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FISHERIES RESEARCH AND DEVELOPMENT CORPORATION

Final Report to Project

"Experimental and Clinical Studies of Mannitol in the Treatment of Ciguatera"

by

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Queensland Department of Primary Industries, 1994

SUMMARY

Mannitol has been shown to be useful in the treatment of ciguatera. It is found to be most effective at reversing the neurological disturbances, particularly in severe cases when given early. The most important contraindication to the use of mannitol is dehydration. The response of patients to mannitol infusion is often dramatic, with some symptoms abating during the infusion. Other patients report a slower improvement, with some having a relapse about a day after treatment. In these latter cases, a second infusion of mannitol often has further beneficial effects. In Australia, not all ciguatera sufferers (<~25%) respond to the mannitol treatment. The reasons for a poor response of some people have not been determined. Despite these clear indications that mannitol is effective in humans, experimental models [mice, cats, piglets *in vivo* models and isolated phrenic nerve, heart (including human atria) and smooth muscle models] of human ciguatera did not reveal how mannitol acted to reverse the signs of ciguatera in humans. However, a number of likely modes of action have now been eliminated. Amongst informed medical practitioners mannitol has gained acceptance as the treatment of choice for acute ciguatera in Australia. This therapy has the ability to dramatically reduce the suffering and duration of ciguatera.

INTRODUCTION

Until the introduction of the intravenous mannitol therapy, treatment for ciguatera was only symptomatic and supportive. Mannitol was first used in the Marshall Islands (Palafox *et al.*, 1988). While not controlled or independently replicated, their initial study indicated that mannitol provided dramatic and sustained improvement for all of the victims of ciguatera treated in their study. Given the potential for the mannitol therapy to improve management of ciguatera in Australia, the present project was initiated with FRDC support to investigate the interaction between mannitol and ciguatera both clinically and experimentally.

OBJECTIVES OF ORIGINAL PROPOSAL

The objectives of the original proposal were to:

(i) Clinically assess the efficacy of mannitol in the treatment of ciguatera with the aim of providing an effective treatment for ciguatera;

(ii) Develop *in vivo* and *in vitro* models of ciguatera able to assess the protective effects of mannitol with the aim of optimising the use of mannitol in the treatment of ciguatera;

(iii) Determine the mechanism(s) underlying the protective effects of mannitol against ciguatera;

(iv) Use mannitol as a "lead" compound in the search for other potential therapeutic agents with the aim of further improving the treatment for ciguatera.

This project will refine the treatment regimen and facilitate adoption by Australian medical practitioners.

METHODS

CLINICAL STUDIES

Details on the approach and methodology used for the clinical studies are described in Appendix I.

EXPERIMENTAL STUDIES

Details on the approach and methodology used for the experimental studies are described in the Methods sections of Appendices II–VI.

RESULTS AND DISCUSSION

CLINICAL TRIALS

Our initial results with the use of mannitol as a therapy for ciguatera clearly indicated that mannitol is effective in cases of ciguatera. This work is described in Pearn *et al.* (1989). In summary, low doses of mannitol infused slowly (0.5 g/kg infused over 1 hour) were ineffective (a fortuitous control result), whereas a high dose (1.0 g/kg) infused over ~30 minutes provided dramatic improvement. Mannitol treatment is indicated when ciguatera is diagnosed on the basis of symptoms typical of ciguatera appearing within 24 hr of eating a suspect fish. The most important contraindication to the use of mannitol is dehydration. Patients must be adequately hydrated prior to any infusion of mannitol. The response of patients to mannitol infusion is often dramatic, with some symptoms abating during the infusion. Other patients report a slower improvement, with some having a relapse about a day after treatment. In these latter cases, a second infusion of mannitol often has further beneficial effects.

Additional conclusion from this study are that:

(i) early-treated cases respond best (first 1-5 days of the disease during the acute phase). Treatment of ciguatera appears most affective when given in the acute phase before the onset of recovery, particularly in severe poisoning episodes. We estimate that ~75% of such cases responds to mannitol. The mannitol treatment regimen for ciguatera is shown in Figure 1.

(ii) Many cases of ciguatera are still not treated with mannitol, either because cases are not brought to the attention medical practitioners in time, ciguatera is diagnosed but the mannitol therapy is not implemented through ignorance of considered medical opinion, or ciguatera is not diagnosed. Some patients prefer not to undergo the treatment despite the indicated benefits (fear of needles/infusions etc).

We investigated the possibility of conducting a double-blind clinical study of mannitol in Australia to assist with the rapid acceptance of the therapy by general practitioners. Unfortunately, such a trial is now viewed as withholding treatment. Also, the slow rate of victims accepting treatment would make such a trial protracted and costly. A double-blind clinical trial has been running for two years in Pacific Island countries severely affected by ciguatera but result are still not available. To increase awareness of this treatment, an updated ciguatera pamphlet (Appendix XIV) was mailed to all medical practitioners in Queensland (the cost of publication was met by the Queensland Fish Management Authority). Unfortunately, it is estimated than fewer than 20% of medical practitioners may have benefited from the mail-out (even now, only an estimated 15% of doctors are sufficiently aware of the disease to make a correct diagnosis).

EXPERIMENTAL STUDIES

We tested the interaction between mannitol and ciguatoxin both in whole animals and in isolated tissues. *In vitro* studies concentrated on the phrenic nerve (a motor nerve) following the discovery that ciguatoxin causes marked oedema of Schwann cells surrounding motor nerves (Allsop *et al.* 1990), as well as on rodent heart and smooth musculature and human heart tissue. *In vivo* studies have investigated mouse, cat and piglet *in vivo* models for ciguatera. The implications on the mannitol study of finding multiple ciguatoxins in moray eel have also been investigated.

Isolation, extraction and purification of ciguatoxin

Moray eels were purchased from the Kiribati. Toxic Queensland fish were collected following outbreaks of ciguatera. The extraction and purification were performed using procedures established during the FIRTA funded antibody project (Lewis *et al.*, 1991). Pure ciguatoxin-1 (as determined by HPLC) was used for all studies, except that partially pure ciguatoxin was fed to cats and given orally to mice.

Whole animal studies

Mice: Mice injected with ciguatoxin (i.p.) did not respond to i.v. or i.p. mannitol, irrespective of whether mannitol was given before, during or 15 minutes after ciguatoxin. This result is based on observation of signs, measurement of body temperature and death times. Mice were also dosed orally with ciguatoxin. Again, mannitol was without observed benefit.

In mice given a sublethal dose of ciguatoxin (i.p.) no growth was observed initially (0-4 days), but subsequent growth (4-30 days) was accelerated compared to controls. Mannitol (i.v., 15 minutes after ciguatoxin) did not influence either of these two phases. The details of these studies on mice have been published (Lewis *et al.,* 1993) and are given in Appendix III.

Cats: We commenced studies on cats following the apparent unsuitability of the *in vivo* mouse model for assessing mannitol. In collaboration with Dr W. Reynolds (Veterinary Sciences, University of Queensland) we assessed if mannitol can successfully treat laboratory cats fed ciguatoxin 24 hours previously. These studies indicate that cats do not receive any benefit from the mannitol therapy. Ethics approval was withdrawn when this conclusion was reached.

Piglets: We have commenced studies on the action of ciguatoxin on anaesthetised piglets in collaboration with Dr J. Tibballs (Royal Children's Hospital, Melbourne). Ciguatoxin is remarkably potent at arresting respiration in pigs. Mannitol given during the acute phase of ciguatoxin's effect in part reversed its effects on respiration only in one of three piglets trialled to-date. Mannitol had no effect on control piglets. Initial results indicate that ciguatoxin, at the doses affecting respiration, does not cause any oedema of Schwann cells surrounding peripheral nerves, as it apparently does in humans, suggesting that the pig model may also not be appropriate as a model for human ciguatera.

Studies on isolated preparations

Peripheral motor nerve studies: The rat phrenic nerve-diaphragm preparation was used to assess if mannitol can reverse the effects of ciguatoxin on myelinated peripheral motor nerves. We observed partial protection by mannitol against nerve block caused by ciguatoxin when mannitol was administered before but not after ciguatoxin. This result has been published (Wong Hoy and Lewis, 1992), and details are given in Appendix II. The basis for the protective action of mannitol remains to be established. As this protective effect of mannitol was only seen when mannitol was applied prior to ciguatoxin, this result can only partly explain the apparent ability of mannitol to treat ciguatera in humans. The rat preparation was used to assess if local anaesthetics can influence the effects of ciguatoxin. Such drugs have considerable theoretical attraction but none were able to reverse the effects of ciguatoxin in this model (Wong Hoy and Lewis, 1992).

We have also studied ciguatoxin's effects on the mouse phrenic nerve-diaphragm (Lewis *et al.*, 1993). Preparations removed from control and ciguatoxin injected mice were compared. Only at death did ciguatoxin impair nerve responsiveness. At this stage mannitol had a significant action to reverse the effect of ciguatoxin; however, it was concluded that such beneficial effects may relate only to mannitol's effects the terminal condition (acidosis etc) which contribute to death. In collaboration with Dr B. Campbell (Consulting Pathologist, Sullivan and Nicolaides, Brisbane) the ultrastructure of the phrenic nerve was investigated to determine if oedema is associated with reduced nerve responsiveness following ciguatoxin injection. These preliminary studies did not reveal such an oedema (Lewis *et al.*, 1993). Taken together, these studies indicate that the lethal effects of ciguatoxin are of central origin.

Cardiac tissue studies: The initial finding on guinea-pig atria was that mannitol could reverse ciguatoxin-induced positive inotropy (Lewis, 1988). Unfortunately, we could not repeat these results in more recent experiments. We subsequently investigated the potential for mannitol to reverse ciguatoxin effects in human heart tissue in collaboration with Dr D. McGiffin (Prince Charles Hospital, Brisbane), and in mouse atria. The conclusion from these studies is that mannitol is unable to reverse the effects of ciguatoxin in human (Lewis *et al.*, 1992; Appendix IV) or mouse atria (unpublished results).

Smooth muscle studies: Assessment of the interaction of mannitol and CTX in vas deferens revealed unexpected actions of ciguatoxin that required further investigation before such preparations could be used as a model for ciguatera. These studies indicated that ciguatoxin causes only indirect effects on the contractility of smooth musculature, and previously reported direct effects were absent. Mannitol was able to reverse the effects on contractility on the vas deferens but only at unphysiologically high concentrations (100 mM) but not at 50 mM (the maximum peak concentrations expected clinically).

Implications of finding multiple ciguatoxins in moray eels: As part of a previous FIRTA project, we found that moray eels are contaminated with multiple ciguatoxins. A possible explanation for the lack of benefit of mannitol may be that we were using the wrong ciguatoxin (mostly ciguatoxin-1) in these studies. This would be especially significant if the three major ciguatoxins had significantly different pharmacology. These possibilities were excluded since (a) we found that Queensland fish have a similar ratio of the three ciguatoxins as is found in moray eels and these ciguatoxins were the major toxins present in Queensland fish (Lewis and Sellin, 1992; Appendix V) and (b) these ciguatoxins had quite similar (though not identical) modes of action on isolated smooth, cardiac and neural tissues (Lewis and Wong Hoy, 1993; Appendix VI).

CONCLUSIONS

Five major conclusion are forthcoming from this study. Conclusions (i)-(iv) relate to the project objectives, while conclusion (v) is a general conclusion from the study.

(i) Clinical trials emphasised the importance of early treatment. A double-blind clinical trial was not considered feasible. Though acceptance of the treatment has increased, most ciguatera victims are not treated with mannitol. Most medical practitioners are not aware of ciguatera and the possibility to treat it successfully with mannitol.

(ii) Several potential *in vitro* and *in vivo* models of ciguatera were investigated. These studies indicate that mannitol (a) does not directly displace ciguatoxin from it site of binding on the sodium channel, (b) does not chelate ciguatoxin directly and (c) is unlikely to be acting as an hydroxyl free-radical scavenger. Unfortunately, these models did not reveal a significant effect of mannitol. It is possible that the effects of ciguatoxin that mannitol acts upon are unique to primates.

(iii) The precise mechanism of the beneficial action of mannitol in cases of ciguatera remains to be elucidated. The most likely explanation is that mannitol reverses an oedema of Schwann cells that has been observed in persons severely affected with ciguatera. Hyperosmotic mannitol, through its water drawing action would reduce this cell swelling and thereby effectively reverse the course of this often distressing and debilitating disease. To explain the long term effectiveness of mannitol it is hypothesised (i) that mannitol prevents long-term nerve damage (eg. lesions, anoxic zones) which is probably a sequelae of this oedema and (ii) that ciguatoxin is relatively quickly excreted, remaining bound only for a few days. Consequently, the longer-term effects of ciguatoxin relate to nerve damage. These hypotheses also explain why mannitol would be most effective when given early in the course of the illness.

(iv) Because of our inability to develop a good animal model for mannitol treatment of ciguatera, we were not able to use mannitol as a "lead" compound to assess the potential of other drugs (in particular the local anaesthetics and other orally active drugs) as potential therapeutic agents for the treatment of ciguatera.

(v) Further studies are required to find *in vivo* models more appropriate for the study of ciguatera in humans. Orally effective treatments for ciguatera would gain greater acceptance than the intravenous mannitol therapy.

PUBLICATIONS FROM THE STUDY

Six publication were forthcoming from this study. These are given in Appendices I–VI.

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APPENDICES

Appendices I–VI are journal publication forthcoming from this study. Appendices VII–XI are additional recent publications by QDPI that relate to the treatment of ciguatera. Appendix XII is the trip report, Appendix XIII is the distrubuted pamphlet on ciguatera and its treatment, Appendix X1V is the original grant application to FIRTA, and Appendix XV is the proceedings of the Ciguatera Management Workshop, Bribie Island 1993. This latter publication contains a number of articles on the mannitol therapy and ciguatera clinically.

Appendix I

Ciguatera and mannitol: experience with a new treatment regimen

Ciguatera and mannitol: experience with a new treatment regimen

John H. Pearn, Richard J. Lewis, Tilman Ruff, Michael Tait, John Quinn, William Murtha, Geoffrey King, Anthony Mallett and Noel C. Gillespie

ABSTRACT **Ciguatera is a distressing, hitherto-untreatable and not rare disease which results from the eating of ciguatoxin-contaminated fish from tropical and subtropical waters. We report here the results of a pilot study to assess the efficacy of mannitol therapy in ciguatera poisoning. Twelve adult patients (six men) have been treated, five of whom — who were ill acutely — experienced a significant benefit from this therapy, in three cases, with a hitherto-unexperienced dramatic reversal of symptoms. We conclude that an intravenous infusion of 1.0 g/kg of mannitol which is given over 45 minutes, after rehydration if required, can be of significant benefit to at least some acutely intoxicated victims. We postulate either a reduction of axonal oedema, or a scavenger effect, or both, as the mechanism of the beneficial effects of mannitol. Ciguatoxin is rich in hydroxyl groups, and causes microscopic oedema of neural tissue. If our conclusion of the beneficial effects of mannitol therapy is confirmed, this will offer the first effective therapy for acute phases of this disease, and has promise of preventing much long-term morbidity.** **(Med J Aust 1989; 151: 77-80)**

Ciguatera poisoning is a rare,[1] distressing,[2] and enigmatic intoxication which results from the eating of certain fish that are associated with coral reefs.[3] Its pleomorphic clinical effects include gastrointestinal,[1,2] neurological,[1,4] cardiac and other manifestations.[1,3] It presents in diverse ways and the clinical features are of a variable duration. A typical severe case manifests a subacute or chronic course. A distressing recrudescence of symptoms can occur after inadvertent rechallenge with the offending ciguatoxin.[1] Recent experimental studies have demonstrated that the toxic effects are a result of the opening of sodium channels in the cell membranes of excitable tissue[5] such as nerve, cardiac[6,7] and skeletal-muscle cells.[1,8]

No effective treatment hitherto has existed for this condition. Victims experience a self-limiting (but distressing) subacute or chronic course which may be incapacitating. In the past, calcium supplements,[1] amitriptyline,[9] lidocaine analogues[10] and other drugs[1] have been suggested as helpful therapy, but those which have been assessed independently have been proved to be disappointing in practice. None of these drugs has been subjected to a controlled clinical trial. Folk-medicine cures abound in many parts of the world.

At the start of this pilot study, a then unpublished report from colleagues in the Marshall Islands had suggested that an intravenous infusion of mannitol (0.5 g/kg to 1.0 g/kg over 30 minutes) was beneficial in reversing the acute clinical symptoms permanently. These results now have been published.[11] One of the authors (R.J.L.), while pursuing laboratory studies, had demonstrated an apparent modification of the cardiotoxic effects of ciguatoxin by mannitol with isolated guinea-pig atrium as the test system.[12]

We report here our experience with seven subacute cases of ciguatera poisoning that were treated with a low-dose infusion of mannitol, and with a further five acute cases of this condition which we believe have been treated successfully.

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Methods

Cases

The Department of Primary Industries' Fisheries Research Branch, together with one of the authors (J.H.P.) had written in early 1987 to selected doctors in high-risk areas, seeking expressions of interest in a collaborative pilot study on the efficacy of mannitol therapy in human ciguatera poisoning. Twelve cases were treated collaboratively after this overture. The 12 cases occurred in six clusters of ciguatera poisoning. The cases were not consecutive, and a significant degree of self-selection (by both patients and doctors) was inescapable.

Ethics

The nature of the study was explained fully to each adult patient who was poisoned. No children were included in any of the pilot studies. The open-ended nature of the possible results (neutral, positive, or negative) was explained. All except one patient signed a consent form, and were informed that they were free to withdraw at any time, without prejudice. Besides the 12 patients who are reported here, a further five patients were approached to be subjects in this study, but they declined. The project was approved by the Royal Children's Hospital and Princess Alexandra Hospital's Ethics Committee.

Dosage

Initially, we used a cautious dose of 0.5 g/kg of mannitol (a 20% solution in three cases, Cases 1–3, and a 10% solution in the three Victorian cases, Cases 5–7; Osmitrol, Travenol Laboratories, Sydney) which was administered intravenously over one hour, after the correction of any dehydration with a 4.0% glucose in 0.18% saline solution. In the last four cases (Cases 9–12), we employed a dose of 1.0 g/kg of mannitol which was administered intravenously over 45 minutes, and repeated this within 24 hours in two cases (Cases 10 and 11).

Clinical records

The case details are summarized in the Table. Some illustrative features of some of these cases are noted below.

Case 1

An 18-year-old woman was one of 30 adults who was poisoned after a Rugby League party with a fish course that was held at Hervey Bay in Queensland. Twenty-one days after the initial intoxication, she still was lethargic and weak subjectively, had headaches, and complained of persistent itchiness and dysaesthesiae. Two infusions of mannitol (on Day 21 and Day 24) were administered without any subjective or objective decline in symptoms. Review on Day 47 revealed that all nausea had disappeared and that the patient subjectively was much improved. This time-course did not differ significantly from the convalescence of other patients who were not treated with mannitol in this cluster of cases.

Cases 5, 6 and 7

These cases were three of five Fijian men who were poisoned on October 11, 1987. They were admitted to Fairfield Infectious Diseases Hospital on October 12, 1987. All worked on board a gas-transport freighter which had left Suva, Fiji, on September 21, 1987, and arrived at Western Port Bay, near Melbourne on October 12, 1987.

The fish that was implicated in this outbreak of ciguatera was *Lutjanus bohar* or the red bass, which is known in Fiji as "bati damu". *Lutjanus bohar* is recognized as one of the most-toxic fish species in the Pacific, and for this reason is not accepted for sale by the Queensland Fish Board.[1] Its sale also is banned at the main fish market in Suva, but it commonly is sold at other outlets in that city.

The fish in question had been purchased at a supermarket in Suva before the ship's departure and had been kept frozen on board the ship. Three times a week the crew had eaten various other fish that had been stored in a similar manner without suffering any ill effects. On the afternoon of October 11, 1987, four reef fish including two *Lutjanus bohar* (each of which weighed about 2 kg) were thawed and then were boiled by the cook who prepared a fish stew. The cook ate no "bati", and nine other crew members also consumed none, having picked the bati out of the stew carefully. Another crew member who previously had suffered from ciguatera poisoning tasted the stew and decided to eat no more. In fact, he developed only mild dysaesthesiae of the fingers and was not admitted to hospital.

