis,1979; Lewis,1984; Yasumoto et al.,1984), 4.2/000 in the Virgin Islands (Olsen et al.,1984), and 3–10/000 at Saint Barthelemy Island (Vernoux, this memoir) and in the Saintes Islands (Czernichow et al.,1984). With the increasing use of air travel, tropical reef fish or consumers (inhabitants or tourists) are constantly moving and ciguatera is being documented in temperate regions (Lange et al.,1992). Thus, ciguatera is a world health problem and there is a need for a practical screening method for ciguatera toxins. Amounts of ciguatoxin in fish that pose a public health problem are very low. One approach to the estimation of dangerous levels of toxin in fish tissue is to dose it in those portions that had elicited human ciguatera poisoning. Some authors did this using mouse or mosquito bioassays (Yasumoto et al.,1984; Chungue et al. 1984; Bagnis et al.,1987; Vernoux, this memoir). Assuming that 500ng CTX can kill c.1000g of mouse (i.p. LD_{50} of CTX into mice=0.45μg/kg), these studies show that the minimum threshold for the human pathogenic dose is c.50–100ng. This level corresponds to 100–200g of mouse killed by a 200g fish portion i.e. the flesh sample has a specific toxicity of 0.5–1g of mouse killed per g of flesh). So only trace quantities of ciguatoxins are needed to elicit human poisoning (0.25ppb). Therefore only those detection methods capable of detecting as little as 250–500pg of CTX per gram of fish flesh need be considered. HPLC and immunological methods could be appropriate methods. Unfortunately, until now the detection of these extremely low levels of multiple ciguatoxins (>20, Legrand et al.,1990) has delayed the use of HPLC methods. Immunological methods that possess the desired sensitivity and specificity have already been developed (Hokama,1990; Park et al.,1992). However, the potential of these methods for large scale use remains to be demonstrated, at least in part due to the possibilities of multiple ciguatoxins in fish contamination. A biological assay which encompasses all the different toxins and gives total toxicity could be used as a robust method for public health control of fish. Two bioassays having the desired sensitivity are the mosquito injection bioassay (Chungue et al., 1984) and the mouse injection bioassay (Hoffman et al.,1983). The latter is preferred since it is more convenient, simple, specific and has been widely used. After having used it for 20 years, we present some recent developments in its use as screening method.

METHODS

Flesh Extraction

Raw or cooked minced flesh can be used since ciguatoxins are heat resistant. If cooking, use a boilable cooking pouch filled with raw minced sample and boil in water for 30 minutes.

A typical procedure to extract 50g of flesh is:

TABLE 1. Testing of fish for consumption by the mouse bioassay
1, prepare LR for 50g of flesh and dilute with 2ml of 1% Tween 60 saline at 37°C and homogenize thoroughly.
2, inject i.p. into 2 male mice (18–24g) at dosage d=0.04ml/g of mice (i.e. 1g equivalent of flesh per gram of mouse).
3, Observe symptoms during 4hr and conclude for ciguatoxin presence (=penile erection) for neurotoxin presence (=respiratory distress) or for okadaic acid or fatty acid presence (crawling gait, slow breathing and general cyanosis).
4, note death after 24hrs and weigh the survivors. Use the following table to indicate edibility.

Observed mortality after a 1g.eq. of flesh injection/g of mouse	Toxin concentration in flesh	Loss of weight at 24hr (>5%)	Interpretation
2/2	≥ 1MUg/g.e.f	-	not edible
1/2	0.5 to 1 MUg/g.e.f	yes	not edible
0/2	<0.5 MUg/g.e.f	yes no	bordeline edible

1, homogenise 3 min in a Waring blender with 150ml of acetone, 2, filter onto a buckner funnel and wash the remaining cake with 30ml of 80% acetone; discard the cake, 3, remove acetone on a rotary evaporator under reduced pressure and reduce the volume of the remaining aqueous solution to 30ml (add water if below), 4, add 10ml of ethanol, shake and extract twice with 40ml of diethyl ether, 5, remove diethyl ether and residual water under reduced pressure (addition of ethanol allows to remove quickly residual water), 6, dissolve the diethyl ether residue in 25ml of 80% methanol and wash twice with 50ml of hexane; discard the hexane solubles, 7, remove methanol and water under reduced pressure; resulting dry residue is called lipid-soluble residue (LR), 8, if weight of LR is >75mg dissolve in 10ml of 80% methanol and wash again twice with 20ml of hexane, 9, emulsify LR in 2ml of 1% Tween 60 saline and keep at –20°C until use.