The four crew members who ate substantial proportions of the bati stew all developed the classic symptoms of ciguatera poisoning. One of the four

TABLE: Summary of 12 clinical cases of ciguatera poisoning, which were treated with mannitol infusions during the symptomatic phase*

ase no.	Age (years)/sex	Symptoms/signs	Time between ingestion of toxic fish and mannitol infusion	Dose of mannitol	Comments
1	18/F	Lethargy, itchiness, headaches, dysaesthesiae	21 d	0.5 g/kg (20% solution), infused over 1 h	Consumed fish from Hervey Bay, putatively (but unconfirmed to be) the blotched javelin fish. No change in symptoms over ensuing 2 d. Significant recovery eight weeks after ingestion of toxic fish
2	24/F	Abdominal cramps, itching, burning hands, myalgia, headache	5 d	0.5 g/kg (20% solution), infused over 1 h. Second infusion given	Same batch of fish as in Case 1. No change within 12 h after mannitol infusion. Progressive reduction of symptoms over ensuing days
3	21/F	Abdominal pain, myalgia, headaches, perioral parasthesiae. Dental pain, burning sensation from cold water on skin	4 d	0.5 g/kg (20% solution), infused over 1 h. Second infusion given	Same batch of fish as in Case 1. No change within hours of mannitol infusion. Symptoms declined gradually over ensuing 24-h period, after a second infusion of mannitol
4	38/F	Vomiting, diarrhoea and dehydration. Muscle weakness. Numbness and dysaesthesiae of hands, feet and mouth. Headaches, dizziness, sweating and tremor	5 d	0.5 g/kg (20% solution), infused over 1 h. Second infusion given	Consumed a portion of narrow-barred Spanish mackerel — part of an epidemic of over 60 cases of poisoning. No immediate improvement. Over subsequent 4–5 d perioral paraesthesiae disappeared. Other symptoms disappeared slowly over many days
5	31/M	Diarrhoea, anorexia, limb pain, weakness, dysaesthesiae, headache	29 h	0.5 g/kg (10% solution), infused over 1 h	Consumed fish stew of *Lutjanus bohar* (red bass), caught in Fiji. Mild and transient elevation of levels of: alanine aminotransferase, 75 U/L (normal, 10–40 U/L); γ-glutamyl transpeptidase, 65 U/L (normal, less than 50 U/L); creatine phosphokinase, 165 U/L (normal, less than 50 U/L); and erythrocyte sedimentation rate, 71 mm/h in the first hour (normal, 3–5 mm/h). No immediate effect of mannitol infusion. Symptoms resolved gradually over the ensuing 6 d at which time recovery was complete
6	26/M	Abdominal pain, diarrhoea, vomiting, faintness and vertigo, generalized aching, headache, dysaesthesiae, lethargy. Sinus bradycardia (55 beats/min)	30 h	0.5 g/kg (10% solution) infused over 1 h	Poisoned by *Lutjanus bohar*. Erythrocyte sedimentation rate, 34 mm/h. One litre of normal saline administered over 1 h before infusion of mannitol. Over the ensuing 24 h, some progression of proximal muscle weakness (of neck flexion, shoulders and hips); areflexia persisted. Symptoms resolved gradually over ensuing days, resolving completely by Day 5 when deep tendon reflexes had returned
7	22/M	Restlessness, dizziness, vertigo, skeletal pain, dysaesthesiae. Weak (unable to stand)	29 h	0.5 g/kg (10% solution), infused over 1 h	Consumed *Lutjanus bohar* fillet in fish stew (same poisoning cluster as Cases 5 and 6). Alanine aminotransferase level, 55 U/L. No immediate effect of mannitol infusion. Gradual resolution of symptoms over the ensuing 5 d by which time convalescence was complete
8	37/F	Dyspnoea, muscle weakness, numbness of limbs, numbness and paraesthesiae of face, lips and tongue. Vomiting and diarrhoea. Itching, abnormal sweating, abnormal temperature perception, headache and prostration	27 h (15 h after onset of symptoms)	1.0 g/kg (20% solution), infused over 1 h, followed by 1 L of saline with 20 mmol of potassium chloride	Consumed two bowls of raw, marinated Spanish mackerel. Family cat also affected very severely. Significant decline in symptoms during mannitol infusion. Patient asymptomatic 5 h after infusion of mannitol ceased. Cat not treated with mannitol, remained ill, under veterinary care, for a further 4 d
9	28/M	Abdominal pain, vomiting, abnormal temperature perception, perioral paraesthesiae, shivering, bradycardia and hypotension	10–12 h (10 h after onset of symptoms)	0.5 g/kg (20% solution), infused over 30 min, preceded by rehydration with 500 mL of 4% glucose in 0.18% saline solution	Consumed an entire redfin (caught on reef off Bremer Island). Considerable symptomatic improvement over 30 min, with exception of perioral tingling. Discharged from hospital after 36 h. Readmitted 3 d later, after symptoms had redeveloped gradually — perioral paraesthesiae persisted for a further two weeks
10	37/M	Sensation of burning of skin, especially of penis. Perioral numbness — sensation of tongue swelling. Headache, reversal of temperature perception. Diarrhoea, itchiness of upper torso. Severe arthralgia of all upper limb joints. Tremor	5.5 d	1.0 g/kg (10% solution), infused over 45 min, followed by glucose/saline. Second infusion, 24 h later	Ate 10 meals of a large coral trout over 4 d. Immediate reduction of symptoms during mannitol infusion (but no effect during preceding glucose/saline rehydration). Arthralgia, itchiness and headache disappeared within 1 h. Tremor decreased. All remaining symptoms (except for paraesthesiae) disappeared within 1 h of the second infusion

TABLE (cont.): Summary of 12 clinical cases of ciguatera poisoning, which were treated with mannitol infusions during the symptomatic phase*

Case no.	Age (years)/sex	Symptoms/signs	Time between ingestion of toxic fish and mannitol infusion	Dose of mannitol	Comments
11	21/M	Headaches, nausea, abdominal pain and diarrhoea	8 d	1.0 g/kg (20% solution), infused over 45 min, preceded and followed by glucose/saline. Repeat infusion, 24 h later	Ate a meal of large coral trout (same fish as in Case 10). All symptoms disappeared during mannitol infusion; symptoms recurred over ensuing 12 h. All symptoms disappeared within 12 h of second infusion
12	39/F	Bursting headache, nausea and vomiting, profuse diarrhoea, severe arthralgia, muscle cramps, chills and fever and "felt she was going to die"	7 h	1.0 g/kg (20% solution) infused over 45 min. No other rehydration required.	Patient ate a meal of a large reef "stripey", and another unidentified fish, caught off Southern Cross Reef, near Bowen. Symptoms commenced 6 h later. Two of her children affected also and three other friends, all affected mildly. Dramatic reduction of symptoms at the end of the infusion time. All joint pains gone within 30 min. Headache and nausea ceased within 2 h after infusion ceased

*Cases 1–4 occurred as one poisoning cluster in Hervey Bay, Queensland; Cases 5–7 occurred as one poisoning cluster on a Fijian fishing-boat; Cases 10 and 11 were both a result of eating one reef fish (a large coral trout), which was caught near Port Douglas.

members, a 48-year-old man, suffered an illness that was characterized by vomiting and watery diarrhoea, skeletal pains and dysaesthesiae which was precipitated by the exposure of his hands to cold water. He had recovered completely within 24 hours and it was felt that no therapy was indicated. He served as a fortuitous "control" subject for the interpretation of the convalescence of his three poisoned colleagues.

The three other victims of ciguatera poisoning all were significantly symptomatic when admitted to hospital approximately 24 hours after ingesting the fish, and all gave informed consent to a trial of mannitol infusion. Each of these three patients also received amitriptyline by mouth in two doses of 25 mg each; the initial dose was given eight to 10 hours after the mannitol infusion had been completed, and the second dose was given 12 hours after the first dose.

Patient 7 serves as a representative of these three subjects. He was a 22-year-old Fijian-born Indian deck-officer, who previously had been in excellent health. At 3.50 p.m. on October 11, 1987, he had consumed a belly fillet of "bati" together with yams. By 10.00 p.m. he had developed pain in both knees and both ankles. On October 12, 1987 at 1.00 a.m. (eight hours after ingestion), he felt restless, and became dizzy and vertiginous with movement. Drinking a glass of cold water at this time resulted in an uncomfortable sensation that his tongue had been "poked".

By 9.00 p.m. on October 12, 1987, he had noticed increasing skeletal pain (in the thighs, calves, shoulders and pectoral areas). The dysaesthesiae had persisted in his tongue, and he felt weak and cold, and was unable to leave his bed. His symptoms declined gradually later that day except for the dysaesthesiae which involved his tongue and feet (walking bare-foot produced a feeling of walking on needles).

On examination at 8.00 p.m. on October 12, 1987, he was alert with a regular pulse of 80 beats per minute; supine and erect blood pressures of 140/80 mmHg and 120/100 mmHg, respectively; an oral temperature of 37.0°C; and a vital capacity of 4.3 L. His weight was 62 kg. Ankle dorsiflexion resulted in bilateral calf pain. His neck flexion was weak and mild proximal weakness was present in both upper and both lower limbs; the patient was unable to stand from the sitting position without using his arms. Bulbar function, reflexes, coordination and gait were normal. Dramatic dysaesthesiae was produced by touching ice to his finger-tips.

Investigations included serum sodium, potassium, urea, creatinine, calcium, inorganic phosphate and C-reactive protein levels, and the erythrocyte sedimentation rate, which all were normal. The alanine aminotransferase level was elevated minimally at 55 U/L but other liver-function tests gave normal results. The creatine phosphokinase level was elevated minimally at 58 U/L (normal range, less than 50 U/L). Both these abnormalities were transient. He had a mild peripheral eosinophilia (1.4×10^9/L; normal range, $0.04–0.4 \times 10^9$/L).

Mannitol was infused 29 hours after the ingestion of the toxic fish, and 24 hours after the onset of symptoms. He also received subsequently a single by-mouth dose of 60 mg of codeine and 1 g of paracetamol, in addition to 25 mg of amitriptyline. No subjective or objective change occurred within eight hours after the mannitol infusion. Over the ensuing days, his symptoms declined gradually, and the muscle weakness and dysaesthesiae had disappeared by Day 5 of his illness.

Case 10

This 65-kg 37-year-old bricklayer from Mossman, Queensland, had consumed 10 meals from a very-large coral trout, over a period of four days. Within 36 hours of the first fish meal, he developed dramatic penile pain, which subsequently was made worse by urination (a recognized symptom). He progressively became incapacitated, and developed a numb mouth, headache, reverse thermal sensation, severe arthralgia of both shoulder-joints and all upper-limb joints, itchiness of the upper torso, diarrhoea, and a tender abdomen.

While these symptoms were evolving, he (naïvely) continued to consume the offending fish fillets (nine further pieces) on a further two occasions. Within 96 hours of the onset of the first symptoms he was prostrated, and weeping with the penile pain that was exacerbated by micturition. After an infusion of 4.0% glucose in 0.18% saline solution (with no effect) he was given 1.0 g/kg of mannitol by the intravenous route over 45 minutes, with an immediate and dramatic decline in symptoms.

During the infusion, his headache, arthralgia and itchiness disappeared. The pain on micturition was reduced dramatically. Perioral dysaesthesiae were reduced but still were present. Some symptoms reappeared over the ensuing 12 hours. A second infusion, which was given 24 hours after the first, resulted in the loss of all symptoms except perioral dysaesthesiae. He was discharged from hospital and subsequently has remained well.

Discussion

Ciguatoxin opens the voltage-dependent sodium channels in the cell-membranes of excitable tissues. At the histopathological level, striking changes have been observed with marked oedema of the axonal Schwann-cell cytoplasm.[4] Therefore, the use of mannitol — an osmotic diuretic agent — in ciguatera poisoning has some theoretical attraction.

Persuasive evidence exists to suggest that mannnitol may exert some of its effect as a scavenger of hydroxyl radicals.[13] The ciguatoxin molecule is known to possess a number of hydroxyl groups. Whether the putative therapeutic effects of mannitol act by way of this mechanism as a scavenger molecule, or are related to its osmotic effects, must remain conjectural. Such possibilities open the way for further in-vitro experimentation.

However, the clinical use of mannitol does have some potential hazards, as numbers of patients are dehydrated as a result of the vomiting and diarrhoea which may be part of the clinical syndrome,[1] and others may be hypotensive from vasomotor and/or cardiac involvement. In spite of the widespread and extensive clinical use of mannitol to reduce raised intracranial pressure, and its obvious safety, we felt it prudent to proceed cautiously in this open-ended pilot study. Mannitol is believed to alter microcirculatory dynamics and to decrease cerebrovascular resistance, in addition to its water-drawing (osmotic) effects.[14]

The "fortuitous" occurrence of seven poisoned victims in one cluster, with three treated (Cases 5, 6 and 7) patients and the rest acting as control subjects, has provided one important piece of negative evidence. That is, a single low dose (0.5 g/kg) of mannitol that is infused slowly (over one hour) in long-standing cases (symptoms for longer than 24 hours) does not appear to be effective. Such

patients (seven cases) who were treated in this way in our study did not experience an accelerated convalescence when their cases were considered with those of all the other poisoned victims in an overall perspective.

We are heartened by the apparently dramatic response of five of our acutely ill patients to a higher-dose (1.0 g/kg) infusion of mannitol. We are very conscious of the claims for mannitol therapy in other diseases (multiple sclerosis, for example[15]) in which it has proved to be disappointing as a practical form of therapy, and of the dangers in a non-blinded clinical study where symptoms essentially are subjective and where several patients may be affected simultaneously.

However, as a pilot study, we are impressed with the clinical responses of these five of our acutely ill patients who were suffering a hitherto-untreatable illness. We are embarking on a single-blind clinical trial, as a next step, in addition to experimental studies. Our findings support those of Palafox et al. in the Marshall Islands.[11]

The distribution of mannitol after an intravenous infusion results in a two-compartment system. In patients with normal renal function, mannitol doses of up to 1.0 g/kg (which are given as a rapid infusion, that is, in less than 30 minutes) are likely to produce a maximal increase in osmolality of 10–20 mosmol/kg.[16] Peak concentrations, physical osmotic effects and between-compartment distributions vary with different infusion rates and dosages.[16,17]

We believe that an infusion of 1 g/kg of mannitol which is administered over a time-course of not less than 30 minutes (after rehydration, if indicated) is a safe procedure for victims of ciguatera poisoning. A second infusion may be indicated. Our experience is that this will be of benefit to at least some patients, if it is administered in the acute phase of the intoxication.

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Appendix II

The effect of potential therapeutics on ciguatoxin inhibition of the rat phrenic nerve

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THE EFFECT OF POTENTIAL THERAPEUTICS ON CIGUATOXIN INHIBITION OF THE RAT PHRENIC NERVE

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INTRODUCTION

Ciguatera fish poisoning, caused by the consumption of fish that have been contaminated with a class of toxins called ciguatoxins, produces a variable clinical syndrome in poisoned individuals. Typically, neurological symptoms are manifested, although gastro-intestinal and cardiac disturbances may also present. Cases of ciguatera poisoning were first successfully managed in the Marshall Islands with an intravenous infusion of mannitol (Palafox *et al.*, 1988). Later, in an Australian study, Pearn *et al.* (1989) indicated that apparent reversal of the acute clinical signs and symptoms could be attained by intravenous infusion of a 20% mannitol solution (1.0 g/kg over 30 minutes), with lower doses proving less effective. In view of the potential for reversal of the clinical manifestations of ciguatera, the present work was done to reveal any pharmacological bases for interactions between the major ciguatoxin and potential therapeutics including mannitol *in vitro*.

MATERIALS and METHODS

Hemi-diaphragm preparations

Male Wistar rats (250-350g) were stunned by a cephalic blow and bled. The left hemi-diaphragm was excised with its phrenic nerve intact and immediately placed in a Krebs-Ringer solution of the following composition (mM): NaCl, 135.0; KCl, 5.0; $CaCl_2.2H_2O$, 2.0; $MgCl_2.6H_2O$, 1.0; KH_2PO_4, 1.0; $NaHCO_3$, 15.0; glucose, 11.0. Each hemi-diaphragm was trimmed to approximately 15 mm width. Dissection was completed under continual aeration with carbogen (95% O_2/5% CO_2). Preparations were suspended in a 5.0 ml organ bath containing Krebs-Ringer solution, subjected to a resting tension of 9.8 mN, gassed with carbogen and maintained at 37°C. Experimental protocol was performed as described in a short account of part of this work (Wong Hoy and Lewis, 1990). Briefly, alternating electrical shocks were delivered from a Grass stimulator to preparations at 0.1 pulse/sec, indirectly via the phrenic nerve (at 0.2 msec pulse duration and at 150% threshold stimulation voltage) and directly to the diaphragm musculature (at 2.0 msec pulse duration and at voltage sufficient to cause twitch responses of similar height to those produced via indirect stimulation). Contractions were measured via isometric force displacement transducers (Narco Biosystems) with recordings produced on a dual channel Goerz Metrawatt chart recorder. All drug

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concentrations refer to when in the organ bath solution. Preparations were equilibrated for at least 15 min prior to testing.

Effects of cumulative addition of ciguatoxin (9×10^{-14} - 2.3×10^{-10} M) and of single dose application of the toxin were recorded. Mannitol at 25-50 mM was added 30 min before or after exposure to ciguatoxin (2.3×10^{-10} M). Local anaesthetic drugs (10^{-8} - 10^{-5} M) were added after inhibition by ciguatoxin had reached a steady level (~ 30 min). Cumulative concentrations of each of these drugs were added at 15 min intervals or until a new twitch height had stabilized. In some experiments, responses to brief intervals (1-2 sec) of indirect stimulation at pulse frequencies of 0.1, 20, 50 and 100 Hz were recorded. Student's *t*-test was used for statistical analysis of results with level of significance $P < 0.05$. Results are presented as mean ± standard error with n= 4 (unless otherwise indicated).

Ciguatoxin

Acetone extracts from the liver and viscera of moray eels were subjected to two successive liquid-liquid partitionings and four low pressure column chromatographic steps. Ciguatoxin-1 (CTX), the major toxin of the class of ciguatoxins was finally purified by reverse phase H.P.L.C. This material was estimated to be 95% pure by H.P.L.C. monitored at 215 nm. The material had a LD_{50} of 0.25 μg/kg and a MH^+ m/z of 1111.6 (Lewis *et al.*, 1991). Alliquots of a methanol:water stock solution of CTX were diluted with water and sonicated before addition to the bath.

Drugs

The following drugs were used: quinidine, lidocaine, benzocaine, diphenylhydantoin, aminoacridine hydrochloride (Sigma Chemical Co., St. Louis, MO); phentolamine hydrochloride (Regitine) (Ciba-Geigy, Sydney, Australia); tocainide hydrochloride (Astra pharmaceuticals, North Ryde, Australia); mannitol (A.R. grade) (Ajax Chemicals, Auburn, Australia). Local anaesthetics were prepared in aqueous stock solutions before dilution and use. Phentolamine stock was prepared immediately prior to use of the drug in the organ bath. Mannitol (50 and 100mM) was prepared as an aqueous stock solution prior to its addition to the organ bath as a hypertonic solution.

RESULTS

Effects of ciguatoxin

Cumulative addition of ciguatoxin (CTX) producing concentrations of 9×10^{-14} M - 1.4×10^{-10} M did not significantly inhibit twitches elicited via indirect stimulation (0.1 and 50 Hz) (Fig. 1). However, CTX at a cumulative concentration of 2.3×10^{-10} M did cause significant inhibition of twitches (50-60%). An equivalent concentration of CTX applied as a single dose (2.3×10^{-10} M) caused even greater inhibition of

twitches produced by indirect stimulation at 0.1 Hz ($P < 0.05$) (cumulative 47.5 ± 10.7% (n = 11) cf. single dose 78.2 ± 6.3% (n = 13) after 1 hr). This single dose of CTX (2.3×10^{-10} M) caused equivalent inhibition of twitches at frequencies of 0.1, 20, 50 and 100 Hz (see Fig. 2). The onset of inhibitory effects was typically delayed by 10-15 min before progressive abolition of the response. Whilst CTX invariably reduced twitch height, in some preparations the frequency of twitches also became irregular, producing occasional lapses in response before its total abolition. Twitches elicited via direct stimulation of the diaphragm musculature (at 0.1 Hz) were not significantly affected when CTX was added either cumulatively (Fig. 1) or as a single dose (data not shown). In most preparations, an initial increase in twitch height (4.9 ± 1.1%) at 0.1 Hz was observed soon after CTX had been applied ($P < 0.05$; n = 20). Muscle fasciculations were clearly observed 7-20 min after the addition of CTX in three preparations and these events preceded inhibition. Washing of preparations for up to 1 hr did not reverse the inhibition of twitch responses. Twitches diminished more rapidly (a 50.9 ± 5.5% loss within 15 min) in preparations washed 10 min after onset of CTX inhibition than in preparations not washed at that time (29.8 ± 5.4% loss within 15 min; $P < 0.05$) (see Fig. 3).

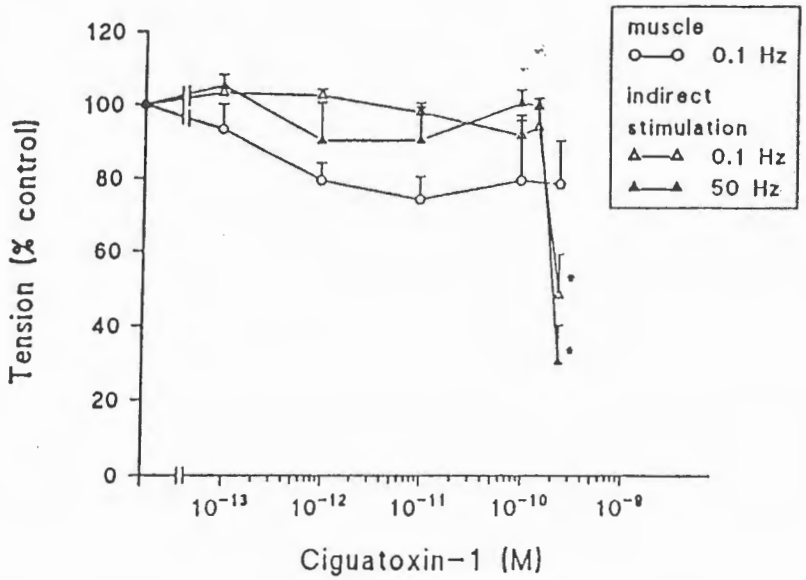

FIG. 1 THE EFFECT OF CUMULATIVE ADDITION OF CIGUATOXIN (CTX) ON THE RAT PHRENIC NERVE-DIAPHRAGM. Data are presented as mean percentages + 1 S.E. (for direct stimulation of muscle, n = 4; for indirect stimulation via the nerve, n = 6-11). Note the steep inhibitory inflection from 1.4×10^{-10} M CTX at either low (0.1 Hz) or high (50 Hz) stimulation rate. Significant difference from control is indicated by * ($P < 0.05$).

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Effect of mannitol

Pre-exposure of preparations to mannitol (50 mM) caused significant protection of the twitch response produced by indirect stimulation (0.1 Hz) against a single dose of CTX (2.3×10^{-10} M) (Fig. 2). Whereas CTX alone caused approximately 80% inhibition of twitch height after 1 hr exposure, prior addition of mannitol significantly reduced inhibition caused by CTX to 46.3 ± 5.0% ($P < 0.05$). No significant initial increase in twitch response was observed after addition of CTX in preparations that were pre-treated with mannitol. CTX inhibition at other stimulation frequencies (20-100 Hz) was not significantly affected by prior exposure to mannitol. Further, the percentage inhibition by CTX after mannitol treatment did not differ significantly between these higher stimulation frequencies. Mannitol itself slightly reduced the control twitch height (to 82.1 ± 4.2%; n = 7) initiated by 0.1 Hz indirect stimulation but had no effect on twitches produced by 20-100 Hz stimulation (Fig. 2). Responses to direct stimulation were also unaffected by mannitol.

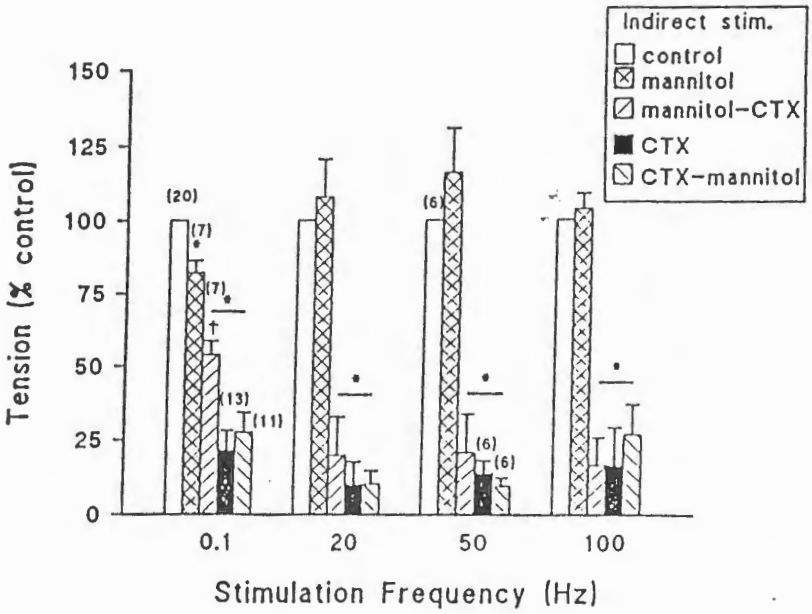

FIG. 2 CIGUATOXIN INHIBITION OF TWITCH RESPONSES PRODUCED BY VARIOUS STIMULATION FREQUENCIES. Mannitol (50 mM) was applied either before or after 60 min exposure to 2.3×10^{-10} M CTX. Control bars were constructed separately for respective frequency groups. Data are presented as mean percentages + 1 S.E. Significant difference from control ($P < 0.05$) is indicated by * or $\underline{*}$ (for a group of data). † denotes significant difference from CTX treatment ($P < 0.05$). No significant restoration of inhibited twitches was observed with post-application of mannitol. Four experiments were performed for each treatment unless otherwise indicated in parenthesis.

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Figure 3 illustrates the significant protective effect of mannitol over time. CTX almost abolished the twitch response at 80 min (12.6 ± 6.2% of the control response remained) whereas with mannitol applied 30 min prior to CTX addition, 39.3 ± 8.8% of the twitch response persisted. Indeed, the time to 50% inhibition was delayed significantly in those preparations that had been pretreated with mannitol (57.6 ± 3.7 min) ($P < 0.05$) as compared with those treated with CTX alone (37.3 ± 3.8 min).

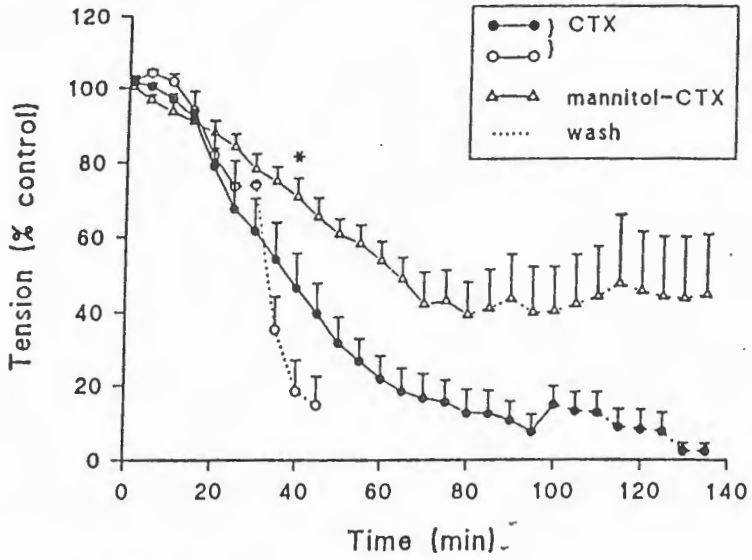

FIG. 3 TIME COURSE OF EVENTS ASSOCIATED WITH CIGUATOXIN INHIBITION OF TWITCH RESPONSES AND WASHOUT. Mannitol (50 mM) was applied 30 min before exposure to 2.3 x 10^{-10} M CTX in preparations stimulated at 0.1 Hz (Δ-Δ, n = 7). Mannitol-CTX curve is constructed using the twitch height after mannitol inhibition as control. Washing of preparations is indicated by (....). In some preparations, washing began after a brief exposure to CTX (10 min after onset of inhibition, o-o ; n = 6). Data are presented as mean percentages + 1 S.E. In those preparations pre-treated with mannitol, twitch responses became significantly different (*) from respective values for CTX alone after 60 min (●-●, n = 13; $P < 0.05$). Tension at 100% is equivalent to 3.2 ± 0.3 g weight (n = 20).

Washing of preparations exposed to CTX (and pre-treated with mannitol) did not cause further reduction of twitch responses. In contrast with the protective effect of mannitol produced when applied prior to CTX (at 0.1 Hz), no reversal of established CTX inhibition was apparent with post-application of either 50 mM mannitol (Fig.4) or 25 mM mannitol (data not shown). Similarly, CTX inhibition was not reversed by mannitol at any other stimulation frequency (see Fig. 2).

Effect of drugs with local anaesthetic activity

Twitch responses produced via indirect stimulation and inhibited by CTX (2.3 x 10^{-10} M) were not restored to any significant extent by the local anaesthetics tested (10^{-8} - 10^{-5} M) (Fig. 5). No significant

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difference was recorded between treatments using local anaesthetics. Control preparations (no CTX) were similarly unaffected by these drugs.

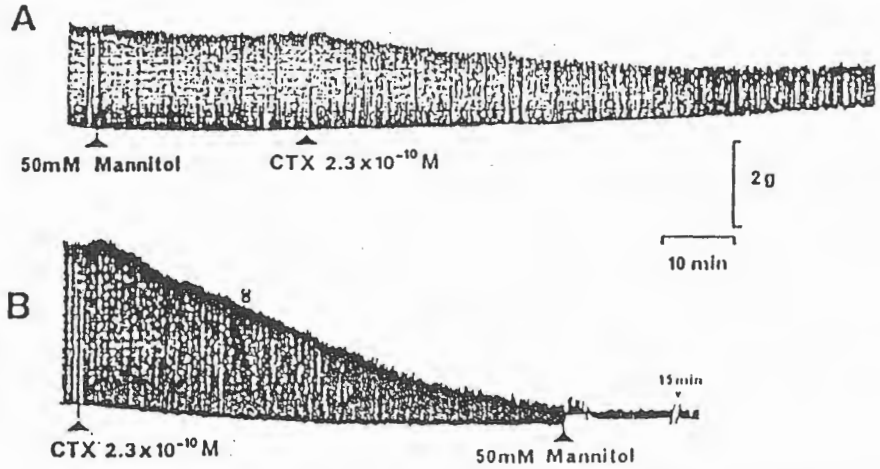

FIG. 4 TRACE RECORDINGS OF TWITCH RESPONSES INDUCED BY INDIRECT STIMULATION IN THE RAT PHRENIC-NERVE DIAPHRAGM AND THE EFFECT OF CIGUATOXIN AND MANNITOL. Mannitol (50 mM) was applied either before (A) or after (B) 2.3 x 10^{-10} M CTX. Twitches were produced at 0.1 Hz stimulation via the phrenic nerve (at 0.2 msec pulse duration and at 150% threshold stimulation voltage). In (A), mannitol itself causes only a small reduction in twitch response before partially protecting against CTX inhibition. In (B), CTX causes a transient increase in twitch height before inhibition.

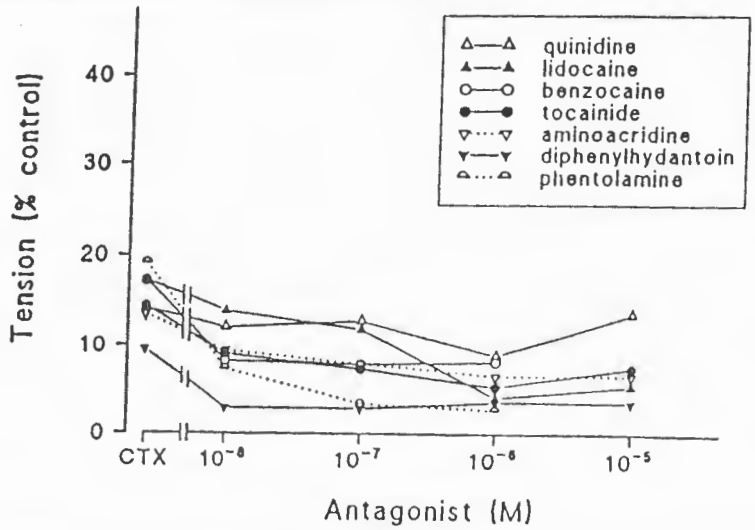

FIG. 5 EFFECTS OF DRUGS WITH LOCAL ANAESTHETIC ACTIVITY UPON CIGUATOXIN INHIBITION OF TWITCH RESPONSES. Drugs were applied after exposure to 2.3 x 10^{-10} M CTX. Data are presented as mean percentages of control (untreated preparations). Treatment with seven drugs having local anaesthetic activity (10^{-8} - 10^{-5} M) did not produce any significant restoration of inhibited twitches. Data is representative of four experiments performed for each drug. Error bars have been omitted for clarity.

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DISCUSSION

The result indicating that low concentrations of CTX cause a small transient increase in twitch height via indirect stimulation is an effect of the toxin that has not been previously described. The increased twitch height may reflect the reported enhancement of quantal transmitter release from motor nerve terminals at low concentrations of toxin (Molgo *et al.*, 1990). The cumulative concentration-response curve for CTX demonstrates an extremely steep inflection, with CTX at 10^{-10} M having no effect on twitches produced via nerve stimulation, while twice this concentration is then sufficient to cause at least 50% inhibition. Enhanced transmitter release (Molgo *et al.*, 1990) may offset inhibition of some neural elements by these low concentrations of CTX. At the higher concentration of 2.3×10^{-10} M, the inhibitory effect of CTX predominates. Indeed, Molgo *et al.* (1990) reported an increase in quantal content as inferred from end plate potential recordings prior to irreversible blockade of nerve evoked transmitter release. However, it remains to be determined as to the cause of the reduced potency of CTX when applied cumulatively as compared with single dose administration. Muscle fasciculations produced after CTX exposure indicates an abnormal excitability of fibres that could be produced by repetitive transmitter release or postsynaptic membrane depolarization (Molgo *et al.* 1990). The action of CTX indicates no significant use-dependence upon relevant ionic channels as evidenced by equivalent inhibition of neurally-evoked responses at 0.1-100 Hz stimulation rates. It is quite apparent that twitch development through neural stimulation is far more sensitive to CTX inhibition than that produced by direct stimulation of the sarcolemma. Moreover, concentrations of purified CTX up to 2.3×10^{-10} M provide a convenient measure of the functional integrity of the nerve only, without the complication of effects of CTX produced directly on the muscle.

This work confirms earlier studies indicating that washing of CTX-treated preparations does not restore twitch responses (Banner *et al.*, 1963; Lewis and Endean, 1983), thereby suggesting a failure to remove CTX. Washout attempts also fail to restore neurotransmitter release in nerve-muscle preparations (Molgo *et al.*, 1990), membrane polarity in neuroblastoma cells exposed to CTX (Bidard *et al.*, 1984) and fail to reduce the positive inotropy caused by CTX in the guinea-pig atria (Lewis and Endean, 1986). In fact, in the present study, washing of CTX-treated preparations resulted in more rapid reduction of twitch height. This is particularly confusing in view of the quasi-irreversible nature of CTX binding. At this stage we can only speculate that washout removes a secondary effect or agent that normally opposes the inhibitory effect of CTX. However, what is certain is that further development of CTX-induced inhibition of responses is not arrested by washing of preparations.

That CTX causes an initial increase in twitch height followed by inhibition of height and frequency in the isolated preparation, is an observation that corresponds with those made in earlier studies using whole

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animals. In those *in vivo* experiments, an injection of CTX, at various degrees of purity, produced changes to respiration, characterized by an initial increase but then followed by a decrease in rate and depth with ultimately total cessation of ventilatory movement (see Banner *et al.*, 1963; Li, 1965; Cheng *et al.*, 1969; Legrand *et al.*, 1982; Legrand *et al.*, 1985). Since respiratory compensation is expected to precede inhibition *in vivo*, it is arguable whether an initial increase in twitch height following exposure of the nerve to CTX (at low concentrations as used in our study) could contribute to the *in vivo* observation. More conclusive evidence is required to determine if CTX causes respiratory arrest by acting only upon central targets since the present results indicate that CTX can affect the functional integrity of the phrenic nerve even at very low concentrations.

CTX causes depolarization that is blocked by TTX in frog skeletal muscle (Rayner, 1972) and in the guinea-pig atria (Lewis, 1988). Earlier, Rayner and Kosaki (1970) showed that increased $^{22}Na^+$ influx was antagonized by TTX in frog skeletal muscle and more recently, Bidard *et al.*(1984) demonstrated that CTX stimulates $^{22}Na^+$influx via sodium channels of neuroblastoma and skeletal myoblasts and that this increased $^{22}Na^+$flux was also abolished by TTX. Further, CTX shifts activation of voltage-dependent sodium channels in the hyperpolarizing direction and causes an increased Na^+ current in the node of Ranvier (Benoit *et al.*, 1986). Consequently, the ability of local anaesthetics to block Na^+ current (Ritchie and Green, 1980) has provided the rationale for the use of these drugs as potential antagonists of CTX. However, none of the local anaesthetics investigated reverse CTX inhibition of the phrenic nerve. In particular, the lack of effect of lidocaine markedly contrasts with the actions of this drug in antagonizing the changes caused by CTX to heart rate and mean arterial pressure in cats (Legrand *et al.*, 1985) and in rapidly reversing the effects of both high and low concentrations of CTX in the isolated guinea-pig atria (Lewis, 1988). Part of the reason for the difference between the effect of local anaesthetics upon CTX responses in mammalian cardiac tissue and that observed in nerves may be because the location of local anaesthetic binding sites could differ between the two types of tissue (Alpert *et al.*, 1989). Local anaesthetics block sodium influx by binding to accessible sites on the cytoplasmic side of excitable membranes of nerves and skeletal muscle (Hille, 1977; Schwarz *et al.*, 1977) but may bind at an additional external binding site on or near sodium channels in cardiac tissue (Alpert *et al.*, 1989). In the phrenic nerve, insensitivity of CTX inhibition to local anaesthetics may reflect the possibility of reduced or delayed access to binding sites in this tissue. In the case of tocainide, which may produce some improvement in neurological symptomology (Lange *et al.*,1988), it is quite possible that any beneficial action is directed predominantly upon central targets (Lindstrom and Lindblom, 1987) rather than on peripheral nerves.

Ciguatoxin causes marked reduction of nerve conduction and prolongation of latency period in human

peripheral nerves (Allsop *et al.*, 1986). This effect has been attributed to compression of the axon caused by oedema of the adaxonal schwann cell cytoplasm. In this respect, the osmotic effects of mannitol may underlie the beneficial effects of its use in the treatment in ciguatera (Pearn *et al.*, 1989). Mannitol itself inhibits nerve function, albeit slightly, but does not inhibit excitation-contraction events of the muscle. In addition, mannitol partly protects the nerve against CTX inhibition, although the basis for this protective effect is still unknown. Nevertheless, it is intriguing that both the slight inhibitory and protective effects of mannitol are evident only at the slow stimulation rate of 0.1 Hz and not at stimulation rates that are more physiologically relevant (20-100 Hz). It is possible to speculate that mannitol, by altering the osmotic gradient, could protect against adaxonal oedema or induce a critical shift in resting membrane potential to produce its protective effect at 0.1 Hz. In small diameter axons, periods of repeated stimulation at higher frequencies may predispose the membrane to a reduction of the Na^+ gradient and this combined with the depolarizing effects of CTX by increasing Na^+ permeability (Rayner, 1972; Bidard *et al.*, 1984) may mitigate against any recovery process facilitated by mannitol. Although mannitol has no protective effect at physiological stimulation rates, it is important to point out that mannitol alone, also has no inhibitory effect at these high rates of stimulation. This observation strengthens the opinion that the use of mannitol with proper rehydration (Pearn *et al.*, 1989) is a safe treatment for ciguatera. The inability of mannitol to readily reverse an established CTX blockade indicates that inhibition by the toxin in the rat phrenic nerve may not stem from oedema of Schwann cells or suggests that irreversible damage has already occurred prior to the addition of mannitol. It is also difficult to explain why control twitches elicited by only low frequency stimulation are affected by mannitol. Mannitol could produce effects in addition to that of reducing adaxonal Schwann cell oedema. Both electrophysiological and ultrastructural studies may prove fruitful in determining the mechanism of mannitol protection. It remains to be determined if the protective effect of mannitol upon peripheral nerves *in vitro* underlies its successful reversal of clinical effects associated with the ciguatera syndrome.

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Appendix III

Ciguatera and mannitol: *in vivo* and *in vitro* assessment in mice

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CIGUATERA AND MANNITOL: *IN VIVO* AND *IN VITRO* ASSESSMENT IN MICE

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R. J. LEWIS, A. W. WONG HOY and M. SELLIN. Ciguatera and mannitol: *in vivo* and *in vitro* assessment in mice. *Toxicon* **31,** 1039–1050, 1993.—Mannitol (1 g/kg i.v.) is currently the treatment of choice for acute ciguatera, but confirmation of this treatment's apparent efficacy awaits further experimental or controlled clinical evidence. In mice, mannitol (1 g/kg i.v.) administered before or after i.p. ciguatoxin did not influence the signs of intoxication or the time to death. The effects of oral ciguatoxin differed from those following i.p. ciguatoxin, but again i.v. mannitol provided no detectable benefit. Development of hypothermia was rapid in mice receiving i.p. or oral ciguatoxin and was unaffected by i.v. mannitol. A sublethal i.p. dose of ciguatoxin initially retarded (day 0–4) but then accelerated (day 4–12) the growth of mice. Mannitol (i.v.) had no influence on these effects of ciguatoxin on the growth of mice. Ciguatoxin inhibited responses of isolated diaphragms to nerve stimulation ($ED_{50} = 9 \times 10^{-11}$ M), while directly stimulated diaphragms were inhibited by five-fold higher concentrations. Mannitol (50 mM) added to the organ bath did not influence the ciguatoxin-induced inhibition of diaphragm responses to nerve stimulation *in vitro*. Responses of isolated diaphragm to nerve stimulation were normal in preparations removed from ciguatoxin-treated mice displaying pronounced dyspnoea (gasping). However, responses to nerve stimulation were reduced in preparations removed from mice immediately following death from ciguatoxin. Mannitol (i.v.) partially protected the phrenic nerve–diaphragm from this effect of ciguatoxin *in vivo*. We conclude that the lethal effects of ciguatoxin in mice probably stem from a central action, and suggest that species differences may account for the absence of any marked beneficial effect of i.v. mannitol in the mouse model for ciguatera in humans.

INTRODUCTION

CIGUATERA results from the consumption of fish contaminated by ciguatoxins. The ciguatoxins arise from the bio-transformation of gambiertoxins produced by certain strains of *Gambierdiscus toxicus* (MURATA *et al.*, 1990; HOLMES *et al.*, 1991; LEWIS *et al.*, 1991). Several ciguatoxins (CTX-1, -2 and -3) have been isolated from the flesh and viscera of fish (MURATA *et al.*, 1990; LEWIS *et al.*, 1991; LEWIS and SELLIN, 1992). These toxins often cause distressing neurological symptoms that can last from several weeks to many months, as well as causing gastrointestinal symptoms of shorter duration. Ciguatoxin acts on sodium channels, shifting the voltage-dependence of activation in the hyperpolarizing

direction to cause depolarization in a range of cell types, especially nerves (RAYNER, 1972; BIDARD *et al.*, 1984; BENOIT *et al.*, 1986; LEWIS and ENDEAN, 1986; MOLGÓ *et al.*, 1990). The effect of ciguatoxin on nerves is similar to that of the brevetoxins (GALLAGHER and SHINNICK-GALLAGHER, 1980; BADEN *et al.*, 1984; TSAI *et al.*, 1991), a class of polyether toxins that compete with ciguatoxin for site 5 on voltage-dependent sodium channels (LOMBET *et al.*, 1987; LEWIS *et al.*, 1991).

Treatment for ciguatera remained symptomatic and supportive until it was found by serendipity that i.v. mannitol could rapidly reduce the apparent severity and duration of ciguatera symptoms (PALAFOX *et al.*, 1988; PEARN *et al.*, 1989; WILLIAMSON, 1990; STEWART, 1991; LEWIS, 1992). Mannitol appears most effective at reversing the neurological effects associated with ciguatera, especially when given as a large dose (1 g/kg) during the acute phase that lasts from 1 to ~ 5 days (PALAFOX *et al.*, 1988; PEARN *et al.*, 1989; LEWIS, 1992). The mechanism of beneficial action of hyperosmotic mannitol may stem from its water-drawing action reversing a ciguatoxin-induced Schwann cell oedema (PEARN *et al.*, 1989). However, the mechanism underlying the mannitol therapy for ciguatera remains unclear, despite investigations using isolated human atria (LEWIS *et al.*, 1992*a*) and rat phrenic nerve–diaphragm (WONG HOY and LEWIS, 1990, 1992).

Mice have been used to assay and characterize the *in vivo* effects of i.p. ciguatoxin (KIMURA *et al.*, 1982; HOFFMAN *et al.*, 1983; LEWIS and ENDEAN, 1984; BAGNIS *et al.*, 1985; LEWIS *et al.*, 1991) including its pathology on the heart, adrenal glands and autonomic nerves (TERAO *et al.*, 1991). In the present study, *in vivo* and *in vitro* models for ciguatera are developed using mice and the ability of mannitol to reverse the effects of ciguatoxin in these models is assessed.

MATERIALS AND METHODS

In vivo *studies in mice*

Mice of 18–22 g (Quackenbush strain of either sex) were used in this study, unless otherwise indicated. Ciguatoxin-1, -2 or -3 (CTX-1, -2 or -3) or a mixture of these ciguatoxins (CTX-m) was administered i.p. or orally after suspension in 0.25–0.5 ml of 1% Tween 60, 0.9% saline (Tween). Unless otherwise stated, mannitol (20% solution) was administered i.v. at 1 g/kg (~ 0.1 ml) over 15 sec, either 3 min before or 5 or 15 min after i.p. CTX-1, or 15 min after oral CTX-m. Mannitol (1 g/kg i.v.) and Tween (0.5 ml) were also administered to control mice. In some experiments, CTX-1 was incubated with 20% mannitol for 15 min prior to i.p. injection of the mixture. For each mouse, a toxicity profile was compiled from observed signs of intoxication over a period of 24 hr after injection. To assess the effect of ciguatoxin on growth, male mice were weighed (between 11 a.m. and 12 noon) on an averaging balance over a 32 day period following injection. At 32 days, these mice were sacrificed, internal organs weighed and a fatty deposits scored on an arbitrary scale (1–3) upon visual examination. Rectal temperature in mice was also measured intermittently with a probe thermometer (type 1604, Comark) for up to 24 hr after injection. For orally administered CTX-1 and CTX-m, the LD_{50} and minimum time to death were estimated from dose *vs.* time to death relationships (see LEWIS and ENDEAN, 1984). Mice were given food and water *ad libitum* and were housed at 23–26°C with 12 : 12 hr light–dark cycle. Animals were used in accordance with National Health and Medical Research Council, Australia guidelines relating to the care and use of animals for experimental purposes.

Phrenic nerve–diaphragm studies

The left hemidiaphragm with intact phrenic nerve was excised from Quackenbush mice of either sex (20–25 g) within 30 sec of being killed by either (i) a blow to the head and exsanguination (control), (ii) 2 mouse units (MU) i.p. CTX-1, with or without i.v. mannitol (1 g/kg) 15 min after toxin, or (iii) a blanket of CO_2 gas. In some experiments, mice treated with i.p. CTX-1 (2 MU) were killed when pronounced dyspnoea (gasping) appeared (typically ~ 15 min prior to death from ciguatoxin). Each hemidiaphragm was trimmed to 8–10 mm width and placed in Ringer of the following composition (mM): NaCl 135.0; KCl, 5.0; $CaCl_2$, 2.0; $MgCl_2$, 1.0; KH_2PO_4, 1.0; $NaHCO_3$, 15.0; and glucose, 11.0. Ringer was bubbled with carbogen (95% O_2/5% CO_2) throughout experiments, with preparations maintained under 1.0 g tension in 5-ml organ baths at 37°C (pH 7.4). Contractile responses to electrical stimulation were recorded on chart recorders via isometric force transducers (F-60, Narco

Biosystems) within 15 min of tissue removal. Preparations were stimulated at 0.1 Hz (0.2 msec duration, 150% of threshold) or directly through the muscle (0.1 Hz with 2.0 msec pulses at a nominal 35 V). Stimuli of 50 Hz were applied for approximately 1 sec at various times during experiments to assess the effect of CTX-1 on the tetanus response of these preparations. *In vitro* responses to cumulative additions of CTX-1 were measured 20–90 min after addition of each dose of toxin. For these experiments, preparations were obtained from mice killed by cervical dislocation and exsanguination.

In vitro *blood–brain barrier model*

Passive transendothelial transport was inferred from lipophilicity, estimated following the approach of VAN BREE *et al.* (1988). Lipophilicity was estimated from the partition coefficient of CTX-1 or pure maitotoxin-2 (a polyether toxin isolated from *G. toxicus* as described by HOLMES *et al.*, 1990) between ethylacetate and HEPES buffered Ringer (1 : 1, 37°C). The concentration of toxin in each phase was determined by mouse bioassay after solvent removal.

Ciguatoxins and mannitol used

Pure CTX-1, -2 and -3 were obtained as described by LEWIS *et al.* (1991). In some experiments, a mixed ciguatoxin fraction (CTX-m, i.p. LD_{50} = 17 μg/kg) containing CTX-1, -2 and -3 was used. CTX-m comprised CTX-1, -2 and -3 in a ratio typically found in the flesh and viscera of ciguateric fishes (LEWIS *et al.*, 1991; LEWIS and SELLIN, 1992), i.e. 90%, 5%, 5% of total i.p. mouse lethality, respectively. One MU is defined as the i.p. LD_{50} dose for a 20 g mouse (equivalent to 5 ng of CTX-1 (LEWIS *et al.*, 1991) or 340 ng CTX-m). A 20% mannitol (A.R. grade, Ajax Chemicals, Australia) solution was used for *in vivo* and *in vitro* experiments.

Statistics

Results are presented as the mean ± 1 S.E. The Student's *t*-test was used to compare means, with $P < 0.05$ considered significant. ED_{50}s were estimated using non-linear curve fitting procedures (FigP software, Biosoft, U.S.A.).