Determination of Toxin Concentration

LR emulsified in 1% Tween 60 saline was heated at 37°C and i.p. injected into male mice weighing 18–24g (2 mice per dose). A series of doses that vary in a geometric progression by a factor of 1.1938 are assayed. Doses are expressed in gram equivalents of flesh per gram of animal (g.e.f/g). They were chosen in succession in the following series of numbers running from 10^{n-1} to 10^n which increase successively by a constant power of 10 (since $1.1938^{13} \times 10^{n-1} = 10^n$). Fourteen numbers in such a series are 0.10, 0.12, 0.14, 0.17, 0.20, 0.24, 0.29, 0.35, 0.41, 0.49, 0.59, 0.70, 0.84, 1.0. One gram equivalent corresponds here to 0.04 ml of LR solution and the volumes to be injected are respectively: 0.04 ml/g of mouse multiplied by the numbers indicated in this series i.e. 0.004 ml/g; 0.0048 ml; 0.0056 ml; 0.0068 ml, etc. Note that the total weight of mouse to be injected requires a total volume below that available in the experiment and the use of mice <20g for the highest dosage may be necessary.

Doses <1g.e.f/g are injected in 0.8ml per 20g mouse by carrying out dilutions directly in the syringe with 1% Tween 60 saline at 37°C. The approach described above allows determination of the LD_{50} and the minimum lethal dose (MLD) which is the lowest dose capable of killing two mice in the two mice group (or one mouse in the one-mouse group) after 24 hr. The toxin content is expressed in terms of Mouse Units gram (MUg) where 1MUg is 1g of mouse killed by the MLD (or the LD_{50}) expressed in g.e.f/g. The toxin concentration is expressed in MUg per gram of flesh (MUg/g.e.f) which is the reciprocal of MLD (or LD_{50}).

The suitability of fish for consumption may be controlled by mouse bioassay using 50g of flesh (Table 1). Quantitative and semi-quantitative conclusions are presented. Two hours are needed to prepare LR. If dissolution of LR is difficult, 0.1 ml of ethanol can be added per 1.9ml of Tween solution. A negative control is run using two mice injected with a blank solution (without LR). Non-toxic fish extracts give negative results (they elicit no symptoms).

Acute toxicity may be determined in two steps (Table 2) with a minimum of animals (Lorke et al.,1983). For this method 200g of flesh should be extracted and four hours are needed to prepare the LR. In the initial investigation, which requires an amount of extract corresponding to about 100g of flesh and 6 mice, an approximate range of doses producing the toxic effects is established. Normally this initial investigation would include doses used in the control of fish for consumption method i.e. injection of 1g.e.f/g as a first step; in

TABLE 2. Determination of the acute toxicity of LR, prepared from 200g of flesh, in two steps.

1st step EXPERIMENTAL				2nd step				RESULTS			
Doses d_1 in g.e.f./g of mouse			Approximate deduced toxin concentration in MUg/g.e.f.	Doses d_2 in g.e.f./g chosen for the second test (2 mice per dose)				Corresponding toxin concentration in MUg/g.e.f. according to the (MLD)			
Lethality[a] in the first test (5 possibilities)											
1[c]	0.49	0.24									
0/2	0/2	0/2	<1	[2][b]	1.7	[1.4][b]		0.5 (2.0)	0.59 (1.7)	0.71 (1.4)	-
1/2	0/2	0/2	>0.5 and <1	1.4	1.2	[0.84]		0.71 (1.4)	0.83 (1.2)	-	-
2/2	0 or 1/2	0/2	≥1 and <2	0.84	0.7	0.59		1.00 (1.0)	1.19 (0.84)	1.43 (0.70)	1.70 (0.59)
2/2	2/2	0 or 1/2	≥2 and <4	0.41	0.35	0.29		2.04 (0.49)	2.44 (0.41)	2.86 (0.35)	3.45 (0.29)
2/2	2/2	2/2	≥4	0.20	0.17	0.14	0.12	4.17 (0.24)	5 (0.20)	5.88 (0.17)	7.14 (0.14)

[a] Number of animals died/number of animals used
[b] One mouse per dose
[c] This dose allows control of fish for consumption.
[ ] Possible only when control of fish for consumption is used instead of the first test.

that case according to the observed lethality 0/2 or 1/2 it may be possible to bypass the 0.49 and 0.24 g.e.f/g injections (but not if a 2/2 lethality is observed). Based on these results, further specific doses are administered to a group of one mouse or two per dose depending on the predicted toxin concentration (<1 or >1MUg/g.e.f). From the results of these two tests, 6 or 7 successive levels of dosage ($d_1 + d_2$) are then assayed. For a two-mice group an LD_{50} can be calculated by the method of Weil (1952) using the equation

$$\log LD_{50} = \log D_a + \log R\ (f + 1)$$

D_a being the lower dosage level, R the geometric factor and f a value given in Tables. This permits a simple and rapid estimation of the LD_{50} with a corresponding confidence interval.

For the one animal group per dose, LD_{50} is estimated as the geometric mean of the doses for which 0/1 and 1/1 are found (Lorke et al.,1983). The minimum lethal dose can be used instead of LD_{50}.

RESULTS AND DISCUSSION

Control of Fish for Consumption

The proposed acetone method is much more economic and is as rapid as the method of Lee et al. (1987) who used methanol as the extracting agent instead of acetone for okadaic acid, a toxin chromatographically related to ciguatoxin. The methanol method is convenient only if the tissue portions to be extracted are <10g. So we prefer the procedure with acetone (Vernoux,1981) and we have been using this method since 1981.