RESULTS

Influence of mannitol on signs in mice intoxicated with ciguatoxin

CTX-1 (2.3 or 0.6 MU) injected i.p. into mice caused toxic signs including loss of activity, laboured respiration, cyanosis, weakness, diarrhoea, piloerection, lachrymation and hypersalivation ($n = 4$). Comparable responses were observed for i.p. CTX-m ($n = 8$). Mannitol (1 g/kg i.v.) administered 15 min after i.p. CTX-1 (2.3 or 0.6 MU) did not alter the appearance of toxic signs ($n = 4$) or the time to death in mice receiving a lethal dose of CTX-1 (Fig. 1A). Recovery from laboured respiration and loss of activity after 0.6 MU of CTX-1 (130 ± 10 and 90 ± 10 min, respectively) were also not markedly altered by i.v. mannitol (100 ± 10 and 140 ± 10 min, respectively) ($n = 4$). Mannitol administered 3 min prior to ($n = 2$) or 5 min after ($n = 3$) i.p. CTX-1 (2.5 MU) also did not alter the toxicity profile or time to death. Mannitol (i.v.) administered at 2 g/kg or as three 1 g/kg doses over 1 hr also did not alter the toxicity profile or time to death for mice given 2.5 MU i.p. CTX-1 ($n = 3$). Mannitol mixed with CTX-1 (2.5 MU) prior to i.p. injection had no influence on the toxicity profile or time to death in mice ($n = 3$). Mannitol (i.v.) or Tween (i.p.) alone had no apparent effect on mice.

In an attempt to model more closely ciguatera in humans, CTX-1 or a mixture of ciguatoxins that typically contaminates ciguateric fishes (CTX-m), were administered to mice by the oral route. Oral administration of CTX-1 or CTX-m (1–20 MU) induced in mice signs similar to those following i.p. administration, except that lethal oral doses of CTX-1 or CTX-m induced hind-limb paralysis (5/7 for each), swollen tongue (3-4/7) and no diarrhoea (0/7 for each), and at half an oral LD_{50} dose induced few effects. Hind-limb paralysis appeared after 45 min and swollen tongue appeared after 1 hr following oral

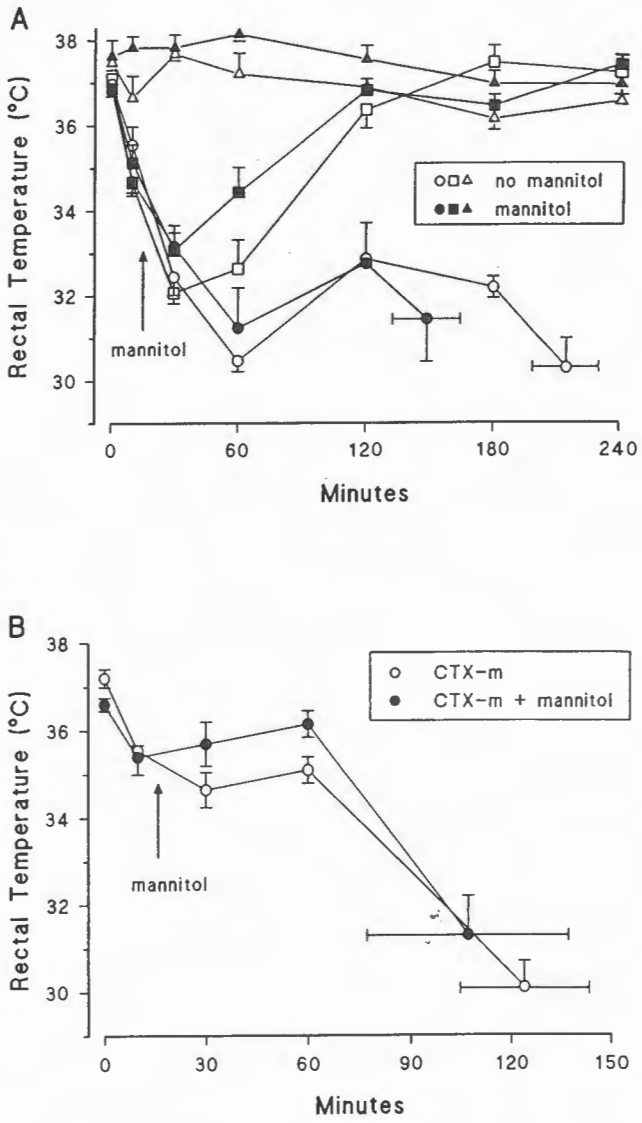

FIG. 1. EFFECTS OF i.v. MANNITOL ON CIGUATOXIN-INDUCED HYPOTHERMIA.
(A) Rectal temperature was measured in male mice receiving Tween (△), mannitol (▲), 0.6 MU i.p. ciguatoxin-1 (CTX-1) (□), 0.6 MU i.p. CTX-1 followed by mannitol (■), 2.3 MU i.p. CTX-1 (○), or 2.3 MU i.p. CTX-1 followed by mannitol (●) ($n = 4$). (B) Rectal temperature of mice (either sex) receiving 5 MU oral CTX-m (○) or 5 MU oral CTX-m followed by mannitol (●) ($n = 3$–4). Mannitol (1 g/kg i.v.) was administered as a bolus dose 15 min after CTX-1 or CTX-m. Data are means ± 1 S.E. Biaxially labelled means indicate rectal temperature at death and the average time to death.

administration of either CTX-m or CTX-1. For lethal oral doses of CTX-m, initial signs of inactivity were followed by an asymptomatic phase (lasting 40–145 min), with signs of intoxication again appearing 110–190 min after toxin administration (for 5/7 mice). Such a period of recovery was not observed for i.p. dosed mice (0/8). Mannitol (1 g/kg i.v.)

administered 15 min after oral CTX-m did not influence the toxicity profile or the time to death (Fig. 1B).

The dose *vs.* time to death relationship (where t = time to death in hr) and minimum time to death (min) for oral CTX-m [log MU = 7.8 log $(1+t^{-1})$; 60 min] indicate a slower action than i.p. CTX-m [log MU = 2.3 log $(1+t^{-1})$; 37 min; LEWIS and ENDEAN, 1984; LEWIS *et al.*, 1992*b*]. Similarly, the equation and minimum time to death for oral CTX-1 [log MU = 8.0 log $(1+t^{-1})$; 81 min] indicate a slower action than i.p. CTX-1 [log MU = 3.3 $(1+t^{-1})$; 37 min]. On the basis of LD_{50} estimates in mice, oral CTX-1 (0.22 μg/kg) was similar in potency to i.p. CTX-1 (0.25 μg/kg; LEWIS *et al.*, 1991), whereas oral CTX-m (34 μg/kg) was half the potency of i.p. CTX-m (17 μg/kg).

Ciguatoxin-induced hypothermia and the influence of mannitol

Lethal and sublethal i.p. doses of CTX-1 (2.3 and 0.6 MU) induced an immediate, rapid fall (−0.15°C/min) in mouse rectal temperature to below 32°C (Fig. 1A). Rectal temperature fell to as low as 25°C in some mice. Similar responses, including the rapid fall in rectal temperature, were recorded following i.p. CTX-2, CTX-3 or CTX-m (n = 6–8). After an initial delay of 60 min, hypothermia also developed (~ −0.1°C/min) after administration of lethal oral doses of CTX-m (Fig. 1B). For i.p. or orally dosed mice, the onset of rapidly developing hypothermia coincided with the appearance of signs of intoxication (n = 4). The development of hypothermia following i.p. CTX-1 or oral CTX-m was not significantly influenced by i.v. mannitol (Fig. 1A and B). Recovery from hypothermia after a sublethal i.p. dose of CTX-1 (0.6 MU) occurred over a 90 min period (Fig. 1A), with attainment of normal temperatures coinciding with recovery from laboured respiration and loss of activity (n = 4). The recovery phase was unaffected by i.v. mannitol (Fig. 1A). Hypothermic mice did not display signs of vasodilation but often displayed piloerection.

Effect of ciguatoxin on mouse growth and the influence of mannitol

The influence of i.p. CTX-1 (0.6 MU) on the growth of mice was assessed over a 32 day period (Fig. 2). Despite complete recovery from signs of intoxication within 2 hr of 0.6 MU CTX-1, growth in the first 4 days after injection was less than control but greater than control from day 4–12. CTX-1 treated mice were significantly heavier (and visibly larger) than control mice from day 12 onward. Mannitol did not reduce the growth of control or CTX-1 treated mice (Fig. 2). At the end of the experiment, the weights of liver (5.4 ± 0.1, 5.8 ± 0.2, 4.7 ± 0.2 and 5.3 ± 0.3% of total body weight) and gastrointestinal tract (11.9 ± 0.3, 10.7 ± 0.4, 10.8 ± 0.5 and 10.8 ± 0.3% of total body weight) were not significantly different between Tween (control) and i.p. CTX-1 or between i.v. mannitol and i.p. CTX-1 plus i.v. mannitol treated mice, respectively (n = 4). There was also no difference between the fatty tissue score for control, CTX-1, mannitol, or CTX-1 plus mannitol treated mice. These results indicate that the increase in weight does not stem from increases in the weight of internal organs or from increases in fat deposits.

Influence of mannitol on phrenic nerve–diaphragm treated with ciguatoxin

CTX-1 induced a concentration-dependent inhibition of responses of mouse diaphragm to 0.1 Hz nerve stimulation (ED_{50} = 0.020 ± 0.001 MU/ml) and direct stimulation (ED_{50} = 0.10 ± 0.02 MU/ml) (Fig. 3A). Responses to 50 Hz stimulated nerves (tetanus) were inhibited by the same range of concentrations that inhibited responses to 0.1 Hz nerve

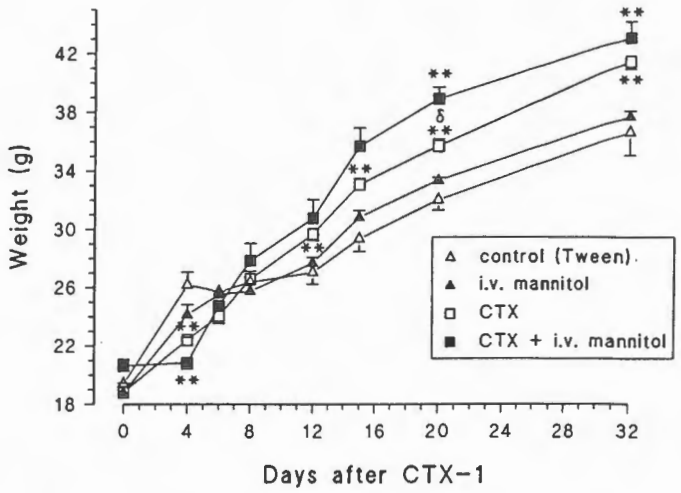

FIG. 2. EFFECT OF i.p. CIGUATOXIN-1 (CTX-1) AND i.v. MANNITOL ON MOUSE GROWTH. Weight (g) was measured for male mice receiving Tween (△), mannitol (▲), 0.6 MU i.p. CTX-1 (□) or 0.6 MU i.p. CTX-1 followed by mannitol (■). Data are means ± 1 S.E. ($n = 4$). $P < 0.01$ (**) indicate a significant increase in the weight of CTX-1 *vs.* control mice or for CTX-1 plus i.v. mannitol *vs.* mannitol treated mice. $P < 0.05$ (δ) indicates a significant difference between weights of CTX-1 treated mice, with or without mannitol (1 g/kg i.v. administered 15 min after CTX-1).

stimulation (Fig. 3A). Inhibition was often preceded by a transient increase in twitch height and a period of spontaneous muscle fasciculation (8 of 16 preparations) (see Fig. 3B). A 60 min wash with Ringer partially restored responses to 0.1 Hz nerve stimulation (to 13–20% of control) in three of eight preparations treated with CTX-1. Pre-application ($n = 2$, Fig. 3B) or post-application (30–60 min after CTX-1, $n = 8$) of mannitol (50 mM) had little influence on the CTX-1-induced inhibition of responses to nerve stimulation. Repeated application and washout of mannitol also had little effect on these inhibited responses.

The *in vivo* effects of ciguatoxin on diaphragm responses were also assessed (Fig. 4). Isolated diaphragms removed from control mice, from i.p. CTX-1 treated mice showing pronounced gasping, or from mice killed by CO_2 responded similarly to nerve stimulation, whereas diaphragms removed from mice immediately after death from i.p. CTX-1 were significantly less responsive to nerve stimulation (0.1 Hz and 50 Hz). Administration of i.v. mannitol to mice 15 min after i.p. CTX-1 protected diaphragm responses to 0.1 Hz nerve stimulation from this inhibitory action of CTX-1 *in vivo* (Fig. 4). The responses to direct stimulation of diaphragm musculature were not significantly different among these treatments ($n = 4$).

In vitro *blood–brain barrier model*

The partition-coefficient, log (ethyl acetate/Ringer ratio), was > 2.0 for CTX-1 and -1.7 for maitotoxin-2, indicating that CTX-1 was more lipophilic than maitotoxin-2 in this model. Based on these lipophilicity estimates, CTX-1 should cross the blood–brain barrier, whereas this is unlikely for maitotoxin-2.

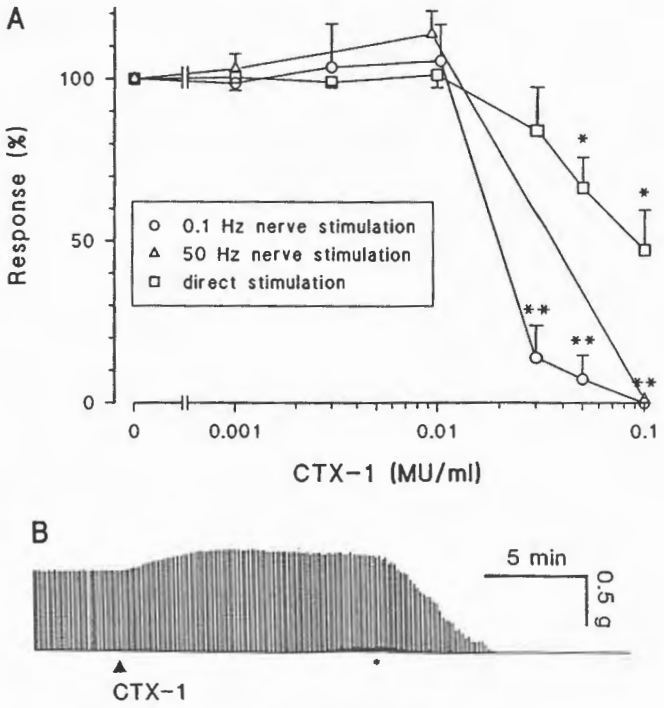

FIG. 3. EFFECT OF CIGUATOXIN-1 (CTX-1) ON MOUSE DIAPHRAGM *in vitro*.
(A) Concentration-dependent effect of CTX-1 on response of diaphragm to phrenic nerve stimulation at 0.1 Hz ($n = 7$–17) or 50 Hz ($n = 4$–5) or to direct stimulation of diaphragm musculature ($n = 4$–6). CTX-1 was applied cumulatively and the response measured within 90 min of addition of each dose. $P < 0.05$ (*) and $P < 0.01$ (**) indicate significant inhibition of contractile response (paired t-test on untransformed data). (B) Time-course of the effect of 0.05 MU/ml CTX-1 on diaphragm responses to 0.1 Hz nerve stimulation. Responses are measured in gram-tension and time is in min. This preparation was pretreated with 50 mM mannitol 60 min before CTX-1. The asterisks indicates a period of muscle fasciculation. Note the initial increase in twitch contractions that preceded the inhibition phase. Similar responses were obtained for another mannitol pretreated preparation and eight other preparations not pretreated with mannitol.

DISCUSSION

The signs induced by i.p. or orally administered CTX-1 or CTX-m (a purified fraction containing a mixture of ciguatoxins typically found in ciguateric fish) have been characterized. For each route of administration, no difference in signs was apparent between mice administered either CTX-1 or CTX-m. However, signs induced by oral administration of CTX-1 or CTX-m, including hind-limb paralysis, swollen tongue, delayed effects and the absence of diarrhoea, do not occur following i.p. administration. Such differences were not evident in previous studies on the effects of orally administered ciguatoxin (OGURA *et al.*, 1968; TERAO *et al.*, 1991). Despite these differences, the ciguatoxins were similarly potent whether administered i.p. or orally. Mannitol (1 g/kg i.v.) administered before or after i.p. CTX-1 or after oral CTX-m did not influence the development of toxic signs or the time to death in mice. The signs displayed by adult cats (including loss of activity, hypersalivation, hind-limb paralysis and laboured respiration) 8–24 hr after oral CTX-m (20 MU/kg) were also not altered by a bolus dose of mannitol (1 g/kg i.v.) (unpublished

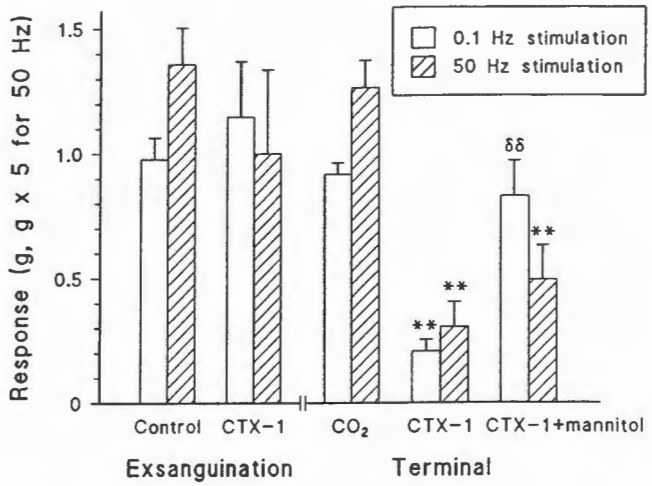

FIG. 4. INFLUENCE OF i.v. MANNITOL ON MOUSE PHRENIC NERVE–DIAPHRAGM EXPOSED TO CIGUATOXIN-1 (CTX-1) *in vivo*.

Responses (g) to nerve stimulation (0.1 or 50 Hz) were recorded in preparations from (i) control mice; (ii) CTX-1 (2 MU i.p.) treated mice displaying pronounced gasping; (iii) immediately following death from CO_2; and (iv and v) immediately following death from 2 MU i.p. CTX-1, with or without i.v. mannitol (1 g/kg) administration 15 min after CTX-1. Data are means ± 1 S.E. (n = 4–6). $P < 0.01$ (**) indicate a significant reduction in response compared with control. $P < 0.01$ ($\delta\delta$) indicates significant improvement of the CTX-1 inhibited response by i.v. mannitol. Responses to direct stimulation were not significantly different among these treatments (data not shown).

result). Incubation of CTX-1 with mannitol prior to i.p. administration of the mixture to mice resulted in signs of intoxication and times to death that were the same as for i.p. CTX-1 alone, indicating that mannitol is unlikely to detoxify ciguatoxin through direct effects such as chelation.

An immediate, rapid fall (−0.15°C/min) in mouse rectal temperature followed the administration of i.p. CTX-1, -2, -3 or CTX-m. Hypothermia to below 32°C was not uncommon and such hypothermia remained until death. Mice receiving a sublethal i.p. dose of CTX-1 (0.6 MU) developed transient hypothermia with recovery to normal temperatures within 2 hr. In contrast, SAWYER *et al.* (1984) reported that sublethal i.p. doses of ciguatoxin (a crude extract from Caribbean fish) induced hypothermia that developed over 2–6 hr and abated at least 24 hr later. Contaminants or additional toxins in their crude extract may account for such delayed effects. Oral doses of CTX-1 or CTX-m caused little initial effect on body temperature, but body temperature dropped rapidly to ~ 30°C prior to death. The onset of signs of intoxication coincided with the onset of the rapid fall in rectal temperature for all intoxicated mice, irrespective of the route used to administer the ciguatoxin. Similarly, recovery from hypothermia coincided with recovery from signs of intoxication. Thus hypothermia in mice provides a guide to the extent of intoxication by ciguatoxin. Mannitol (1 g/kg i.v.) administered 15 min after i.p. CTX-1 or oral CTX-m did not alter the development of hypothermia.

Hypothermic mice often displayed piloerection, indicating that these mice may have perceived the feeling of cold associated with the hypothermia. This suggests that an altered set point for control of body temperature was probably not involved. The absence of visible vasodilation in ciguatoxin-treated mice indicates that the fall in body tempera-

ture is unlikely to involve an increased loss of heat from the skin. In this regard, the ciguatoxins are similar to the brevetoxins which did not cause hypothermia through increased heat loss from the skin of rats (TEMPLETON *et al.*, 1989). In contrast, oral ethanol apparently lowers the set point to cause hypothermia (GORDON *et al.*, 1988), while angiotensin II causes hypothermia via an increased loss of heat from the tail (WILSON and FREGLY, 1985). A reduced metabolic rate may underlie the hypothermia that develops after ciguatoxin, but the precise mechanism remains to be elucidated.

Mice that received a sublethal i.p. dose of CTX-1 recovered from signs of intoxication and hypothermia after 2 hr but had gained less weight than control mice after 4 days. Laboured respiration, diarrhoea, lachrymation and hypersalivation would contribute to water loss in the first 2 hr, and perhaps contribute to this reduced growth, although it is possible that other effects of ciguatoxin that lead to reduced growth may persist in the absence of signs of intoxication. Surprisingly, growth from day 4 to 12 was greater for CTX-1 treated mice than for control mice, with these mice becoming significantly larger than control mice from day 12 onward. Ciguatoxin is known to stimulate in a variety of tissues the release of β-agonists such as noradrenaline (OHIZUMI *et al.*, 1981; LEWIS and ENDEAN, 1986; LEWIS *et al.*, 1992*a*). β-agonists can promote lean tissue disposition possibly through effects on metabolism (HANRAHAN, 1987; FORSBERG *et al.*, 1989). A sustained rise in the levels of circulating β-agonists following i.p. ciguatoxin may underlie the phase of enhanced growth in mice. Mannitol had little influence on growth of control or CTX-1-treated mice, suggesting that any rise in circulating β-agonists is not suppressed by mannitol.

In the isolated diaphragm, CTX-1 inhibited twitch responses elicited via 0.1 Hz stimulation of the phrenic nerve (ED_{50} = 0.02 MU/ml or 9×10^{-11} M) in a concentration-dependent manner. Responses to 50 Hz stimulation (tetanus) were similarly affected, while five-fold higher concentrations of CTX-1 (ED_{50} = 0.1 MU/ml or 5×10^{-10} M) were required to inhibit responses to direct stimulation of the diaphragm. Prior to the inhibition of nerves, CTX-1 enhanced twitch responses and caused muscle fasciculation. Similar responses were reported previously for the rat phrenic nerve–diaphragm preparation (LEWIS and ENDEAN, 1983; WONG HOY and LEWIS, 1992), except that the rat nerve is 2.6-fold less sensitive to the inhibitory action of CTX-1 (WONG HOY and LEWIS, 1992). Enhanced quantal release of acetylcholine and/or an increase in the frequency of miniature end plate potentials (MOLGÓ *et al.*, 1990) may underlie these effects.