In our method the LR yield must be below 0.15% of the flesh since the less impurities present the more marked the symptoms in mice for a given dose. Doses received by mice with this method do not exceed 1.5 mg of LR/g of mouse. Our proposed interpretation of symptomatology and lethality includes:

- the unique propensity of ciguatoxin to induce penile symptoms i.e penile cyanosis and/or transitory and incomplete erection (sometimes even reaching priapism i.e. complete and permanent erection seen following sub-lethal doses (Vernoux & Bagnis,1976; Vernoux et al.,1985). This symptom was recently confirmed by Terao et al., (1991) who pointed out the penis as a target organ for ciguatoxin.
- the symptoms in mice after i.p injection of okadaic acid (Vernoux & Moulin,1989) or fatty acids (Vernoux,1981) are different from that elicited by CTX but resemble the effects of maitotoxin, a toxin never detected in fish flesh (Yasumoto et al.,1984).
- the existence of a narrow range of doses (d - 2d) between 0% and 100% lethality (Hoffman et al.,1983; Lewis & Endean 1984; Vernoux & Moulin,1989).
- the general observation of a minimum pathogenic dosage only 1/4 to 1/3 the LD_{50} dosage and the link between the pathogenic dosage and loss of weight (Chungue et al.,1984; Vernoux,1988).

- additional observations suggest a 5 MUg or 10 MUg dosage/g of mice if survival time is respectively about 1 hour and half an hour but it may vary considerably with fish species (Vernoux and Tahla,1989). The relationship between the mouse response and the quantity of toxin present is given in Table 1 and 2.

ACUTE TOXICITY DETERMINATION

Extracting 200g is convenient for investigating fish for consumption and for quantifying the toxin concentration in the 0.5–7.14MUg/g.e.f range. This range is sufficiently wide to include toxin concentrations found in fish in the Australia, Pacific area or the Caribbean. There is no upper limit for the determination of toxin concentration, since the more toxic the flesh the less RL consumed in the test. Unlike the method for the control of fish for consumption, acute toxicity determination takes more than one day to conduct. Fortunately, stability of toxins in 1% Tween 60 saline is complete when samples are stored at –20°C for up to 6 months.

The geometric factor R = 1.1938 was chosen to provide a closely spaced series of dosage levels. This geometrical series of numbers increases successively by a constant power of 10 as already mentioned above. Furthermore, as $(1.1938)^4$ = 2.0 another geometric factor R = 2 can be used and numbers of the corresponding series are therefore included in the first one. The two-step method shown in Table 2 was developed using these series. Since the first one is a closely spaced series, this enhances the precision of the MLD determination. In this case we observed that the MLD values obtained with two animal groups were equivalent to the LD_{50} values obtained with four animal groups (Vernoux and Tahla,1989). This experimental correlation can be easily explained since the slope of the dose response curve for ciguatoxin is high with a narrow dose range (d–2d) between 0% and 100% lethality, thus including MLD and LD_{50} values (Vernoux and Moulin,1989). Nevertheless the two-mice group or even one-mouse group also give reliable LD_{50} values (Weil,1952; Lorke,1983). Here with the two-mice group, to calculate LD_{50} we use the method of Weil (1952) since it is easier and more rapid than the method of Litchfield & Wilcoxon (1949) and the former approach allows the confidence interval to be estimated. However, we prefer MLD determination to LD_{50} calculations since a greater accuracy is not necessary in view of the range of variation from one dose to another.

It might be thought that MLD or LD_{50} could be determined from the curve of dose (d) versus survival time (t) particularly since the test can be conducted in one day. Nevertheless this method is convenient only if the toxin concentration in flesh is ≥ 2MUg, thus limiting its application. The relationship is $d = LD50\ (1 + 1/t)^b$ or $d/LD50 = (1 + 1/t)^b$ i.e. number of MUg/g.e.f = $(1 + 1/t)^b$. Unfortunately b is fish-species dependent and it varies from 2 to 3 (Vernoux,1991) thus complicating the situation. So this method is of limited interest in controlling fish for consumption.

CONCLUSIONS

Presence of multiple ciguatoxins in fish flesh led us to propose here a simplified mouse ciguatoxin bioassay. Exhaustive description of the method should allow it to be use to control fish by any unspecialised hygiene laboratory. The limited quantity of flesh used (200g) is convenient for investigating fish for consumption and it allows to determine toxin concentration in all situations. The fixed method should replace the multiple mouse ciguatoxin bioassay methods for which toxicological bases are not very clear. Standardisation of the mouse strain could be realised with a known toxin having a similar physiological effect, brevetoxin for example. We hope that our proposals will gain wide acceptance, since the mouse ciguatoxin bioassay proposed here provides both a qualitative and semi-quantitative bioassay for ciguatoxins in fish.

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CIGUATERA IN THE FRENCH WEST INDIES

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Ciguatera fish poisoning was investigated on the Island of Saint-Barthelemy, Leeward Islands, Caribbean Sea, from 1979 to 1989. Clinical features include gastrointestinal and neurological disorders. 440 fish caught in fish-pots or by hook and line were checked by mouse and chicken bioassays. Jacks (*Caranx* spp.) and barracudas were highly ciguatoxic. Weight and toxicity were not correlated except for the most toxic species *Caranx latus*. Small carnivorous fishes classified as invertebrate feeders are likely involved in the transfer of ciguatoxin in the food chain since they contained significant levels of toxin. Herbivores (e.g. surgeonfishes or parrotfishes) which are not locally implicated in ciguatera, contained sometimes low levels of ciguatoxin. *Gambierdiscus toxicus* occurred in coastal waters of Saint Barthelemy but this species may not directly produce ciguatoxin.

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In the Caribbean, ciguatera poisoning is known from the Bahamas (Pesty,1975) to Martinique (Menger,1979) including Florida (Lawrence et al.,1980) Cuba (Bagnis,1979a) and Puerto Rico (Gilman,1942; Payne & Payne,1977) with a higher incidence in small Islands such as the Saintes, the Virgin Islands (Czernichow et al.,1984; Hanno,1981; Morris et al.,1982) and the Leeward Islands (Morice,1965). One of the Leeward Islands, Saint Barthelemy, is a small (c.25km), arid (without river or spring), tropical Island at 17°55'N, 62°50'W (Fig.1). The 3 adjacent islands of Saint Barthelemy, Saint Martin and Anguilla are surrounded by a wide shelf (max. depth 60m), with fringing reefs which continue across the shelf. These submerged reefs often form scattered coral-shoals 10–20m underwater. At the edge of the open sea the shelf falls abruptly to >200m. This coral reef ecosystem shelters a great variety of reef fishes (Vernoux et al.,1988).

Laboratory research on ciguatera was initiated in this area in the 1980's. It was shown that in fish the poison is lipid-soluble and quite similar to ciguatoxin isolated in the Pacific (Vernoux et al.,1982; Hoffman et al.,1983). Heterogeneity of ciguatoxins extracted from viscera or flesh of Caribbean fish was also demonstrated (Vernoux & Abbad el Andaloussi,1986) and confirmed (Vernoux & Tahla,1989; Gamboa et al.,1990). *Gambierdiscus toxicus* (Bergman & Alam,1981; Besada et al.,1982) was suspected as the ciguatoxin elaborator and as the producer of maitotoxin (Miller et al.,1984). A study of ciguatera poisoning and occurrence of ciguatoxins in fish was carried out on Saint Barthelemy. Results are presented here, together with results from experiments with *G. toxicus* sampled at Tahiti in 1976 (in Dr. Bagnis's laboratory).

MATERIAL AND METHODS

Fish collected at numerous locations on Saint Barthelemy between 1979 and 1989 were identified from Stokes (1980). Most were caught in fish traps on the sea bottom (depth: 30-50m) within the reef habitat. King mackerel (*Scomberomorus cavalla*) and cero (*Scomberomorus regalis*) were collected by trolling, and greater amberjack (*Seriola dumerili*), black jack (*Caranx lugubris*) and african pompano (*Alectis crinitus*) by hook and line. Whole animals or separated tissues were frozen at –20°C for transport to the laboratory and stored until processed.

Lipid-soluble residues (LR) were prepared from flesh or viscera by a routine acetone or methanol method, respectively (Vernoux et al.,1985a). For the qualitative testing, fish liver or LR were fed to chicks as reported elsewhere (Vernoux et al.,1985b; Vernoux & Lahlou,1986). For quantitative testing a mouse bioassay was used (Vernoux et al.,1985a). The toxin concentration was expressed in Mouse Units gram per gram of tissue (MUg/g), where 1 MUg is the weight-specific minimum lethal dose (1MUg=1MU/20). Fish was assumed to be ciguatoxic when typical symptoms of ciguatera (Vernoux, this memoir) were observed in both chick and mouse.

For experiments in Tahiti in 1975 and 1976, a mixture of algae and detritus was scraped from the surface of dead corals collected at the Gam-

FIG.1. The Caribbean region.

bier Islands. This mixture was fractionated by hand according to size and, then by successive passes through sieves of different mesh sizes (Blutex Nylon: 85μm, 36μm and 10μm). Vortex shaking helped to separate associated organisms. Our routine acetone method was used to extract ciguatoxin in each biodetritus fraction. Remaining dry organic matter was treated with boiling methanol (Yasumoto & Endo, 1973) to extract the maitotoxin. This allowed a separation of ciguatoxin (CTX) and maitotoxin (MTX) with little reciprocal contamination, since maitotoxin is poorly soluble in acetone (Yasumoto et al., 1976). These toxins were identified by symptomatology induced in mice and by their chromatographic behaviour on a silicic acid column. For analysing toxins in the gut contents of herbivorous fish the method of Yasumoto et al. (1977a) was used.

Analysis of variance (ANOVA) was done using SPSS-PC software. A non-parametric ANOVA K-W test was also used in parallel.