Mannitol (50 mM), at a concentration that approximates peak serum levels following a 1 g/kg bolus i.v. dose of mannitol (CLOYD *et al.*, 1986), did not influence the effects of CTX-1 *in vitro*, indicating that mannitol is unlikely to influence the binding of ciguatoxin to sodium channels in myelinated nerves of mice. Isolated diaphragms removed from CTX-1-treated mice displaying pronounced gasping responded normally to nerve stimulation, suggesting that gasping was the result of a central effect of ciguatoxin on respiration. Isolated diaphragms removed from mice immediately following death from i.p. CTX-1 did have an impaired response to nerve stimulation. In similar experiments where mice were treated with mannitol (1 g/kg i.v.) 15 min after CTX-1, diaphragms responded normally to 0.1 Hz nerve stimulation. However, this protective action of mannitol was not evident at 50 Hz stimulation. A similar result was obtained using the rat diaphragm, where pre-application of mannitol protected twitch responses from CTX-1 inhibition at 0.1 but not 50 Hz nerve stimulation (WONG HOY and LEWIS, 1990, 1992). The mechanism underlying this difference in the response to low and high frequency nerve stimulation is unclear (see WONG HOY and LEWIS, 1992). The protection by mannitol *in vivo* was not accompanied by

an improvement in survival time, indicating that respiratory arrest through a block of the phrenic nerve was unlikely to be the primary cause of death in these mice. These experiments in unanaesthetized mice support previous findings that central respiratory arrest was the cause of death from ciguatoxin in rats (RAYNER *et al.*, 1968; CHENG *et al.*, 1969), without the potential for added complication from the sodium pentobarbital anaesthetic used in these earlier studies. For other toxicants, sodium pentobarbital anaesthetic can shift the cause of death from peripheral to central origin (CLEMENT, 1984).

The partial beneficial action of mannitol *in vivo* may relate to its therapeutic action in humans, perhaps as a consequence of the hydroxyl radical scavenging or ion conductance blocking actions of mannitol (MAGOVERN *et al.*, 1984; HOCH *et al.*, 1991; RITTER *et al.*, 1991). Alternatively, such actions of mannitol may protect nerve function from secondary effects such as hypoxia or acidosis that are likely to accompany the terminal effects of ciguatoxin. Hypoxia and acidosis accompany the terminal effects of brevetoxin-induced respiratory failure (FRANZ and LECLAIRE, 1989; TEMPLETON *et al.*, 1989). The protective action of mannitol on the phrenic nerve may stem from an ability to reduce nerve pathology (TERAO *et al.*, 1991), or from an ability to reduce the quantity of ciguatoxin bound to nerves. The latter action is considered unlikely in view of mannitol's inability to significantly influence either the time to death from ciguatoxin or the inhibitory effect of ciguatoxin on the phrenic nerve *in vitro*.

The absence of dramatic effects of mannitol on the signs of mice (and cats) or on the survival of mice following administration of ciguatoxin is in contrast with the typically rapid relief mannitol (1 g/kg i.v.) provides against the neurological symptoms associated with acute ciguatera in humans (PALAFOX *et al.*, 1988; PEARN *et al.*, 1989; WILLIAMSON, 1990; STEWART, 1991; LEWIS, 1992). The absence of therapeutic effects of mannitol in animal models for ciguatera studied to date may stem from species differences. Such differences are evident from differences in the estimated lethal oral dose of CTX-m for cats ($\sim$ 15 MU/kg) and mice (100 MU/kg) which are higher than the estimated lethal oral dose for humans ($\sim$ 5 MU/kg) (unpublished observations). Further species differences in response to ciguatoxin include the appearance of prominent hypothermia in mice but not in ciguatera in humans or cats (BAGNIS and LEGRAND, 1987; unpublished observations). It is possible that humans are particularly susceptible to peripheral neural effects of ciguatoxin, e.g. adaxonal Schwann cell oedema (ALLSOP *et al.*, 1986), whereas in mice, toxic effects of central origin may predominate over such peripheral effects. A central action of ciguatoxin on respiration in mice is supported by the finding that ciguatoxin is 25-fold more potent when administered intracerebroventrally to mice than when administered i.p. (BIDARD *et al.*, 1984). The present study confirms that CTX-1 is sufficiently lipophilic to cross the blood–brain barrier. Scanning electron microscopy of transverse sectioned mouse phrenic nerves removed at death from mice treated i.p. with 2 MU of CTX-1 did not reveal adaxonal Schwann cell oedema (unpublished observation), again suggesting that the peripheral effects of ciguatoxin in mice may be less prominent. An absence of peripheral adaxonal Schwann cell oedema in mice following ciguatoxin may account for the absence of any marked beneficial effect of mannitol in this species. Further studies on the mannitol therapy would be facilitated by a better animal model for ciguatera in humans.

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Appendix IV

Action of ciguatoxin on human atrial trabeculae

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ACTION OF CIGUATOXIN ON HUMAN ATRIAL TRABECULAE

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R. J. LEWIS, A. W. WONG HOY and D. C. MCGIFFIN. Action of ciguatoxin on human atrial trabeculae. *Toxicon* **30**, 907–914, 1992.—This report describes the action of ciguatoxin-1, the major ciguatoxin present in fishes that cause ciguatera, on the contractile activity of human cardiac musculature. Ciguatoxin-1 caused a large, sustained and concentration-dependent positive inotropy in human atrial trabeculae that were obtained during coronary artery bypass surgery from otherwise healthy hearts. Atenolol (a β_1-adrenoceptor selective antagonist without local anaesthetic-type activity) or low concentrations of tetrodotoxin abolished the positive inotropy caused by ciguatoxin-1, indicating that ciguatoxin-1 stimulated neural elements present in this tissue to release noradrenaline. The positive inotropic action of ciguatoxin-1 did not stem from a significant direct action on myocardial voltage-dependent sodium channels, nor did it stem from significant α_1- or β_2-adrenoreceptor stimulation. Ciguatoxin-1 caused positive inotropy in preparations stimulated at between 0.02 and 2.0 Hz. Mannitol, currently the treatment of choice for ciguatera, did not significantly reverse the positive inotropy induced by ciguatoxin-1 in human atrial trabeculae.

INTRODUCTION

CIGUATERA is a widespread human disease that results from consumption of tropical and subtropical marine fishes contaminated with lipid-soluble polyether toxins named ciguatoxins (CTX). The structures for three ciguatoxins (CTX-1, -2 and -3) have recently been determined (MURATA *et al.*, 1990; LEWIS *et al.*, 1991). Ciguatera is characterized by a range of neurological and gastrointestinal signs and symptoms (GILLESPIE *et al.*, 1986; LEWIS *et al.*, 1988). Severe poisonings are rarely fatal but may include bradycardia, hypotension and arrhythmias (SOROKIN, 1975; BAGNIS *et al.*, 1979; GILLESPIE *et al.*, 1986). LEWIS *et al.* (1991) found that CTX-1 contributes approximately 90% of the total toxicity in ciguateric fishes (on the basis of mouse lethality), and on this basis CTX-1 is the major toxin involved in ciguatera.

Early *in vitro* studies demonstrated that ciguatoxin had indirect effects (*via* the stimulation of intrinsic nerves) on atria from rabbits and rats (OHSHIKA, 1971). Recent studies have revealed that moderate concentrations of ciguatoxin have both indirect and direct positive inotropic actions on guinea-pig atria and papillary muscles (LEWIS and ENDEAN, 1986; SEINO *et al.*, 1988). At high concentrations ciguatoxin also has a direct negative inotropic action (LEWIS, 1988). The positive and negative inotropic effects of CTX-1 are

mediated *via* activation of voltage-sensitive sodium channels. This study reveals that pure ciguatoxin-1 (CTX-1) has a potent indirect positive inotropic action on human atrial trabeculae *in vitro*.

MATERIALS AND METHODS

Cardiac preparation

Right atrial appendage tissue was obtained from male (11) and female (2) patients (aged 39–75 years) undergoing coronary artery bypass surgery, with approval from the Ethics Committee of The Prince Charles Hospital. Calcium antagonists, β-blockers and nitrates were discontinued 24 hr prior to operation. No patient had left or right ventricular failure at the time of operation. The anaesthetic technique included induction with fentanyl and pancuronium and maintenance with a fentanyl infusion. A 1-cm^2 piece of right atrial tissue was obtained by amputation of the apex of the right atrial appendage above the purse string suture for right atrial cannulation to establish cardiopulmonary bypass.

Immediately after surgical removal, atrial appendage was immersed in chilled (3°C) carbogenated Krebs–Henseleit Ringer solution of the following composition (in mM): NaCl, 115; KCl, 4.7; $CaCl_2$, 1.2; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$ 25.0; glucose, 10.0; pH 7.4 (Lewis and Endean, 1986). The tissue was transported to the laboratory in an insulated vessel where processing began approximately 30 min after excision. Solutions containing atrial tissue were equilibrated with carbogen (95% O_2 and 5% CO_2) during preparation of atrial segments and during experimentation. Several trabecular strips were dissected free as intact bundles and trimmed minimally to produce preparations at least 3 mm in length and < 1.5 mm in diameter. Preparations ($n = 38$) were mounted vertically in 5-ml organ baths containing initially chilled Krebs–Henseleit solution. Before application of drugs or CTX-1 each atrial segment was equilibrated for at least 1 hr with regular exchange of fresh Ringer's solution. During this period the Ringer solution was gradually warmed to 32°C over 30 min. Preparations were maintained under 0.5 g tension during equilibration and experimentation. Electrical field stimulation of preparations was provided by a Grass S44 stimulator, delivering rectangular pulses at 0.5 Hz and of 3–6 msec duration via platinum wire electrodes. During the first 60 min of the equilibration period, pulses were delivered at low voltage strength (< 4 volts) regardless of development of twitches. After this time, however, voltage strength was increased to 20% above threshold in each preparation prior to experimentation. Unless otherwise stated in the Results, atrial segments were stimulated at 0.5 Hz for the duration of experiments. Twitch tension was measured by Narco Biosystems F-60 isometric force transducers and recorded on Goerz Metrawatt (SE 130 and 120) chart recorders. Each atrial segment was challenged with an initial dose of noradrenaline (3×10^{-6} M) and then washed for at least 20 min prior to further testing. The stated molarity of a drug refers to its final concentration in the organ bath. Antagonists were added cumulatively to preparations exposed to a single dose of ciguatoxin (CTX-1). Doses of noradrenaline, tetrodotoxin (TTX), atenolol and prazosin were prepared from dilutions of aqueous stock solutions. Mannitol (50 mM) was prepared as an aqueous stock solution (20% w/v) prior to its addition to the organ bath as a hypertonic solution. The relationship between twitch tension and stepwise increases in stimulation frequency (0.02–2.0 Hz) was also determined, both before and after the addition of CTX-1. Recordings were made after at least 10 min at each frequency from untreated preparations (control) and from the same preparations after exposure to CTX-1 and subsequent washout (75 min) of the toxin.

FIG. 1. EFFECT OF CIGUATOXIN-1 (CTX-1) ON AN ISOLATED HUMAN RIGHT ATRIUM. Preparations were electrically driven at 0.5 Hz. Twitch contractions before and after CTX-1 (7×10^{-10} M) were measured in gram-tension (g) vs time (min; or sec for individual twitch responses). Numbers between panels indicate time elapsed or wash (W) periods (min). The preparation was also exposed to mannitol (50 mM) for 20 min. CTX-1 caused a sustained increase in the force of twitch contractions (positive inotropy). Note that mannitol caused an additional positive inotropic effect without influencing the delayed after-contractions that developed during exposure to CTX-1.

Ciguatoxin-1 (CTX-1)

CTX-1 was extracted from the liver and viscera of moray eels and purified to homogeneity as described previously (LEWIS *et al.*, 1991). Aliquots of a methanol–water (1:1) stock solution were used either neat or diluted with water. All solutions containing CTX-1 were sonicated prior to addition to the organ bath. In separate experiments, the methanol added as vehicle ($< 5\,\mu l$) had no effect on control twitch responses.

Drugs

TTX, prazosin hydrochloride (Sigma Chemical Co., St. Louis, U.S.A.), noradrenaline (Winthrop, Sydney, Australia), 20% mannitol solution (Travenol, Toongabbie, Australia). Atenolol was a generous gift from ICI Pharmaceuticals, Australia.

Statistical method

Student's t-test was used for analysis of results, with $P < 0.05$ considered significant. Results are presented as the mean $\pm$ S.E.

RESULTS

Figure 1 shows the response of an electrically stimulated human atrial preparation to the addition of CTX-1. The increased twitch tension (positive inotropy) developed over 30 min and could not be reversed during a 90 min washout with fresh Ringer's solution (Figs 1 and 2). CTX-1 at concentrations of 4.7×10^{-10} to 7×10^{-10}M caused a concentration-dependent, positive inotropic response in human atrial segments (Fig. 3), increasing the baseline twitch tension from 0.04 ± 0.005 g ($n = 24$) to 0.43 ± 0.07 g ($n = 18$). CTX-1 (7×10^{-10} M) induced positive inotropy similar ($P > 0.15$) to that caused by 3×10^{-6} M noradrenaline (0.56 ± 0.06 g, $n = 18$).

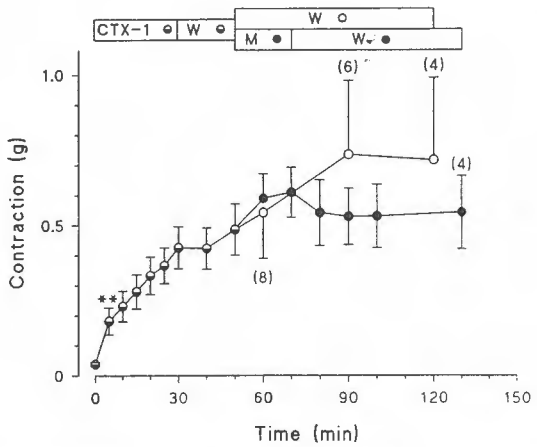

FIG. 2. TIME COURSE OF CIGUATOXIN-1 (CTX-1) INDUCED POSITIVE INOTROPY IN HUMAN ATRIA AND THE INFLUENCE OF WASHOUT (W) AND MANNITOL (M).

Preparations were electrically driven at 0.5 Hz. CTX-1 (7×10^{-10} M) was applied for 30 min and preparations washed for 20 min (◒, $n = 18$). At 50 min some of these preparations were then washed for an additional 70 min (○, n as indicated by numbers in parentheses), while the others were exposed to mannitol (20 min) prior to further washout (●, $n = 9$ except where indicated). Data expressed as means $\pm$S.E. Paired t-test indicated significant ($P < 0.01$) positive inotropy that developed within 5 min of addition of CTX-1 and was sustained to the end of the experiment. Mannitol had little effect on the course of this response.

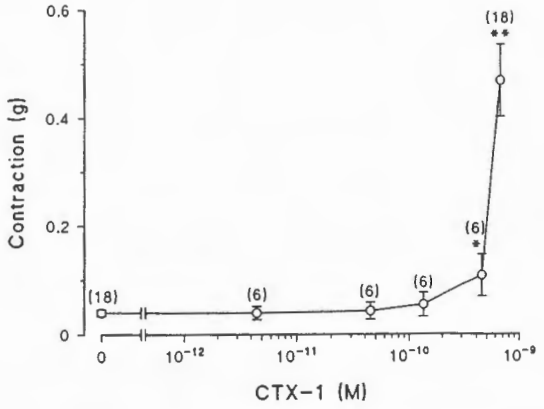

FIG. 3. CONCENTRATION RESPONSE FOR THE POSITIVE INOTROPIC EFFECTS OF CIGUATOXIN-1 (CTX-1) ON ISOLATED HUMAN ATRIA.

Tissues were obtained from patients with coronary artery disease and were electrically driven at 0.5 Hz. Maximum twitch contractions were measured in gram-tension (g) within 45 min of addition of each dose of CTX-1. Data are expressed as means ±S.E., *n* as indicated by numbers in parentheses. $*P < 0.05$; $**P < 0.01$, paired *t*-test for significant positive inotropic effect on control twitches.

The extent of positive inotropy that developed after CTX-1 varied considerably between preparations. Of the 30 preparations that responded to noradrenaline, ten preparations did not respond to CTX-1 at concentrations over the range of 4.7×10^{-10} to 3×10^{-9} M. Of the eight preparations that did not respond to noradrenaline, only one responded to CTX-1 at concentrations over the range 4.7×10^{-10} to 3×10^{-9} M. Seven of the 18 preparations responding to 7×10^{-10} M CTX-1 developed extrasystoles. Some preparations also developed delayed after-contractions (Fig. 1) that were similar to after-

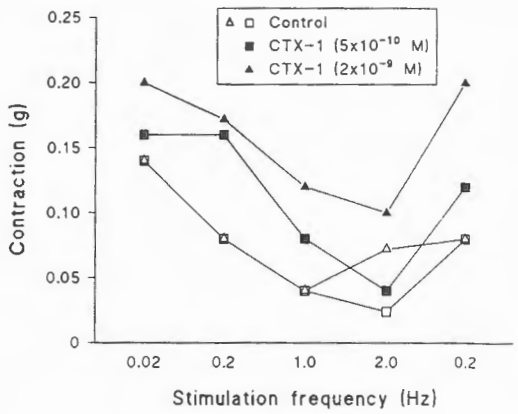

FIG. 4. EFFECT OF CIGUATOXIN-1 (CTX-1) ON THE FREQUENCY–FORCE RELATIONSHIP FOR ELECTRICALLY DRIVEN HUMAN ATRIA.

Twitch contractions were measured in gram-tension (g) after equilibration at each stimulation frequency (descriptive abscissa). Stimulation frequency was increased stepwise from 0.01 to 2 Hz, followed by a return to 0.2 Hz. Responses were measured before (open symbols) or after (closed symbols) addition of CTX-1 in two preparations. Similar responses were obtained for another three control preparations and one toxin treated preparation.

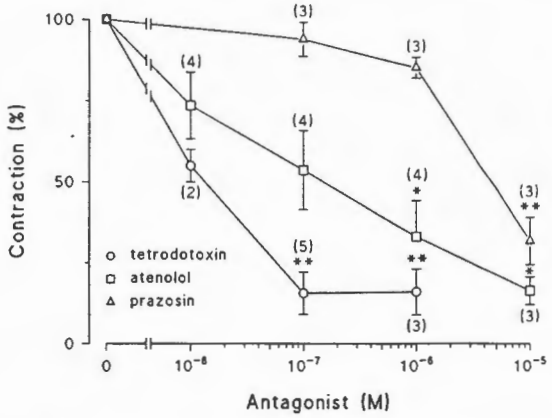

FIG. 5. EFFECTS OF TTX, ATENOLOL AND PRAZOSIN ON CIGUATOXIN-1-INDUCED POSITIVE INOTROPY IN ELECTRICALLY DRIVEN HUMAN RIGHT ATRIA.
Data points are the mean percentages ± S.E. of the maximum response to ciguatoxin-1 (7×10^{-10} M), n as indicated by numbers in parentheses. $^{*}P < 0.05$; $^{**}P < 0.01$, indicate significant inhibition of ciguatoxin-induced positive inotropy (paired t-test on untransformed data).

contractions observed in guinea-pig atria (LEWIS, 1988). CTX-1 enhanced the twitch responses of preparations stimulated at various frequencies between 0.02 and 2 Hz. The enhancement was maximal at intermediate stimulation frequencies (Fig. 4). Recovery of twitch levels at 0.2 Hz following 2 Hz stimulation indicates that these preparations were not significantly damaged at elevated stimulation frequencies by factors such as hypoxia.

Mannitol (50 mM) consistently augmented the responses of control (30%, $n = 8$) and CTX-1 (33%) treated trabeculae (Figs 1 and 2); however, these effects were not significant. The effect of mannitol was rapidly reversed upon washout in control and ciguatoxin-treated preparations. A similar response to mannitol has been observed in guinea-pig atria (BEYER *et al.*, 1986). In four of nine preparations treated with CTX-1, washout of mannitol was followed by a slow reduction in twitch response to below the level established prior to mannitol addition (see Fig. 1). Mannitol had no apparent effect on the delayed after-contractions seen in some preparations exposed to CTX-1 (Fig. 1).

To assess the mechanism of positive inotropy induced by CTX-1, TTX (a toxin selective for neuronal *vs* cardiac Na^+ channels) and antagonist drugs selective for cardiac adrenoreptors were applied to the organ bath after CTX-1. The CTX-1 potentiated twitch responses were partly reversed by atenolol (β_1 antagonist, 10^{-6} M) and prazosin (α_1 antagonist, 10^{-5} M) (Fig. 5). Further, TTX (10^{-7} M) and atenolol (10^{-5} M) completely reversed the positive inotropic effects of CTX-1 to control levels ($P > 0.15$, paired t-test).

DISCUSSION

This study reports the action of ciguatoxin on isolated human cardiac tissue. In the human atrial trabeculae investigated, pure CTX-1 caused a large, sustained and concentration-dependent positive inotropy. Similar potent effects of ciguatoxin on unmyelinated adrenergic and cholinergic nerves have been reported for preparations from other species (LEWIS and ENDEAN, 1984, 1986). The action of CTX-1 was resistant to washout (quasi-irreversible) with toxin-free Ringer. Ciguatoxin has been shown to bind quasi-irreversibly in guinea-pig atria, ilea and cultured neuroblastoma (LEWIS and ENDEAN, 1984, 1986;

BIDARD *et al.*, 1984), indicating that the affinity of CTX-1 for its receptor is similarly high among these mammalian tissues. In human atria, CTX-1 caused positive inotropy at concentrations greater than 1.5×10^{-10} M and this response was often associated with extrasystoles and/or after-contractions.

The positive inotropic action of CTX-1 was abolished by concentrations of TTX below those required to block myocardial sodium channels (HONERJÄGER, 1982; LEWIS and ENDEAN, 1986). This result indicates intrinsic neural elements were involved in the positive inotropic response, rather than direct modulation of cardiac sodium channels by CTX-1. Ten of the 30 preparations that responded to noradrenaline failed to respond to CTX-1, suggesting that these preparations lacked intrinsic neural elements capable of being effectively stimulated by CTX-1. The absence of a positive inotropic response to CTX-1 (up to 3×10^{-9} M) in these preparations further indicates that there is no direct positive inotropic component in human atrial trabeculae.

Atenolol (10^{-5} M), a β_1-selective antagonist that possesses little or no local anaesthetic-type activity (VAN ZWIETEN and TIMMERMANS, 1983), abolished the positive inotropic effect of CTX-1 in human atria. Complete inhibition of the CTX-1 induced response by atenolol indicates that β_1-receptor stimulation is the principal mechanism causing positive inotropy and that there is little, if any, β_2 adrenoreceptor involvement. The significance of β_1-receptors in the mediation of positive inotropy in response to noradrenaline in humans has been noted by MOTOMURA *et al.* (1990). The absence of inhibition by prazosin ($< 10^{-6}$ M) indicates that α_1-adrenoreceptor stimulation is not involved to any significant extent in the positive inotropy caused by CTX-1. The inhibitory action of prazosin at high concentrations is likely to stem from the local anaesthetic-type activity of prazosin (MCNEAL *et al.*, 1985). From these results we conclude that CTX-1 causes positive inotropy in human atrial trabeculae by stimulating intrinsic neural elements to release noradrenaline. The size of the inotropic response to 7×10^{-10} M CTX-1 was equivalent to that following addition of 3×10^{-6} M noradrenaline. Interestingly, the indirect response in human atria is sustained, whereas on guinea-pig atria the response is transient (LEWIS and ENDEAN, 1986).

We have proposed previously that the direct positive inotropic effect of ciguatoxin stems from its ability to reduce the Na^+ gradient across cardiac cell membranes which diminishes the ability of the Na^+/Ca^{2+} exchanger to extrude intracellular Ca^{2+} (LEWIS and ENDEAN, 1986). The absence of detectable direct effects of ciguatoxin indicates that the effect of a reduced sodium gradient may be less for human compared to guinea-pig atria. Alternatively, human myocardial sodium channels may have a lower affinity for ciguatoxin. These results suggest that the cardiotoxic effects associated with ciguatera probably stem from effects on the nerves that innervate the heart.