RESULTS

EPIDEMIOLOGY

With the help of some local physicians, fish poisoning was surveyed at Saint Barthelemy (3000 inhabitants). 10–30 cases were identified per year either from consultations or from patients treated at the hospital. Generally they were fishermen or tourists having consumed small jacks, big mackerels, snappers or seabass. In some cases, poisoning was induced after a second meal of the same fish. Patients complained of sensitivity disturbances (painful tingling about the mouth and throat, hot and cold reversal) nausea and vomiting, abdominal pain, diarrhoea, myalgia, weakness and hypotension. Visual disturbances (blurred vision) and persistent itching were also noted. After symptomatic therapy, complete recovery occurred after a few days, weeks or months. After a primary poisoning some individuals develop a fish feeding allergy and cannot eat any fresh or canned fish. Toxin analysis of fishes implicated in these ciguatera outbreaks revealed ciguatoxins at >1MUg/g of flesh.

DISTRIBUTION OF TOXICITY

During the course of this study we observed that fish only contained toxin in their flesh if their livers were also toxic. Furthermore, ciguatoxin concentration was always higher in viscera than in flesh (at least twice as toxic).

The most toxic species (1–10 in Table 1) are large piscivorous species except for *A. afer* which is a small (<300g) invertebrate feeder.

Intermediate toxicity was detected in species 11–15 (Table 1). Lower toxicity was present in other species (16–30 in Table 1). The above classes include small species (<500g) such as *M. martinicus*, *P. arenatus*, *B. rufus* and *H. radiatus* and *M. plumieri* (<1kg) which feed mainly on benthic invertebrates; these species can be more toxic than some larger piscivorous species. Only one species of herbivorous fish, the ocean surgeonfish (29 in Table 1), had low toxicity. No toxicity was found in other herbivorous fish (31–35 in Table 1). The longjaw squirrelfish (36 in Table 1), a specific crustacean feeder, was not toxic.

Statistical analysis of variance of individual toxicity for species 1–11 (Table 1) (independent of weight) indicated that the means were significantly different ($p<0.01$) for the 11 species group unless *C. latus* and *C. bartholomaei* were removed from this group. Without *C. latus*, the F test was not significant ($p=0.13$) but the K-W test was significant ($p=0.04$). This discrepancy can be explained by the difference in size between the *C. bartholomaei* sample (n=45) and the other

TABLE 1. Flesh ciguatoxicity in various fish species from Saint Barthelemy Island: results presented in decreasing order according to toxicity.

No.	Species	Specimen weight	TOXIN CONCENTRATION IN MUg/G OF FLESH							
			<0.05	0.05–0.49	0.5–0.99	1–1.49	1.5–1.99	2–2.99	3–5	>10
1	*Seriola dumerili*	6–29			1				1	
2	*Caranx latus*	1.3–6		1	8	8	1	2	4	
3	*Caranx ruber*	0.75–2.3		4		1		1		
4	*Caranx bartholomaei*	0.75–4.8		12	14	12	4	2	1	
5	*Alectis crinitus*	3.3–12		5			1	1		
6	*Sphyraena barracuda*	3–10		2	2	1				1
7	*Epinephelus morio*	6.5–8		2	1			1		
8	*Alphestes afer*	0.1–0.25		(16)(11)(12)(2)	(6)	(2)				
9	*Scomberomorus cavalla*	15–20			1	3				
10	*Scomberomorus regalis*	3–4	1	1		1				
11	*Gymnothorax funebris*	3.5–14.5		6	2					
12	*Malacanthus plumieri*	0.3–0.6		(5)(20)(5)(4)(6) (4)(2)(12)	(5)(15)					
13	*Lutjanus jocu*	1.7–2.2		1	2					
14	*Lutjanus griseus*	1.9–2		2	1					
15	*Lutjanus buccanella*	0.3–1.5		(3)1(2)	(2)					
16	*Priacanthus arenatus*	0.4–0.6		(3)(2)						
17	*Bodianus rufus*	0.2–0.4		(2)(15)(12)						
18	*Halichoeres radiatus*	0.5–1		(4)(3)						
19	*Gymnothorax moringa*	1.5–2		2						
20	*Mulloidichtys martinicus*	0.2–0.4		(20)(20)						
21	*Mycteroperca venenosa*	3.2–4.5		3						
22	*Caranx lugubris*	2.5		1						
23	*Lutjanus analis*	4–5		2						
24	*Seriola rivoliana*	2.7–4.5		2						
25	*Mycteroperca tigris*	0.7		(2)						
26	*Epinephalus guttatus*	0.7–0.9		(3)						
27	*Epinephalus adscension*	0.5–0.7		(4)						
28	*Calamus calamus*	0.2–0.4		(6)						
29	*Acanthurus bahianus*	0.05–0.15		(37)(6)						
30	*Balistes vetula*	1.5–2	1	1						
31	*Acanthurus chirurgus*	0.2–0.4	(3)(4)(7)							
32	*Acanthurus coeruleus*	0.2–0.4	(10)							
33	*Scarus coeruleus*	2	1							
34	*Scarus vetula*	0.6	(2)							
35	*Sparisoma viride*	1	1							
36	*Holocentrus ascensionis*	0.1–0.2	(11)							

Specimens were tested individually except where indicated by brackets in which case the number of pooled specimens is given.

samples (n<10). *Caranx latus* had the highest mean toxicity of species examined, suggesting that it is the most dangerous species at Saint Barthelemy.

The relationship between individual toxicity and weight was investigated within each of nine species for which n>2; the smooth curves (Fig.3) were fitted with the linear regression model:

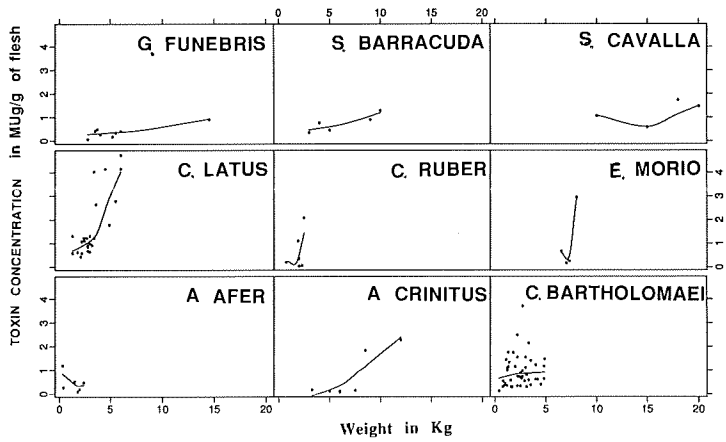

FIG.2. Relation between weight and toxicity for some toxic fish species.

toxicity = $\beta_0 + \beta_1$ weight +E,

where toxicity is in MUg/g and weight is in kg. A significant linear correlation between toxicity and weight was found ($\beta_1/0$) only for *C. latus* ($p<001$; β_0=-0.85502; β_1= 0.79673) and *A. crinitus* ($p=.014$; β_0=-1.31314; β_1=0.29066).

Use of Bio-Indicators

Since jacks occupy the upper part of the ciguatera food chain, we chose *C. bartholomaei* and *C. latus* adults (>1kg) to investigate the degree of bioaccumulation of ciguatoxin per year. From 1979–1985 the median concentration per year of ciguatoxin in their flesh (n=62) was stable around 1MUg/g of flesh. During the same period other bioindicator species such as *M. plumieri* and *A. afer* (benthic fishes) which were thought to feed directly on the ciguatoxin producers were also studied. Their toxicity level per year was constantly 0.2–0.6MUg/g of flesh. So the amount of toxins in the food chain at Saint Barthelemy appeared stable over this period. Nevertheless, in the last years of this study toxicity of *C. latus* was 3–4 times higher (n=10), but no change was found in the toxicity of *C. bartholomaei* (n=15).

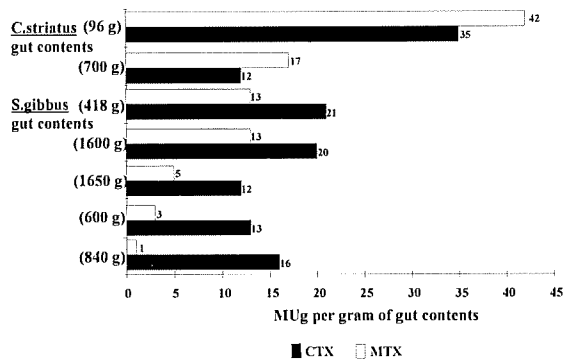

FIG.3. Distribution of toxins in gut contents of surgeonfish *C. striatus* and parrotfish *S. gibbus* caught at the same place in the Gambier Islands.

Distribution of Toxins and *G. toxicus* in Gut Contents of Herbivorous Fishes and in Coral Samples

Extracts of coral samples collected at Saint Barthelemy blanketed with algae did not contain detectable ciguatoxin, even though *G. toxicus* was present in low numbers on these samples.

The maitotoxin and ciguatoxin analysis of gut contents of herbivorous fish, obtained in 1975–1976 from French Polynesia (Fig.3) shows that materials ingested by parrotfish *S. gibbus* or surgeonfish *C. striatus* contained ciguatoxin (fat soluble toxin) and maitotoxin (acetone precipitated toxin). Furthermore, the concentration of ciguatoxin in gut contents of parrotfish was always higher than that of maitotoxin, whereas the ratio of the two toxins was reversed in the gut contents of the surgeonfish. Thus ciguatoxin level appears unrelated to maitotoxin level.

CTX content (in MUg) is quantitatively dominant in scraped coral substrate and in the fraction >85μm (=algae+detritus), four times as much as in the *G. toxicus* fraction (Fig.4A). Attempts to remove CTX from coral substrate by scraping in the presence of a low pressure spray of water were unsuccessful: 30–60% of total CTX content remained attached to the coral substrate (3 experiments). With crude material (not frozen) results were

similar. The MTX content was well correlated with *G. toxicus* (which accumulated in 36–85μm fraction) and the ratio of MTX to CTX was >1 as it was for the gut toxin contents of *C. striatus*.