The relationship between stimulation frequency and the force of twitch contractions in human atrial trabeculae can vary considerably between preparations, but it is often triphasic in nature (LEVY, 1968). The preparations used in the present study showed responses to changes in stimulation frequency comparable to those reported by LEVY (1968). CTX-1 enhanced the twitch responses at all stimulation frequencies (0.02 to 2.0 Hz). In contrast, similar concentrations of ciguatoxin resulted in the abolition of the triphasic frequency–force relationship in guinea-pig atria as a consequence of the direct action of ciguatoxin (LEWIS and ENDEAN, 1986).

Mannitol is currently the treatment of choice during the acute phase of ciguatera. The effects of a i.v. mannitol infusion (1 g/kg over 30 min) are to relieve the neurological symptoms of ciguatera. This action is typically rapid (1–3 hr) and long lasting (PALAFOX *et*

al., 1988; PEARN *et al.*, 1989; WILLIAMSON, 1990). PEARN *et al.* (1989) have proposed that mannitol reverses an oedema of adaxonal Schwann cells that has been observed upon biopsy of a fatal case of ciguatera (ALLSOP *et al.*, 1986). However, other actions of mannitol, including a scavenging of free hydroxyl radicals (MAGOVERN *et al.*, 1984) or involving a direct effect on the ability of ciguatoxin to bind to sodium channels could not be discounted. The present study reveals that mannitol, at levels approximating peak serum levels that would be obtained during ciguatera treatment (CLOYD *et al.*, 1986), has little influence on the action of CTX-1 on human atrial trabeculae. The absence of a marked effect of mannitol on human atrial tissue treated with CTX-1 supports the conclusion that mannitol does not directly displace ciguatoxin from human sodium channels, and further indicates that mannitol is unlikely to reverse the symptoms of ciguatera by an action on peripheral non-myelinated nerves.

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Appendix V

Multiple ciguatoxins in the flesh of fish

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SHORT COMMUNICATIONS

MULTIPLE CIGUATOXINS IN THE FLESH OF FISH

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R. J. LEWIS and M. SELLIN. Multiple ciguatoxins in the flesh of fish. *Toxicon* **30,** 915–919, 1992.—Most cases of ciguatera (fish poisoning) result from consumption of the flesh of fishes contaminated with ciguatoxin(s); however, the relatively low toxicity of ciguateric fish flesh has hindered attempts to identify these ciguatoxin(s). Utilising high performance liquid chromatography, mass spectroscopy and mouse bioassay signs we have determined that ciguatoxin-1 (MH^+ m/z = 1112), ciguatoxin-2 and ciguatoxin-3 are the major ciguatoxins present in the flesh of ciguateric fish. Ciguatoxin-1, -2 and -3 were present in yields of 0.19, 0.09 and 0.02 μg/kg flesh, respectively, in *Scomberomorus commersoni*; 0.08, 0.09 and 0.07 μg/kg flesh, respectively, in *Plectropomus* spp. and; 0.67, 0.61 and 0.06 μg/kg flesh, respectively, in *Pomadasys maculatus*. Two minor toxins, which may be further oxidised analogues of ciguatoxin-1 and ciguatoxin-2, were also identified. The presence of multiple ciguatoxins in fish flesh has important consequences for the detection of ciguateric fish and may be a contributing factor to the observed variability in the symptoms of ciguatera.

CIGUATERA is an illness which affects in excess of 25,000 people annually. It is common in tropical and subtropical areas and is caused predominantly by eating the flesh of ciguateric fish. Neurological and gastrointestinal symptoms peculiar to the illness vary in relative intensity between regions, between ciguateric fish species, as well as between individuals consuming the same fish (GILLESPIE *et al.*, 1986; LEWIS *et al.*, 1988). Ciguatera was initially believed to stem from a single toxin (named ciguatoxin) found in the flesh of moray eels (SCHEUER *et al.*, 1967). Recent studies have determined structures for three ciguatoxins (CTX-1, -2 and -3) in the viscera of toxic moray eels (MURATA *et al.*, 1990; LEWIS *et al.*, 1991). However, the ciguatoxin(s) present in the flesh of ciguateric fish remain to be characterized. This study reports the purification and characterization of the ciguatoxins present in the flesh of three species of fish that often cause ciguatera. Use of a purification scheme optimized for ciguatoxins (LEWIS *et al.*, 1991) facilitated the isolation and characterization of the flesh ciguatoxins.

Since the level of ciguatoxins found in the fish flesh was low, the flesh from 13 narrow-barred Spanish mackerel (*Scomberomorus commersoni*), ten coral trout of the *Plectropomus* spp. complex, and 54 blotched javelin (*Pomadasys maculatus*) were extracted and the crude extracts pooled prior to purification. These flesh samples were collected over a 4-year period and stored at −20°C prior to extraction. The *S. commersoni* and

Plectropomus spp. samples were each implicated in a confirmed case of ciguatera in Australia. *Pomadasys maculatus* have been involved in outbreaks of ciguatera (LEWIS *et al.*, 1988) but samples directly involved in poisoning have not been collected owing to their small size (< 400 g whole weight). The *P. maculatus* used in this study were from batches confirmed to contain toxic individuals. Most of the *Plectropomus* spp. were captured on the Great Barrier Reef. All *P. maculatus* and most *S. commersoni* were caught in Platypus Bay, Queensland (24° 58'S, 153° 29'E), a ciguatera-endemic area (GILLESPIE *et al.*, 1986; LEWIS *et al.*, 1988; LEWIS and ENDEAN, 1983, 1984). *Gambierdiscus toxicus*, the dinoflagellate implicated in the production of the ciguatoxin precursors (gambiertoxins), also occurs in these waters (HOLMES *et al.*, 1991). The flesh from each species was extracted and the ciguatoxins purified using procedures [including PRP-1 (5 μm, Hamilton) and C-18 (5 μm, Merck) high performance liquid chromatography (HPLC)] developed to isolate homogeneous ciguatoxins from moray eel viscera (LEWIS *et al.*, 1991). Ciguatoxins from moray eel viscera were used as internal references in this study. In the absence of an alternative test for the ciguatoxins, the mouse bioassay (using 20 $\pm$ 2 g Quackenbush mice of either sex) was used to locate and quantify the toxic zones during purification. Fractions containing either a mixture of ciguatoxins or unknown ciguatoxins were quantified as mouse units (one MU is the i.p. LD_{50} dose in a 20 g mouse) using the equation for the relationship between dose and time to death: $\log (MU) = 2.3 \log (1 + t^{-1})$, where t = time to death in hr (LEWIS *et al.*, 1991). The total MU in fractions containing known ciguatoxins was estimated using the equation for the dose *vs* time to death relationship specific for that ciguatoxin. Since the quantity of the three major ciguatoxins purified was too small to be accurately weighed, their yield (g) was estimated from total MU, where one MU is equivalent to 5, 48 and 18 ng of CTX-1, -2 and -3, respectively (LEWIS *et al.*, 1991). At least two mice were used for the quantification of each fraction. Mice were used in accordance with the Australian National Health and Medical Research Council animal ethics guidelines. To determine the mol. wt for CTX-1 from flesh, A PE-Sciex (Thornhill, Ontario, Canada) API 111 triple quadrupole mass spectrometer with a pneumatically assisted electrospray (Ionspray) interface was used. C-18 HPLC purified CTX-1 samples from each fish were injected in acidified (0.1% trifluoroacetic acid) acetonitrile–water (1:1) with a syringe infusion pump at 3 μl/min.

The yields and toxicity at each step during purification of the ciguatoxins from three species of fish are indicated in Table 1. The ciguateric *P. maculatus* were on average considerably more toxic (0.26 MU/g) than either *S. commersoni* (0.07 MU/g) or *Plectropomus* spp. (0.04 MU/g), despite *P. maculatus* being a much smaller species. At each chromatographic step prior to HPLC, the flesh toxins eluted as a single toxic zone at the same location as found for the moray eel visceral ciguatoxins (LEWIS *et al.*, 1991). Reverse phase HPLC on the PRP-1 column separated the TSK HW40-S purified fraction into three major toxic zones for each fish analysed (Table 1). Each toxic zone corresponded to a u.v. absorbing peak detected at 215 nm and each eluted at the same time as either reference CTX-1, -2 or -3. Further, each flesh CTX-1 eluted as a homogeneous peak after additional C-18 HPLC at the same time as reference CTX-1. The elution profiles for the purification of the flesh ciguatoxins on the PRP-1 and C-18 columns were comparable to that obtained for the ciguatoxins from moray eels (LEWIS *et al.*, 1991). For the ciguatoxins in fish flesh and moray eel viscera, the areas of the eluting peaks ($A_{215} \cdot ml \cdot ng^{-1}$ of ciguatoxin) were similar (ranging from $6–7 \times 10^{-6}$, $5–6 \times 10^{-6}$ and $1.0–1.1 \times 10^{-5}$ for CTX-1, -2 and -3, respectively), indicating similar purity among the ciguatoxins, irrespective of their source. The bioassay signs in mice for each flesh

TABLE 1. PURIFICATION OF CIGUATOXINS FROM THE FLESH OF *Scomberomorus commersoni* (18.6 kg), *Plectropomus* spp. (9.1 kg) AND *Pomadasys maculatus* (3.6 kg)

Toxic fraction	*S. commersoni* g(MU)*	*Plectropomus* spp. g(MU)	*P. maculatus* g(MU)
Crude extract†	9.22(1350)	4.37(320)	5.80(920)
Silica gel‡	2.75(1320)	0.50(330)	1.01(650)
LH-20§	0.16(1400)	0.02(280)	0.10(600)
LH-20‖	2.7×10^{-3} (n.d.)	7.5×10^{-4} (220)	4×10^{-3} (530)
HW40-S¶	6×10^{-4} (n.d.)	2×10^{-4} (n.d.)	1.6×10^{-3} (n.d.)
PRP-1 HPLC**			
CTX-1	3.5×10^{-6} (690)	7.5×10^{-7} (150)	2.4×10^{-6} (480)
CTX-2	1.7×10^{-6} (36)	8.2×10^{-7} (17)	2.2×10^{-6} (46)
CTX-3	2.9×10^{-7} (16)	6.7×10^{-7} (37)	2.3×10^{-7} (13)

* Yield (g) for partially purified material is expressed as the weight of the dried fraction. Yield is also expressed throughout in total mouse units (MU), where one MU is the i.p. LD_{50} dose for a 20 g mouse, quantified using the appropriate dose *vs* time to death relationship and at least two mice (LEWIS *et al.*, 1991). For HPLC purified material, yield (g) was estimated from total MU, where one MU is equivalent to 5, 48 and 18 ng of CTX-1, -2 and -3, respectively (LEWIS *et al.*, 1991).

† Crude extract (diethyl ether and acetone soluble material) was obtained as described by LEWIS *et al.* (1991).

‡ The chloroform–methanol 9 : 1 fraction was the major toxic fraction.

§ Sephadex LH-20 column (Pharmacia) eluted with dichloromethane-methanol 1 : 1. Toxic zone located by bioassay of 5% of fractions (20 ml) collected.

‖ Sephadex LH-20 column eluted with methanol. Toxic zone located by bioassay of 5% of fractions (20 ml) collected.

¶ TSK HW40-S (Fractogel, Merck) eluted with methanol. Toxic zone located by bioassay of 5% of fractions (10 ml) collected.

** PRP-1 (5 μm, Hamilton) HPLC column eluted at 0.5 ml/min with acetonitrile–water (1 : 1) and eluant monitored at 215 nm with a Waters 481 detector.

CTX-1, -2, -3 = ciguatoxin-1, ciguatoxin-2, ciguatoxin-3. n.d. = not determined.

ciguatoxin were also comparable to the corresponding ciguatoxin from moray eel viscera, and included the presence of hind-limb paralysis for CTX-2 and -3 (LEWIS *et al.*, 1991). Ionspray mass spectroscopy determined that the MH^+ *m/z* was 1112 for each C-18 HPLC purified flesh CTX-1, as well as for reference moray eel viscera CTX-1. These values are comparable to values obtained for CTX-1 (MH^+ m/z = 1111.6) from moray eel viscera using fast atom bombardment mass spectroscopy (MURATA *et al.*, 1990; LEWIS *et al.*, 1991). The flesh ciguatoxins were not isolated in sufficient quantity to allow comparison of ^1H nuclear magnetic resonance spectra.

The yields of CTX-1, -2 and -3 from *S. commersoni* (0.19, 0.09 and 0.02 μg/kg flesh), *Plectropomus* spp. (0.08, 0.09 and 0.07 μg/kg flesh) and *P. maculatus* (0.67, 0.61 and 0.06 μg/kg flesh) (data derived from Table 1) were considerably less than found previously in moray eel viscera (10.1, 5.8 and 2.1 μg/kg viscera) (LEWIS *et al.*, 1991). However, the ratio of CTX-1 to CTX-2 was similar (ranging from 0.9:1 to 2:1) between the flesh and visceral sources. LEWIS *et al.* (1991) have proposed that CTX-1 and CTX-2 may originate from different gambiertoxins (ciguatoxin precursors). As the ratio of CTX-1 to CTX-2 is similar in a range of fish species from different locations it is likely that the ratio of the precursors of CTX-1 and CTX-2 is also relatively constant across ciguatera-prone areas. Recent evidence indicates that a single strain of *G. toxicus* may produce both precursors (HOLMES and LEWIS, 1991). The concentration of CTX-3 (relative to CTX-1) was comparable between *S. commersoni* and *P. maculatus* flesh and moray eel viscera, but the concentration of CTX-3 was considerably higher in *Plectropomus* spp. Factors that may contribute to different ratios of the ciguatoxins in fish include (1) differences in the ratio of

the ciguatoxins and their precursors in the diet of fishes, (2) differences in the ability of fishes to bio-transform the ciguatoxins and their precursors, and (3) differences in the ability of fishes to assimilate and excrete the various ciguatoxins (LEWIS *et al.*, 1992).

Several minor toxins (< 0.5% of total toxicity) that induced ciguatoxin-like signs in mice were also detected. These include a low polarity toxin from *S. commersoni* that eluted from PRP-1 HPLC in an isopropanol–acetonitrile (1:1) wash and toxins eluting at 11.4 min (*P. maculatus*) and at approximately 7.0 min (*S. commersoni*) on C-18 HPLC. These latter two toxins are more polar than CTX-1 (which eluted at 14.5 min) and may represent further oxidised analogues of CTX-2 and -1, respectively. Oxidation of the gambiertoxins to the more polar CTX-1 and -2 (LEWIS *et al.*, 1991), and possibly further, may be a strategy that accelerates the excretion of the ciguatoxins from fish.

This study reveals that CTX-1, -2 and -3 are the major ciguatoxins present in the flesh of fish. Confirmation that CTX-1 is the major toxin (on a mouse toxicity basis) in the flesh of a range of fish species, supports the use of CTX-1 from moray eel viscera for the development of antibody-based tests for the detection of ciguateric fishes. However, antibodies raised using CTX-1 coupled to a carrier protein through its primary hydroxyl may not have high cross-reactivity to CTX-2 or CTX-3, given the structural differences between these ciguatoxins (LEWIS *et al.*, 1991). Each major ciguatoxin possesses a primary hydroxyl to which a fluorophore could be coupled, thereby facilitating the selective detection of the flesh ciguatoxins. Considering that CTX-1, -2 and -3 have different effects *in vivo* (LEWIS *et al.*, 1991), the presence of different relative amounts of the three major ciguatoxins in the pooled flesh of *S. commersoni* compared with *Plectropomus* spp. may account for the different patterns of ciguatera that stem from consumption of these two species (LEWIS *et al.*, 1988). It remains to be determined to what extent the ratio of the three major ciguatoxins varies between ciguateric fish of the same species, and if the presence of multiple ciguatoxins has implications for the treatment of ciguatera.

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Appendix VI

Comparative action of three major ciguatoxins on guinea-pig atria and ilea

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COMPARATIVE ACTION OF THREE MAJOR CIGUATOXINS ON GUINEA-PIG ATRIA AND ILEA

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R. J. LEWIS and A. W. WONG HOY. Comparative action of three major ciguatoxins on guinea-pig atria and ilea. *Toxicon* **31**, 437–446, 1993.—The actions of pure ciguatoxin-1, ciguatoxin-2 and ciguatoxin-3 were assessed on the contractile activity of isolated guinea-pig left atria and ilea. Low concentrations of each ciguatoxin caused transient positive inotropy, whereas moderate concentrations induced transient and sustained positive inotropic phases. The transient positive inotropic phase was inhibited by tetrodotoxin or atenolol, indicating this phase stems from indirect effects of the ciguatoxins via the stimulation of intrinsic adrenergic nerves. On atria pretreated with atropine and α- and β-adrenoceptor antagonists to block neural actions of the ciguatoxins, moderate concentrations of each ciguatoxin induced only slowly developing, sustained positive inotropy. ED_{50}s for the indirect positive inotropic phase were 2.7×10^{-11}, 1.6×10^{-10} and 1.4×10^{-11} M and for the direct positive inotropic phase were 1.6×10^{-10}, 1.4×10^{-9} and 1.5×10^{-9} M for ciguatoxin-1, -2 and -3, respectively, indicating that their effects on neurons are 10-fold (ciguatoxin-1 and -2) to 100-fold (ciguatoxin-3) more potent than those directly on the myocardium. High concentrations of each ciguatoxin additionally induced sustained negative inotropy which could be reversed by lidocaine. On guinea-pig ilea, each ciguatoxin induced a transient contracture which could be abolished by atropine. Each ciguatoxin significantly reduced the contractile response of ilea to nicotine, without affecting the contractile response to acetylcholine. We conclude that ciguatoxin-1, -2 and -3 activate similarly the voltage-dependent Na^+ channels in neuronal and myocardial tissues, but vary in their relative affinity for the Na^+ channels in these tissues.

INTRODUCTION

CIGUATERA is the disease that results from consumption of fish contaminated with lipid-soluble toxins called ciguatoxins. Its clinical effects can vary considerably but typically include neurological, gastrointestinal and cardiovascular disturbances (GILLESPIE *et al.*, 1986; LEWIS *et al.*, 1988). The neurological and cardiovascular signs and symptoms have been successfully treated with intravenous mannitol (PALAFOX *et al.*, 1988; PEARN *et al.*, 1989). Three major ciguatoxins (CTX-1, -2 and -3) separable by reverse-phase HPLC have been isolated from the viscera of moray eels (MURATA *et al.*, 1990; LEWIS *et al.*, 1991). CTX-1 (MH^+ $m/z = 1111$) has one additional hydroxyl compared with both CTX-2 and -3 (MH^+ $m/z = 1095$) and CTX-2 differs from CTX-3 by a stereochemical modification (LEWIS *et al.*, 1991). These structural differences between CTX-1, -2 and -3

significantly influence mouse lethality (i.p. LD_{50}s of 0.25, 2.3 and 0.9 μg/kg, respectively) and neuronal Na^+ channel binding affinity (ED_{50}s of 0.23, 0.85 and 0.43 ng/ml, respectively) (LEWIS *et al.*, 1991). A recent finding indicates that these ciguatoxins are present in variable levels in the flesh of ciguateric fish, a factor which may contribute to the variability in the symptomology of ciguatera (LEWIS and SELLIN, 1992).

Previous studies have investigated the direct and indirect (via intrinsic nerves) actions of ciguatoxin on cardiac tissue (OHSHIKA, 1971; LEGRAND and BAGNIS, 1984; MIYAHARA *et al.*, 1985; LEWIS and ENDEAN, 1986; LEWIS, 1988; SEINO *et al.*, 1988; LEWIS *et al.*, 1992) and have assessed the mode of action of ciguatoxin on smooth muscle (OHIZUMI *et al.*, 1981, 1982; LEWIS and ENDEAN, 1984) and skeletal muscle and associated nerves (RAYNER, 1972; LEWIS and ENDEAN, 1983; BENOIT *et al.*, 1986; MOLGÓ *et al.*, 1990; WONG HOY and LEWIS, 1992). These previous studies reveal that ciguatoxin (typically CTX-1 or a mixture of ciguatoxins of varying purities) activates voltage-dependent Na^+ channels. In the present study we determine the mode of action of pure CTX-2 and CTX-3 on the contractile activity of isolated guinea-pig left atria and ilea and compare these actions with those of pure CTX-1.

MATERIALS AND METHODS

Isolated preparations

Procedures for studying contractile activity of guinea-pig ilea and electrically stimulated left atria follow those described previously (LEWIS and ENDEAN, 1984, 1986). Preparations were placed in carbogenated (95% O_2/5% CO_2) Ringer's solution of the following composition (mM): 115 NaCl, 4.7 KCl, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 glucose (atria), *or* 137 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 0.5 KH_2PO_4, 11.9 $NaHCO_3$, 5.6 glucose (ilea). Atria were suspended under 1 g pre-load in 5-ml organ baths maintained at 31–33°C and stimulated at 1 Hz. Isometric (F-60 transducer, Narco Biosystems) tension changes (g) were recorded on chart recorders. Control responses to noradrenaline (6×10^{-7} M) were obtained for each atrium. Ilea were suspended under 0.5 g pre-load in 5-ml organ baths maintained at 36–38°C and isometric tension changes again recorded. For ilea, the maximum contractile response (including spike activity) time to maximum contracture and duration of contracture (disregarding spike activity) were measured. Control phasic responses to acetylcholine (10^{-6} M) and nicotine (4×10^{-6} M) were obtained for each ileal segment. Atria and ilea were allowed to equilibrate until responses to the control agonists were reproducible (typically 30–60 min after initial set-up).

Ciguatoxins

CTX-1, CTX-2 and CTX-3 were purified to homogeneity as described by LEWIS *et al.* (1991). Each ciguatoxin was dissolved in a small volume of methanol-water (1 : 1) which was sonicated immediately prior to use. The volume of methanol added (< 5 μl) had no effect on control responses in either preparation. Concentrations of the ciguatoxins are expressed in moles/litre (M). In some experiments, the action of a bolus dose of the ciguatoxins [0.15 (atria) or 0.25 (ilea) mouse unit/ml] was assessed [one mouse unit (MU) is the LD_{50} dose for a 20 g mouse and is equivalent to 5 ng CTX-1, 48 ng CTX-2 and 18 ng CTX-3 (LEWIS *et al.*, 1991)].

Drugs

The following toxin and drugs were used: tetrodotoxin (TTX), prazosin hydrochloride, propranolol hydrochloride, atropine sulphate, acetylcholine hydrochloride, nicotine hydrogen tartrate, lidocaine (Sigma, St. Louis, U.S.A.) and noradrenaline (Winthrop, Sydney, Australia). Atenolol was a generous gift from ICI Pharmaceuticals, Australia. Drugs were applied either 20–30 min before, or after addition of the ciguatoxins.

Statistical method

Results are presented as the mean ± 1 S.E. Student's *t*-test was used to compare means, with $P < 0.05$ considered significant. ED_{50} ± S.E. values for the ciguatoxins on atria were estimated by fitting a sigmoid curve to the responses measured over a range of ciguatoxin concentrations using non-linear curve fitting procedures (Fig. P software, Biosoft, U.S.A.).

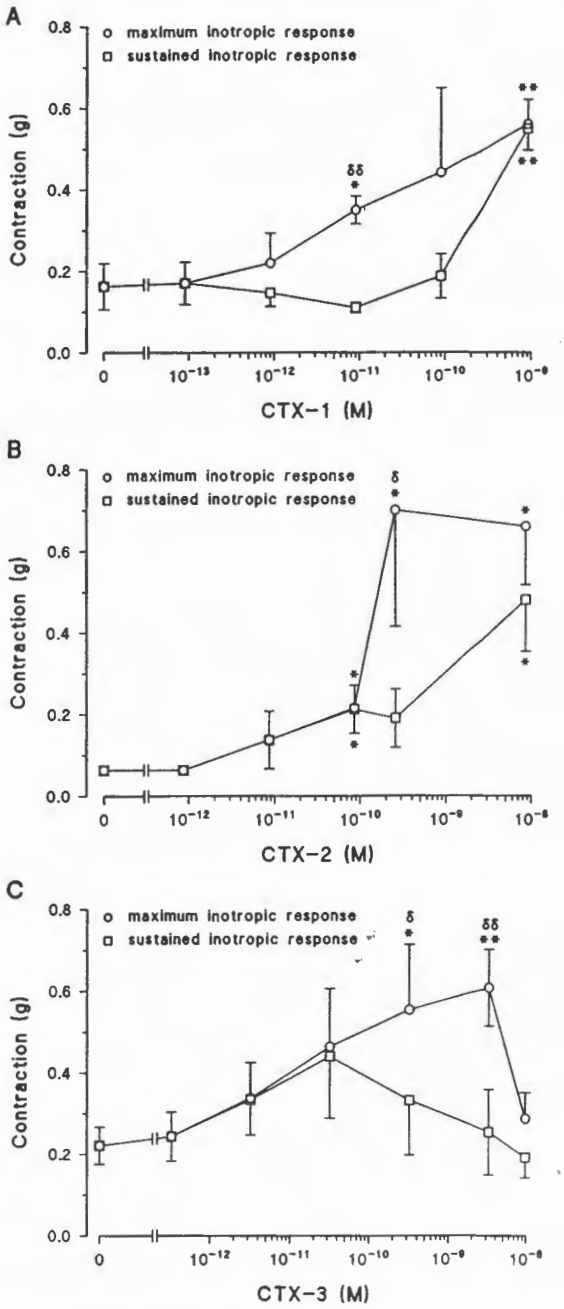

FIG. 1. POSITIVE INOTROPIC EFFECTS OF CIGUATOXINS ON ATRIA: (A) CTX-1, (B) CTX-2, (C) CTX-3. Each ciguatoxin was added cumulatively to isolated guinea-pig left atria electrically stimulated at 1 Hz. After addition of each dose of ciguatoxin, the maximum positive inotropic response (○, typically 1–5 min after ciguatoxin) and the sustained positive inotropic response (□, typically 30–90 min after the maximum response) were measured in gram-tension (g). Data are expressed as means ± S.E., $n = 4$–5. Control responses to noradrenaline (6×10^{-7} M) were similar (0.83 ± 0.12, 0.71 ± 0.12 and 0.95 ± 0.08 g for preparations testing CTX-1, -2 and -3, respectively). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant positive inotropy (paired t-test). $^{\delta}P < 0.05$, $^{\delta\delta}P < 0.01$ indicate significant difference between the maximum positive inotropy and the sustained inotropy at comparable ciguatoxin concentration (two-tailed t-test).

RESULTS

Guinea-pig left atria

In electrically stimulated left atria, CTX-1, -2 and -3 each caused concentration-dependent, transient positive inotropy (increased twitch contraction) that at low concentrations gradually declined to control levels over 30–90 min (Fig. 1). The estimated ED_{50}s for the transient positive inotropic phase were $2.7 \pm 2.4 \times 10^{-11}$, $1.6 \pm 1.0 \times 10^{-10}$ and $1.4 \pm 0.3 \times 10^{-11}$ M for CTX-1, -2 and -3, respectively (estimated from the maximum inotropic response data in Fig. 1). In these preparations, moderate concentrations of CTX-1, -2 and -3 caused positive inotropy that was sustained only for CTX-1 and -2 (Fig. 1). A bolus dose of CTX-1, -2 or -3 (each added at 0.15 MU/ml) induced positive inotropy that commenced in 10–20 sec of addition of CTX-1 and -3 and 20–60 sec of addition of CTX-2, and peaked after 2–5 min before gradually declining (Fig. 2). The maximum size of the positive inotropic responses attained with cumulative (Fig. 1) or bolus (Fig. 2) doses was similar for each ciguatoxin. Washing with toxin-free Ringer had little effect on the positive inotropic response (Fig. 2B). The early phase of this positive inotropy was interrupted by a brief failure of positive inotropy (Fig. 2A). This brief effect was observed consistently for CTX-2 (4/4) and less frequently for CTX-1 (1/4) and CTX-3 (2/4), was most prominent 50 sec, 40 ± 10 sec and 160 ± 30 sec after addition of CTX-1, -2 and -3, respectively, and was not observed in preparations pretreated with 10^{-6} M atropine. In

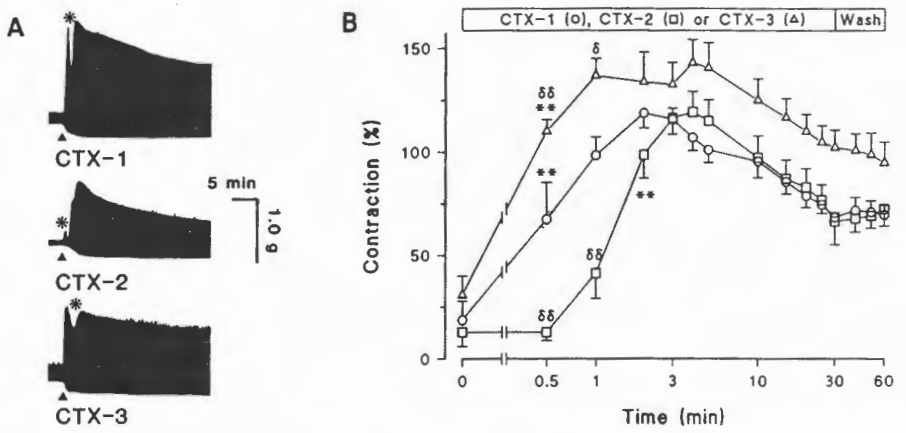

FIG. 2. ACTION OF THREE CIGUATOXINS (CTX) ON ATRIA: (A) ORIGINAL RECORDINGS, (B) TIME-COURSE OF EFFECT.

The actions of bolus doses of CTX-1, -2 or -3 were determined on isolated guinea-pig left atria electrically stimulated at 1 Hz. Preparations were exposed to the ciguatoxins for 30 min, followed by 30 min wash with toxin-free Ringer. Ciguatoxins were added at 0.15 MU/ml (equivalent to 7×10^{-10}, 2.5×10^{-9}, and 7×10^{-9} M CTX-1, -2 and -3, respectively). (A) Representative recordings of the effect of CTX-1, -2 and -3 on atria. Twitch contractions are measured in gram-tension (g) and the time base is min. These traces show the brief failure of positive inotropy (*) and the large positive inotropic component that gradually declined after reaching a maxima. (B) Time-course of the inotropic response. Data are presented as mean percentage ± S.E. of the response to 6×10^{-7} M noradrenaline ($n = 4$). Noradrenaline responses were similar (1.16 ± 0.18, 1.14 ± 0.16 and 0.71 ± 0.16 g for preparations testing CTX-1, -2 and -3, respectively). $^{**}P < 0.01$ indicates the onset of significant positive inotropy (paired t-test). $^{\delta}P < 0.05$, $^{\delta\delta}P < 0.01$ indicate significant differences between CTX-1 responses and those of CTX-2 or CTX-3 at equivalent times after their addition (two-tailed t-test).

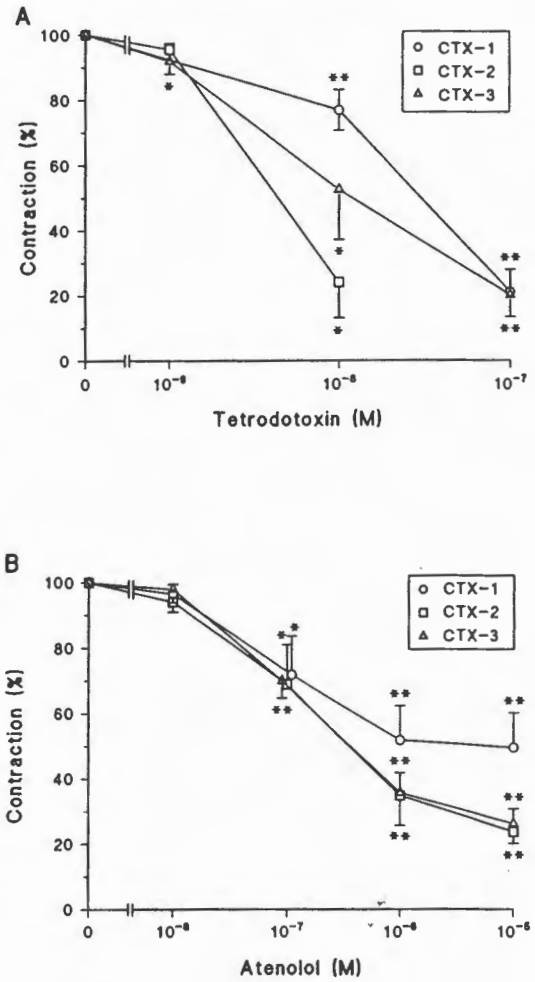

FIG. 3. EFFECT OF TETRODOTOXIN AND ATENOLOL ON CIGUATOXIN-INDUCED POSITIVE INOTROPY: (A) TETRODOTOXIN, (B) ATENOLOL.

Tetrodotoxin and atenolol were added cumulatively to guinea-pig left atria approximately 30 min after exposure to 0.15 MU/ml ciguatoxin (equivalent to 7×10^{-10}, 2.5×10^{-9} or 7×10^{-9} M CTX-1, -2 and -3, respectively). Data are the mean percentages $\pm$ S.E. of the ciguatoxin responses prior to drug addition; $n = 4$–7 in panel (A) and $n = 6$–8 in panel (B). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant inhibition of the ciguatoxin-induced positive inotropy (paired t-test on untransformed data).

two of four preparations, CTX-2 transiently reduced the twitch response to below the control level.

TTX (a voltage-dependent Na^+ channel blocker selective for neuronal *vs.* myocardial channels) and atenolol (a β_1-adrenoceptor blocker) each rapidly and concentration-dependently reversed a significant component of the positive inotropic action of moderate doses of each ciguatoxin (Fig. 3). For each ciguatoxin, this action of TTX or atenolol was reversed upon washout with Ringer to again reveal the ciguatoxin-induced positive inotropy, indicating that these antagonists acted non-competitively.

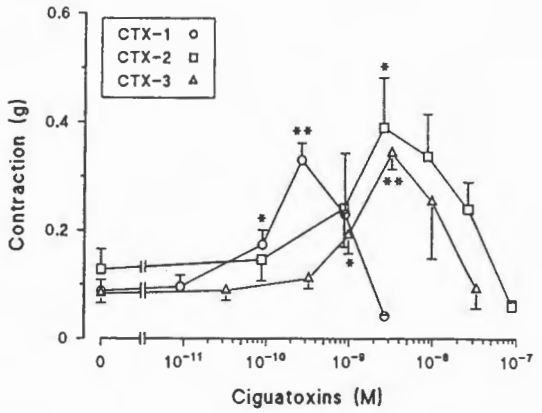

FIG. 4. DIRECT ACTION OF THREE CIGUATOXINS (CTX) ON ATRIA.
CTX-1, -2 or -3 were applied cumulatively to electrically stimulated guinea-pig atria pre-treated with atenolol (10^{-5} M), propranolol (2×10^{-7} M), prazosin (10^{-7} M) and atropine (10^{-6} M) for 20 min to abolish the action of neurotransmitters released by the ciguatoxins. The positive inotropic response which stabilized within 30–45 min of toxin addition was measured in gram-tension (g). Data are expressed as means $\pm$ S.E., $n = 4$. Control noradrenaline (6×10^{-7} M) responses obtained prior to the addition of antagonists were similar (0.80 ± 0.03, 0.75 ± 0.1 and 0.69 ± 0.05 g for preparations testing CTX-1, -2 and -3, respectively). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant positive inotropy (paired t-test).

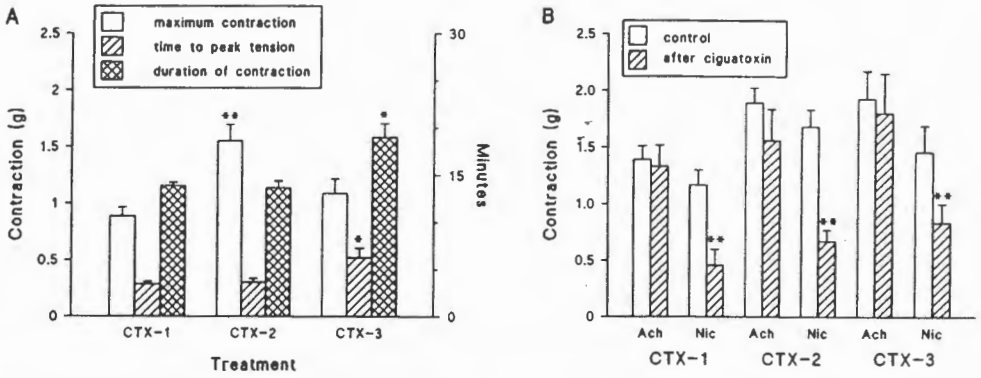

FIG. 5. ACTION OF THREE CIGUATOXINS ON ILEA: (A) CONTRACTILE RESPONSE (B) INFLUENCE ON ACETYLCHOLINE AND NICOTINE RESPONSES.
(A) The actions of a bolus dose of CTX-1, -2 or -3 was determined on isolated guinea-pig ileum. The ciguatoxins were added at 0.25 MU/ml (equivalent to 1.1×10^{-9}, 1.1×10^{-8} and 4.1×10^{-9} M CTX-1, -2 and -3, respectively). Maximum contractile response (g), time to maximum contracture (min) and the duration of the contracture (min) were measured ($n = 6$). $^{**}P < 0.01$ indicates a significant difference between maximum responses produced by CTX-2 compared to those produced by CTX-1 or -3 (two-tailed t-test). $^{*}P < 0.05$ indicates an increase in the time to maximum contracture and the duration of contracture for CTX-3, compared with CTX-1 and -2 (two-tailed t-test). (B) Responses (g) to acetylcholine (Ach, 10^{-6} M) and nicotine (Nic, 4×10^{-6} M) were obtained before (control) and after addition of the ciguatoxins (test response, $n = 6$). Test responses to Ach were obtained 30 min after addition of ciguatoxin and prior to its washout. Test responses to Nic were obtained 15 min after washout of the acetylcholine-ciguatoxin combination. $^{**}P < 0.01$ indicates the ciguatoxins significantly reduced the nicotine responses (paired t-test).

To compare quantitatively the direct effects of ciguatoxin on myocardium, atria were pretreated with a combination of atenolol, propranolol (a β_1- and β_2-adrenoceptor blocker), prazosin (an α_1-adrenoceptor blocker) and atropine (a muscarinic acetylcholine receptor blocker) to abolish the indirect inotropic effects of ciguatoxin. In these preparations, moderate doses of each ciguatoxin caused concentration-dependent positive inotropy (Fig. 4) that was characterized by its delayed onset (1.3 ± 0.1, 2.9 ± 0.7 and 0.6 ± 0.1 min for CTX-1, -2 and -3, respectively, $n = 4$) and its slow development (maximum positive inotropy attained after 30 min). The estimated ED_{50} for the direct, positive inotropic action of CTX-1 ($1.6 \pm 0.1 \times 10^{-10}$ M) was 10-fold lower than the ED_{50}s for CTX-2 and -3 ($1.4 \pm 0.1 \times 10^{-9}$ and $1.5 \pm 0.1 \times 10^{-9}$ M, respectively, $n = 4$), whilst the maximum positive inotropy attained as a result of the direct effects of CTX-1, -2 and -3 were similar (0.41 ± 0.07, 0.47 ± 0.06 and 0.41 ± 0.13 g, respectively, $n = 4$). High concentrations of each ciguatoxin additionally caused dose-dependent negative inotropy (Fig. 4) that developed slowly over a 45 min period and could not be reversed upon washout with toxin-free Ringer. Lidocaine and elevated concentrations of Ca^{2+} have been suggested as potential therapies (RAYNER, 1972; LEWIS, 1988). Addition of calcium chloride (3.2 mM final concentration) and lidocaine (5×10^{-5} M) reversed the negative inotropic effects of each ciguatoxin ($n = 3$), an effect similar to that reported for a reverse-phase HPLC purified ciguatoxin (LEWIS, 1988). A higher concentration of lidocaine (5×10^{-4} M) caused a marked negative inotropic action in these preparations ($n = 3$).

Guinea-pig ilea

A bolus dose of 0.25 MU/ml of each ciguatoxin caused a large, transient contracture of ileal segments, overlayed with spikes of contractile activity. This response is similar to the transient contractions (isotonically recorded) induced by a reverse-phase HPLC purified ciguatoxin (LEWIS and ENDEAN, 1984). CTX-2 induced the largest response and CTX-3 was the slowest acting (Fig. 5A). CTX-2 (0.05 MU/ml) induced a response (0.68 ± 0.14 g, $n = 3$) that was significantly smaller than that induced by 0.25 MU/ml CTX-2 ($P < 0.01$) but was comparable in size with contractions induced by 0.25 MU/ml CTX-1 or -3. Each ciguatoxin significantly inhibited the contractile action of the ganglion stimulant nicotine, whereas acetylcholine-induced contractions were unaffected by the presence of the ciguatoxins (Fig. 5B). CTX-2 (0.25 MU/ml) was without effect on the contractile activity of ilea pretreated with atropine (2×10^{-6} M, $n = 3$).

DISCUSSION

Ciguatoxins -1, -2 and -3 each caused a large, rapidly developing, concentration-dependent increase in the force of twitch contractions (positive inotropy) of electrically stimulated guinea-pig left atria. For low concentrations of each ciguatoxin, positive inotropy was transient, gradually declining to control levels over 30–90 min. A significant component of this positive inotropy was rapidly reversed by atenolol (a β_1-adrenoceptor blocker without local anaesthetic-type activity) or low concentrations of TTX, indicating that this component stemmed from the release of noradrenaline via the stimulation of intrinsic adrenergic neuronal elements by each of the ciguatoxins (indirect effect). An order of magnitude higher molar concentration of CTX-2 was required to induce indirect

positive inotropy, compared with CTX-1, or -3. The indirect action of each ciguatoxin was resistant to washout, showing that each binds in a quasi-irreversible manner to its binding site on neuronal Na^+ channels.

CTX-1, -2 or -3 induced rapidly developing positive inotropy that was often interrupted by a brief failure of positive inotropy. Such a pattern was not observed in atropine pretreated preparations. This result suggests that the ciguatoxins may cause the release of acetylcholine via the stimulation of intrinsic cholinergic nerves in guinea-pig atria. This phase was most prominent after addition of CTX-2 and may account for the delay in the onset of positive inotropy observed for CTX-2, compared with CTX-1 and -3. The appearance of an acetylcholine-induced negative inotropy after the addition of ciguatoxin has been reported previously for rabbit and rat atria (OHSHIKA, 1971; LEGRAND and BAGNIS, 1984).

In addition to the transient positive and negative inotropic phases, moderate concentrations of each of the ciguatoxins induced a sustained, concentration-dependent positive inotropic phase. This phase was quantified in atria pretreated with muscarinic and α- and β-adrenoceptor blockers to abolish the indirect effects of the ciguatoxins. The direct positive inotropy was produced by similar concentrations of CTX-2 and -3 and an order of magnitude lower concentration of CTX-1. Concentrations of each ciguatoxin exceeding those which produced maximum positive inotropy caused concentration-dependent negative inotropy, despite these preparations being pretreated with atropine. The ED_{50}s for the negative inotropy were approximately an order of magnitude greater than their respective ED_{50} for the direct positive inotropy for each ciguatoxin. Lidocaine, a local anaesthetic with voltage-dependent Na^+ channel blocking activity that has been proposed to block selectively the ciguatoxin-modified channel (LEWIS, 1988), could reverse this negative inotropic phase for each ciguatoxin. The negative inotropic effect of a high concentration of each ciguatoxin was resistant to washout, indicating that each ciguatoxin binds in a quasi-irreversible manner to myocardial Na^+ channels. In contrast to the above findings for guinea-pig atria, CTX-1 induced only an indirectly mediated positive inotropy in human atria that was sustained for several hours (LEWIS *et al.*, 1992).

The contractile responses of guinea-pig ilea to each of the ciguatoxins were qualitatively comparable. Each of the ciguatoxins inhibited contractions elicited by nicotine to a similar extent, presumably through the inhibitory effects on cholinergic nerves of ciguatoxin-induced depolarization (LEWIS and ENDEAN, 1984), whereas contractions elicited by acetylcholine were unaffected by the ciguatoxins. The latter finding suggests that the ciguatoxins do not have a direct effect on ileal smooth musculature. In contrast, MILLER (1991) has reported that extracts containing ciguatoxin cause reversible inhibition of the ileal response to acetylcholine, an effect suggested as the basis for the development of an assay for ciguatoxin. The contractile response to CTX-2 was abolished by atropine, indicating that this response was mediated entirely via the effects of acetylcholine.

Ciguatoxin-1, -2 and -3 share a common binding site (site 5) on neuronal voltage-dependent Na^+ channels that overlap the brevetoxin binding site (LEWIS *et al.*, 1991). We propose, on the basis of the present results, that the binding of these ciguatoxins to myocardial or neuronal Na^+ channels leads to similar activation of voltage-dependent Na^+ channels. On atria, the relative potency (ED_{50}^{-1}) of CTX-1, -2 and -3 for the indirect positive inotropic effects (1 : 0.17 : 1.9) differed from the relative potency for their respective direct effects (1 : 0.11 : 0.11). Specifically, CTX-3 was two orders of magnitude more potent on the intrinsic neuronal elements of the heart (indirect effect) than when causing positive inotropy directly on the myocardium, whereas CTX-1 and -2 were only one order

of magnitude less potent at eliciting direct compared with indirect responses. A less oxygenated analogue of the ciguatoxins (a gambiertoxin) resembles CTX-3 in that it is also considerably more potent on the intrinsic neuronal elements of atria than directly on the myocardium (HOLMES *et al.*, 1991). These results suggest that the relatively small structural differences between the ciguatoxins (LEWIS *et al.*, 1991) may underlie significant differences in their relative affinity for neuronal compared with myocardial Na^+ channels. If this is indeed correct, these differences may contribute to the differences in the symptoms they induce in mice (LEWIS *et al.*, 1991) and to variability in the symptomology of ciguatera in humans.

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Appendix VII

Mannitol reverses the action of ciguatoxin *in vitro*

MANNITOL REVERSES THE ACTION OF CIGUATOXIN *IN VITRO*

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(Introduced by J. Wanstall)

Ciguatera is prevalent in Australia and is caused by the ingestion of fish that have accumulated toxic levels of ciguatoxin. Ciguatera is notable for its long-term debilitating effects and the insufficiency of present treatments (Gillespie et al., 1986). A new treatment using an i.v. infusion of mannitol has been used successfully to treat ciguatera in the Marshall Islands (N. Palafox & L. Jain, personal communication). In this study the interaction between mannitol and ciguatoxin *in vitro* has been examined using the isolated guinea-pig left atria. Guinea-pigs were killed and left atria rapidly removed and suspended in Ringer solution (1.2 mmol l^{-1} Ca^{++}). Preparations were electrically stimulated at 1 Hz and twitch contractions recorded as described previously (Lewis & Endean, 1986). Ciguatoxin was applied to atria after the washout (30 min) of a test dose of mannitol (n=3). Mannitol (50 mmol l^{-1}) alone caused a small positive inotropic response that was reversed after wash out of mannitol, as shown previously (Beyer et al., 1986). These preparations were then exposed to ciguatoxin (4×10^{-10} mol l^{-1}) which induced a long lasting positive inotropic response, an action shown previously to stem from the ability of ciguatoxin to open tetrodotoxin-sensitive sodium channels in myocardial cells (Lewis & Endean, 1986). This action was not reversed by extended washing with toxin-free Ringer solution. However, the positive inotropic action of ciguatoxin was reversed (over approximately 1 hr) when the Ringer solution was subsequently made hypertonic by the addition of mannitol (50 mmol l^{-1}), an effect that remained after the washout of mannitol. The persistance of this antagonism by mannitol contrasts with effects of other antagonists of the action of ciguatoxin on atria, including tetrodotoxin, lidocaine and propranolol. These latter are effective only while atria are directly exposed to these drugs, indicating these antagonists do not act by displacing ciguatoxin from its binding site (Lewis & Endean, 1986; Lewis, in press).