Specific CTX content (in MUg/g of dry matter) seems to reside not only with the *G. toxicus* fraction but also on the other size fractions, particularly the fraction <36μm (particles + washing water) (Fig.4b).

G. toxicus thecal structure can be extremely resistant to different conditions: freezing, water dilution, acetone extraction and ultra-turrax sonication.

DISCUSSION

The involvement of fat soluble ciguatoxins in ciguatera in the Caribbean has been confirmed by chemical studies on the toxins in *C. bartholomaei* (Vernoux et al.,1982), *B. rufus*, *M. martinicus*, *M. plumieri*, *E. morio*, *G. funebris*, *S. barracuda*, *S. cavalla*, *S. dumerili* (Vernoux & Abbad,1986), and *A. crinitus*, *C. latus* (Vernoux & Talha,1989). In the Virgin Islands, similar species were clinically found to cause ciguatera poisoning (Brody, 1971; Morris et al.,1982; Engleberg et al.,1983) and ciguatoxin from *Lutjanus buccanella* has been well documented (Hoffman et al.,1983).

At Saint Barthelemy the same species of fish were ciguatoxic as in the Pacific (Randall,1958; Halstead,1978). Nevertheless, jacks are much more toxic in the Caribbean (Arcisz,1950) than in the Pacific Ocean (*Caranx ignobilis* was the only species suspected in the Pacific by Bagnis (1981)). The importance of feeders on small benthic invertebrate feeders in the ciguatera food chain at Saint Barthelemy is worth pointing out since this suggests that ciguatera transmission begins primarily at the invertebrate level. Invertebrates appear to be less important in the Pacific area, though some feeders on invertebrates, such as *Lethrinus kallopterus* at the Marshall Islands (Randall,1980), *Cheilinus undulatus* at Tahiti (Bagnis,1968) and other Lethrinidae at New Caledonia (Bagnis,1979a) are ciguatoxic.

In the French West Indies all ciguateric species were shore fish associated with reefs. With the exception of two semi-pelagic open water species, the king mackerel and cero, they were bottom dwelling species generally found at a depth of <50m (>100m for black jack and greater amberjack). Ciguatoxins are therefore well correlated with the benthic fish. Further illustration is provided by *Caranx ruber* which is dangerous at Saint Barthelemy when caught in fish traps

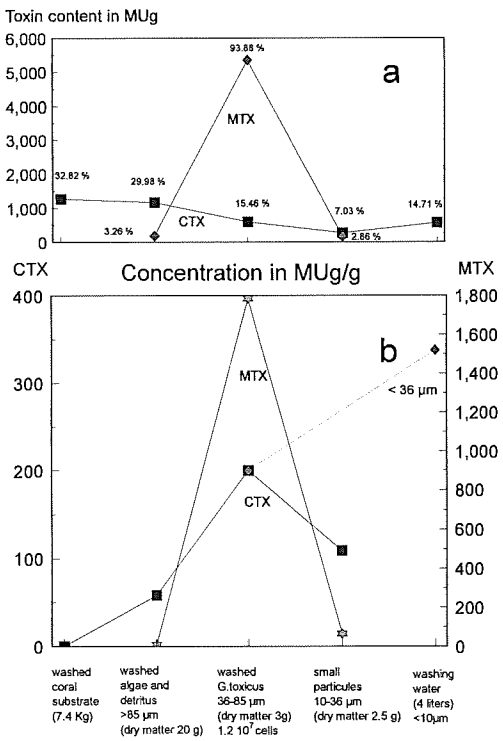

FIG. 4. Distribution of toxins MTX and CTX in fractionated material from the Gambier Islands either considering total toxin content (a) or toxin concentration (b).

(sedentary individuals) but not in nets (migrating shoals of fish).

Unlike the Pacific, at Saint Barthelemy herbivorous fish are regularly consumed without suspicion. Similar observations have been reported in other areas of the Caribbean (Bagnis 1979a; Czernichow et al.,1984) in New Caledonia (Bagnis,1979b) and in the Indian Ocean (Lebeau & Telmar,1978). Nevertheless detectable ciguatoxicity in the surgeonfish *A. bahianus* indicates that low (subsymptomatic) ciguatoxin levels may be present in some Caribbean herbivorous fish species, illustrating that fish edibility depends on toxin level as already described (Bagnis & Vernoux,1975). Here we cannot exclude the possibility that ciguatoxin is present in the other herbivorous fishes, though ciguatoxin levels are probably (extremely) low. The higher toxicity of *A. bahianus* suggests a different diet. We studied 3 Caribbean surgeonfishes (Stokes,1980; Randall,1983) that have different feeding habits (Randall,1967): *A.*

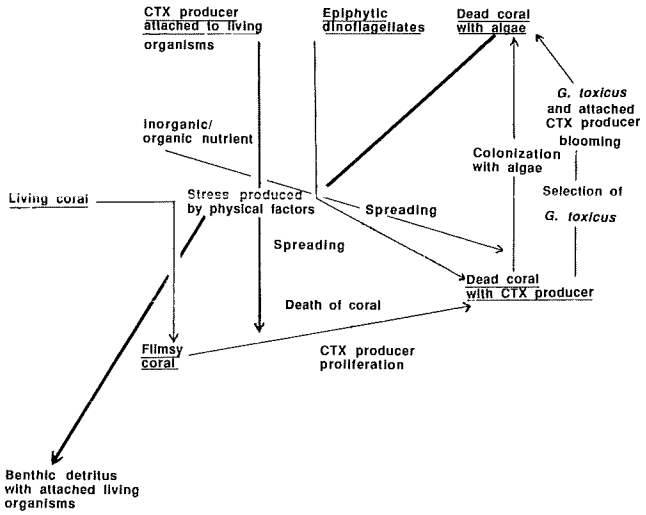

FIG. 5. A proposed mechanism for the initiation of CTX producer proliferation.

chirurgus and *A. bahianus* has a thick walled gizzard-like stomach and ingests inorganic sediment (sand, small shells etc.) with the algae they crop from solid substrata (especially *A. bahianus*) (in Randall,1967; and personal observations); and *A. coeruleus* has a thin walled stomach in which we have observed a higher content of green algae and it does not usually ingest sand. The former are therefore grazers whereas the latter is a browser following the definition of Lobel (1981). In the West Indies, parrotfishes are also grazers that feed mainly on algae attached to dead coral (Randall,1967), except for large *Sparisoma viride* and occasionally large *Scarus vetula* which graze on live coral (Frydl,1979). Taking into account these feeding habits and the corresponding toxicity results, we deduce that the source of the toxin in the food could be an organism linked to bottom detritus. The ciguatoxin producer *G. toxicus*, found on dead coral (Bagnis et al.,1980) and closely associated with reef sediments or with macroalgae (Taylor,1979; Yasumoto et al.,1979) may be the ciguatoxin-producer at Saint barthelemy. This conclusion is consistent with the presence of *G. toxicus* (Bagnis,1981; Besada et al.,1982; Bourdeau & Durand-Clement,1991) and with previous studies (Yasumoto et al., 1977b; Bagnis et al.,1980) which have implicated *G. toxicus* as the ciguatera produced in the Pacific. Nevertheless, the somewhat conflicting results obtained in the study of scraped coral and discussion concerning ingested toxins in gut contents of herbivorous fish at Tahiti have not previously been considered.

Chanteau (1978) studied scraped coral as well as other fractions for toxicity. She obtained toxin partitioning results similar to ours, even though scraping was practised with a metallic brush, i.e. MTX content was highly correlated with the *G. toxicus* containing fraction and <10% of total MTX was present in scraped coral substrate, and in this substrate the proportion of MTX to CTX were inverted (<1). These observations and ours demonstrated that: MTX is a *G. toxicus* marker, especially since it is never excreted out of the dinoflagellate (Yasumoto et al.,1979b). However, CTX contamination level was independent of the amount of *G. toxicus* in these studies. This latter statement was corroborated by analysis of toxins in the gut contents of herbivorous fish: our results and those of others (Yasumoto et al.,1975; Yasumoto et al. 1977a) showed that the relative proportions of MTX and CTX are inverted in surgeonfish (*C. striatus)* compared with parrotfish (*S. gibbus)* caught in the same area. The difference in proportion corresponds to the difference in their feeding habits: the former is a browser which ingests chlorophyll-bearing material (10%) as well as detritus and numerous *G. toxicus* were found in its stomach and gut contents, while the parrotfish is a coral feeder exclusively (103–260μg of algae/100g of ingested sample according to Yasumoto et al. (1977a) and no *G. toxicus* was visible in its stomach or gut contents (Vernoux,1981). Thus ciguatoxin producer could be associated also with living coral (perhaps the zooxanthellae?). Bagnis et al. (1980) stated that they could not find any indication that CTX (or MTX) was excreted into the culture medium, while Shimizu et al., (1982) and Campbell et al. (1987) using fluorescence labelled sheep anti-ciguatoxin antibody both found that the outer wall of a certain percentage of *G. toxicus* contained ciguatoxin and/or ciguatoxin-like compounds. Thus the ciguatoxin producer could be a very small organism dependent (or not) on certain epiphytic dinoflagellates such as *G. toxicus* and perhaps zooxanthellae. The findings that (i) ciguatoxin inhibits cellular multiplication of unicellular marine algae (Durand et al.,1985), (ii) coral death often seems necessary to induce

ciguatera production (Bagnis,1981), (iii) dead coral provides new surfaces for dinoflagellates implicated in ciguatera fish poisoning (Kohler & Kohler,1992) and (iv) the presence of CTX in dead corals with *G. toxicus* together suggest that the ciguatoxin producer could contribute to the death of living coral which could in turn enhance *G. toxicus* proliferation (Fig.5).

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