In conclusion, this study shows that *in vitro* mannitol causes long lasting reversal of the action of ciguatoxin on the atria. Mannitol may increase the dissociation of ciguatoxin from its binding site, possibly through the dehydrating action of mannitol. Studies are in progress to determine the mechanism(s) underlying the mannitol-ciguatoxin interaction. This study is compatible with the recent finding that mannitol reduces the severity and duration of ciguatera in human sufferers.

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Appendix VIII

Ciguatoxin-2 is a diastereomer of ciguatoxin-3

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CIGUATOXIN-2 IS A DIASTEREOMER OF CIGUATOXIN-3

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R. J. LEWIS, R. S. NORTON, I. M. BRERETON and C. D. ECCLES. Ciguatoxin-2 is a diastereomer of ciguatoxin-3. *Toxicon* **31,** 637–643, 1993.—Ciguatoxin-2, a major ciguatoxin present in the flesh and viscera of ciguateric fishes, has been shown by ^{1}H nuclear magnetic resonance studies (2-dimensional homonuclear Hartman Hahn, nuclear Overhauser effect and decoupling difference experiments) to be a diastereomer of ciguatoxin-3, differing only in stereochemistry at carbon 52 (a quaternary carbon). This difference accounts for the significant changes in the chemical shift of resonances for protons in this region of ciguatoxin-2. Differences between ciguatoxin-1, -2 and -3 involve modifications at only one end of the ciguatoxins (ring M) and modest differences in potency, indicating that this ring contributes to, but is not critical for, high affinity binding of the ciguatoxins to voltage-dependent sodium channels. It is proposed that ciguatoxin-2 originates from a different precursor to the precursor (presumably gambiertoxin-4b) for ciguatoxin-1 and -3, and that both precursors are produced by a common biosynthetic pathway in *Gambierdiscus toxicus*.

INTRODUCTION

CIGUATERA is an often distressing disease caused by the consumption of warm-water fish contaminated with ciguatoxins. The ciguatoxins (CTX) presumably arise through the oxidation of precursors (gambiertoxins) produced by *Gambierdiscus toxicus*, and are characterized by high affinity binding to voltage-dependent sodium channels (MURATA *et al.*, 1990; HOLMES *et al.*, 1991; LEWIS *et al.*, 1991). Three major ciguatoxins are typically found in the flesh and viscera of ciguateric fishes in relative abundance CTX-1 > CTX-2 > CTX-3 (LEWIS *et al.*, 1991; LEWIS and SELLIN, 1992). Structures for CTX-1 and -3 have been proposed on the basis of ^{1}H nuclear magnetic resonance (NMR) and mass spectroscopy (MURATA *et al.*, 1990; LEWIS *et al.*, 1991). In contrast to CTX-1, CTX-3 is missing a hydroxyl at carbon 54 and is presumably an intermediate in the metabolism of a gambiertoxin (GTX-4b) to CTX-1 (LEWIS *et al.*, 1991). CTX-2 has the same mol. wt (m/z $MH^+ = 1095$) as CTX-3 but its structure remains to be fully characterized (LEWIS *et al.*, 1991). Structural difference(s) between CTX-2 and CTX-3 contribute to a significant reduction in activity of CTX-2, including a three-fold reduction in lethality to mice and a

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two-fold reduction in affinity for neuronal voltage-dependent sodium channels (LEWIS *et al.*, 1991). In this study we report the structure and configuration of CTX-2 (Fig. 1) and discuss structure–activity relationships among the ciguatoxins and the origin of CTX-2.

METHODS

Ciguatoxin-2 (CTX-2)

CTX-2 was purified to homogeneity in 1991 as described previously (LEWIS *et al.*, 1991). All experiments were performed on a 0.28 mg sample of CTX-2 in 0.45 ml of pyridine-d_5.

^{1}H nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR spectra of CTX-2 were all obtained in pyridine-d_5 (99.96% deuterium, Cambridge Isotope Laboratories) at 500 MHz in 5 mm spinning tubes. The two-dimensional homonuclear Hartman Hahn experiment (HOHAHA) was performed as described by BAX and DAVIS (1985) on a Bruker AM-500 spectrometer at 25°C (436 t_1 values, each with 2048 complex data points over a sweep width of 5208 Hz) with a mixing time of 53.6 msec. Data were zero-filled to produce a 4K × 1K matrix. To improve resolution and signal to noise, a 9 Hz Gaussian deconvolution was applied to t_2 data, and a Hanning filter applied to t_1 data. The HOHAHA experiment was processed using FTTOOL (Centre for Magnetic Resonance, University of Queensland) and analysed on a SUN SPARCstation-2. One-dimensional ^{1}H NMR was performed as described previously (LEWIS *et al.*, 1991). The configuration of CTX-2 was established from coupling patterns and HOHAHA connectivities. One-dimensional nuclear Overhauser effect (NOE) difference experiments obtained on the AM-500 at 25°C (without solvent degassing) provided additional information on the structure of CTX-2. Vicinal couplings between protons on rings L and M were confirmed from ^{1}H NMR decoupling difference spectra measured on a Bruker AMX-500 at 30°C. Chemical shifts are given in ppm downfield of tetramethylsilane using the pyridine resonance at 7.21 ppm as the internal reference.

RESULTS AND DISCUSSION

The structure of CTX-2 from ciguateric moray eels has been determined using 500 MHz ^{1}H NMR on a 0.5 mM sample. Comparison of one-dimensional (see LEWIS *et al.*, 1991) and two-dimensional ^{1}H NMR spectra of CTX-2 (Figs 2 and 3) to corresponding spectra of CTX-1 (MURATA *et al.*, 1990; our unpublished results) revealed that most HOHAHA connectivities, coupling patterns and chemical shifts for proton resonances were the same for these two toxins. The HOHAHA experiment confirmed that OH-54 was absent from CTX-2, accounting for the 16 mass units difference between CTX-2 and CTX-1. This hydroxyl is also missing in CTX-3 and GTX-4b (MURATA *et al.*, 1990; LEWIS *et al.*, 1991).

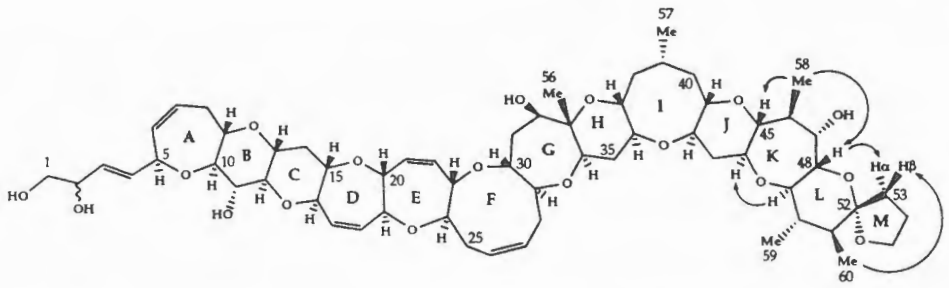

FIG. 1. STRUCTURE AND CONFIGURATION OF CTX-2.
NOE difference experiments in pyridine-d_5 at 25°C detected NOEs from irradiated protons (arrow tail) to observed protons (arrow head) on rings K, L or M. Structure and configuration of rings A to J of CTX-2 were determined by comparison with corresponding spectra of CTX-1. Nomenclature follows that proposed for ciguatoxin (MURATA *et al.*, 1990). CTX-3 differs from CTX-2 only in stereochemistry at carbon 52.

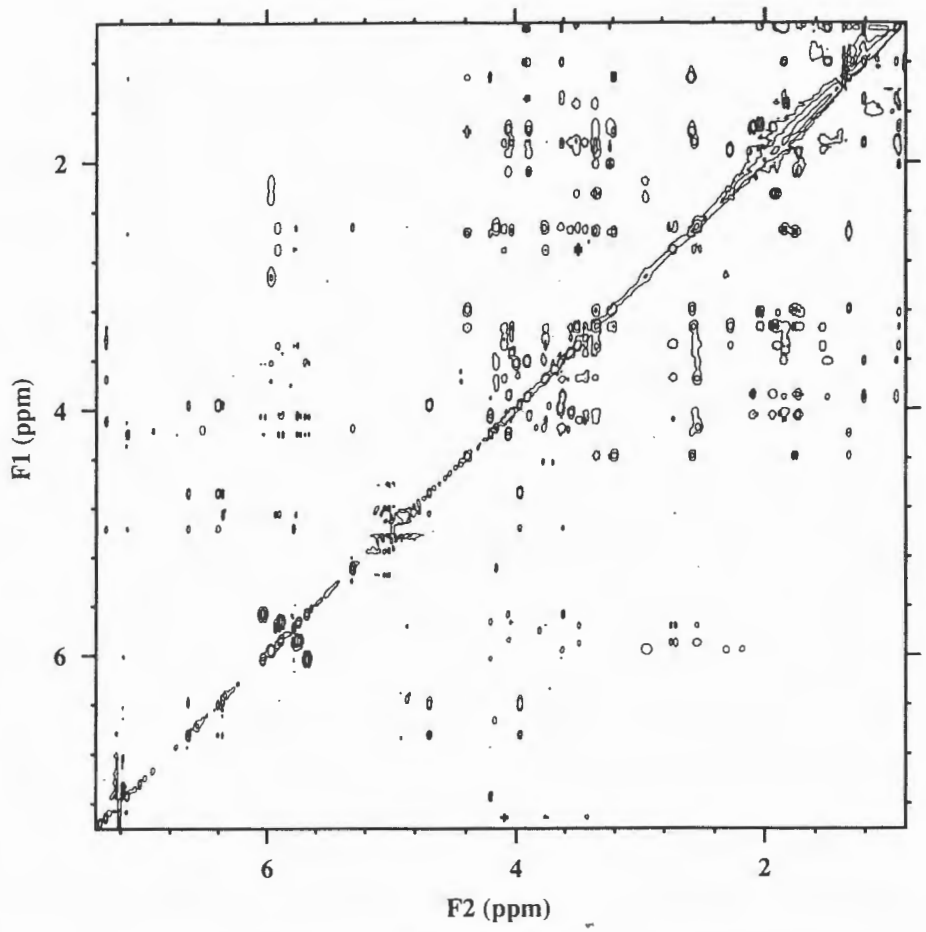

FIG. 2. TWO-DIMENSIONAL HOHAHA SPECTRUM OF CTX-2 IN PYRIDINE-d_5 AT 500 MHz (25°C). Exchange between hydroxyl protons of CTX-2 and H_2O was detected in the HOHAHA (mixing time ~ 54 msec) as direct and relayed cross-peaks to the H_2O resonance (4.96 ppm).

On the premise that the structure proposed for CTX-1 (MURATA *et al.*, 1990) is correct, we assign protons H-1 to H-43 (i.e. all protons associated with rings A to J), methyl 56 (Me-56), Me-57 and all hydroxyl protons (except OH-47) to the positions proposed for CTX-1 (Fig. 1). HOHAHA connectivities (Figs 2 and 3) and coupling patterns overlapped and resonances for these protons were within 0.02 ppm of those reported for CTX-1 in pyridine-d_5 at 25°C (MURATA *et al.*, 1990; our unpublished results).

For CTX-2, prominent NOEs were detected from the irradiated proton H-12 (3.43 ppm) to H-16 (4.03 ppm), from H-15 (3.55 ppm) to H-20 (4.21 ppm), from H-24 (3.63 ppm) to H-19 (4.06 ppm), from H-30 (3.63 ppm) to Me-56 and H-23 (3.99 ppm), and from H-37 (3.50 ppm) to Me-56. These NOEs are expected if the configuration for rings A to J of CTX-2 is the same as that proposed for CTX-1 (MURATA *et al.*, 1990). Irradiation of the H-1 protons (3.96 ppm) collapsed the resonance for 1-OH at 6.32 ppm (d,d,9,9 Hz) to a singlet, confirming that CTX-2 possesses a primary hydroxyl.

In CTX-2, however, the chemical shifts of resonances for many of the protons assigned to rings K, L and M differed significantly from those of GTX-4b (Table 1), CTX-3 (LEWIS

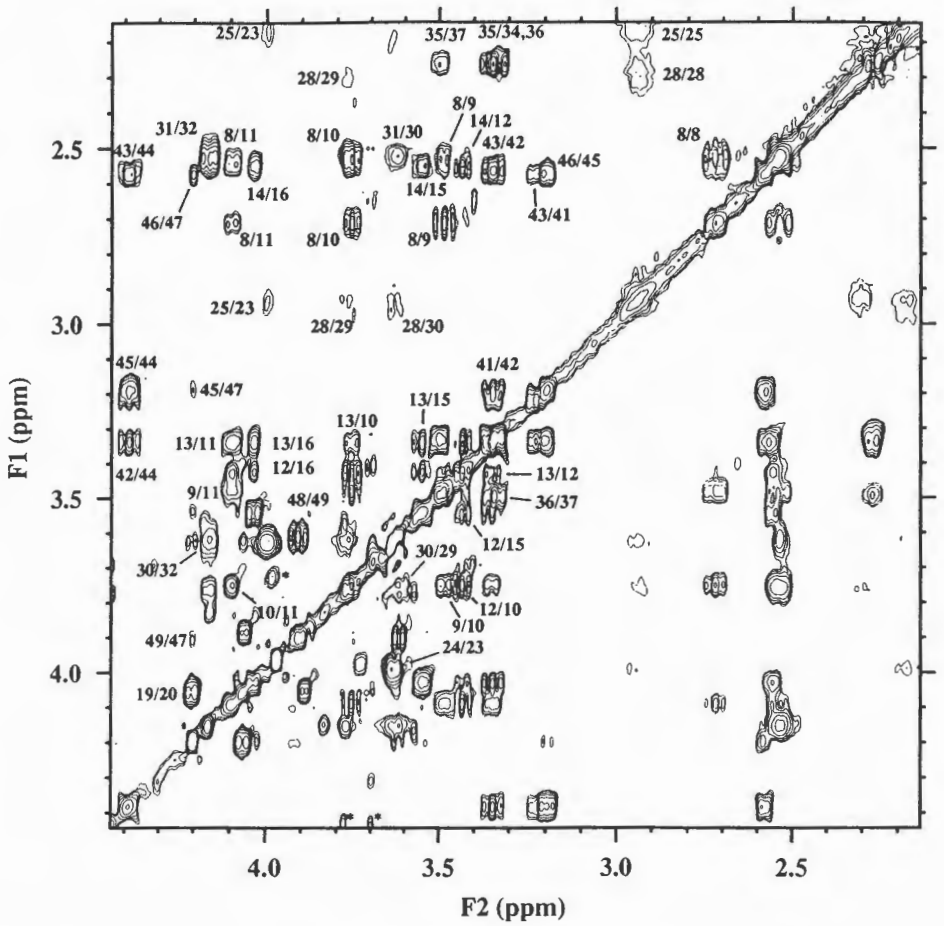

FIG. 3. SELECTED REGION OF THE HOHAHA SPECTRUM OF CTX-2.
Numbers indicate the carbon number (see Fig. 1) for short-range and long-range coupled protons detected in this region, e.g. 25/23 indicates δ 2.96 ppm in F1 and δ 3.99 ppm in F2. Asterisks (*) indicate impurities.

et al., 1991) or CTX-1 (MURATA *et al.*, 1990). The relative position of protons on rings K, L and M of CTX-2 was indicated by HOHAHA connectivities and confirmed by decoupling difference spectra, which showed that Me-59 (irradiated at 1.22 ppm) was vicinal to H-50, H-49 (irradiated at 3.91 ppm) was vicinal to H-48 and H-50, and H-53 (irradiated at 2.09 ppm) and H-55 (irradiated at 3.89 ppm) were vicinal to the H-54 protons. It is notable that the coupling patterns for protons H-44 to H-51 of CTX-2 remained the same as those for comparable protons of GTX-4b (Table 1), indicating similar orientation for protons on rings K and L of CTX-2 and GTX-4b. NOEs detected in CTX-2 from the irradiated proton H-49 to H-44 and H-51, from H-48 to H-47 and from Me-58 to H-45 and H-48 supported this conclusion (Fig. 1). For CTX-2, prominent NOEs were also observed from H-48 to H_{α}-53 (2.09 ppm) and from Me-60 to H_{β}-53 (1.94 ppm). These latter NOEs indicated that carbon 52 was axially substituted in CTX-2 (Fig. 1), in contrast to CTX-1, CTX-3 and GTX-4b, which are equatorially substituted (MURATA *et al.*, 1990; LEWIS *et al.*, 1991).

TABLE 1. SELECTED ^{1}H NMR CHEMICAL SHIFTS (ppm) AND COUPLING CONSTANTS (Hz) FOR CIGUATOXIN-2 AND GAMBIERTOXIN-4B IN PYRIDINE-d_5 AT 25°C*

Position†	CTX-2 ppm(pattern)‡	GTX-4b ppm(pattern)	Position	CTX-2 ppm(pattern)	GTX-4b ppm(pattern)
44	4.39(11,9,5)	4.47(11,9,5)	54	1.75(—)	1.68(—)
45	3.19(9,5)	3.19(9,5)		1.76(—)	1.90(—)
46	2.59(—)	2.59(—)	55	3.89(13,6)	3.87(—)
47	4.20(3)	4.20(3,2)		4.06(13,6)	3.88(—)
48	3.61(9)	4.03(9,1)	56	1.38(s)	1.37(s)
49	3.91(10,10)	3.94(10,10)	57	0.92(7)	0.92(8)
50	1.52(q,d,d,6,9,9,)§	1.94(q,t,6,11)	58	1.34(8)	1.29(8)
51	1.86(—)	1.60(q,d,7,11)	59	1.22(6)	1.28(6)
53	1.94(—)	1.85(—)	60	0.95(7)	0.97(7)
	2.09(—)	1.93(—)	47-OH	7.16(3)	6.76(3)

*Data for protons on rings K, L and M of CTX-2 (500 MHz) and GTX-4b (400 MHz; MURATA *et al.*, 1990).
†Position of protons on CTX-2 refers to the number for the carbon they attach to (see Fig. 1).
‡Pattern (s, singlet; d, doublet; t, triplet; q, quartet) and coupling constants are indicated.
§Obtained from decoupling difference spectra in pyridine-d_5 at 30°C.
CTX-2 = ciguatoxin-2 (LEWIS *et al.*, 1991); GTX-4b = gambiertoxin-4b (MURATA *et al.*, 1990). A dash (—) indicates that the coupling pattern was not assignable.

Construction of molecular models suggests that H-48, H-50 and Me-59 are further from the ether oxygen between carbons 52 and 55 in CTX-2 than in GTX-4b and thus further from the deshielding influence of this oxygen. Conversely, H-51 is closer to this oxygen in CTX-2 than GTX-4b. The altered chemical shifts of resonances for these protons (Table 1) reflect effects on these protons of increased or decreased deshielding. The altered chemical shifts (compared with GTX-4b) for H-44, H-49, OH-47 and Me-58 (Table 1) are likely to reflect a slightly altered conformation for the seven membered ring K that has little influence on the relative orientation of the protons on this ring. Resonances for H-44 and H-49 of CTX-2 are shifted only modestly upfield compared with GTX-4b, suggesting that the aromatic solvent effect reported in CTX-1 and GTX-4b (MURATA *et al.*, 1990) also acts upon these protons in CTX-2. This is evidence that 47-OH has an α-orientation in CTX-2, as it does in GTX-4b, CTX-1 and CTX-3. The observed weak cross-peak between H-47 and H-48 of CTX-2 (Fig. 3) is expected, as molecular models show for this configuration that the dihedril angle between these protons is $\sim 90°$ and consequently coupling between them would approach zero. On the basis of the above results, we propose that the relative configurations of rings A to L of CTX-2 are the same as that proposed for GTX-4b by MURATA *et al.* (1990) and that CTX-2 is a diastereomer of CTX-3 that differs only in its stereochemistry at carbon 52 (a quaternary carbon). The absolute stereochemistry of CTX-1 has been proposed on the basis of comparison of CD spectra of GTX-4b and a enantioselectively synthesized portion of GTX-4b (SUZUKI *et al.*, 1991). CTX-2 is expected to have the same absolute stereochemistry as CTX-1.

This study highlighted the usefulness of the HOHAHA experiment which, at ~ 54 msec mixing time, enabled connectivities to be observed between broadened resonances (e.g. H-23 to H-31; see Fig. 3). Previously, connectivities between these broadened resonances have been reported only at temperatures below $-20°C$ (MURATA *et al.*, 1990). The HOHAHA experiment also detected all hydroxyl protons of CTX-2 as cross-peaks to the H_2O resonance (Fig. 2). Such cross-peaks are likely to arise from chemical exchange between the hydroxyl protons of CTX-2 and H_2O. Relayed cross-peaks to the H_2O resonance were also observed, e.g. from the 1-H protons (3.96 ppm). Similar hydroxyl

exchange cross-peaks were also observed in HOHAHA spectra (at similar mixing times) of CTX-1 in pyridine-d_5 (unpublished result). The observation of HOHAHA cross-peaks arising from direct and relayed effects of chemical exchange between different chemical species in solution has been reported previously (FENG and RODER, 1988).

We have shown that the differences in the structures of CTX-1, -2 and -3 are restricted to the orientation or substitution at ring M. These differences underlie modest differences in the relative potency of CTX-1, -2 and -3 to mice (1:0.11:0.28, respectively) and affinity for voltage-dependent sodium channels (1:0.27:0.53, respectively) (LEWIS *et al.*, 1991). Thus, ring M contributes to, but is not critical for, high affinity binding of the ciguatoxins to voltage-dependent sodium channels. GTX-4b differs from CTX-3 by modifications at carbons 1 and 2 and is approximately four-fold less potent to mice than CTX-3 (MURATA *et al.*, 1990; LEWIS *et al.*, 1991). It would appear that the terminal regions of the ciguatoxins and gambiertoxins are not critical for their high affinity binding to voltage-dependent sodium channels.

The stereochemical difference between CTX-2 and CTX-3 indicates that CTX-2 comes from a precursor that is different from the precursor (presumably GTX-4b) for CTX-1 and -3. *Gambierdiscus toxicus* produces several gambiertoxins in addition to GTX-4b (LEGRAND *et al.*, 1990; HOLMES and LEWIS, 1992) one of which is presumably the precursor of CTX-2. Unlike GTX-4b, which can be oxidized at three positions to yield CTX-1, CTX-2 prescursors are apparently not oxidized at carbon 54, perhaps due to the altered stereochemistry. Alternatively, carbon 54 oxidized CTX-2 may not be easily detected since such a compound is likely to be more polar than CTX-1 (CTX-2 is more polar than CTX-3) and as a consequence could be more rapidly excreted than CTX-1 (LEWIS *et al.*, 1992; LEWIS and SELLIN, 1992). Given the single difference between CTX-2 and -3, it is possible that both arise from a common biosynthetic pathway in *G. toxicus* that yields two gambiertoxins that differ in stereochemistry at carbon 52.

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Appendix IX

Recovery of ciguatoxin from fish flesh

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RECOVERY OF CIGUATOXIN FROM FISH FLESH

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R. J. LEWIS and M. SELLIN. Recovery of ciguatoxin from fish flesh. *Toxicon* **31,** 1333–1336, 1993.—A mouse bioassay, validated for the quantification of ciguatoxin in up to 20 mg of ether extract from fish flesh, revealed that $63 \pm 14\%$ of spiked ciguatoxin was recovered using a standard extraction procedure. Except for extracts from the least toxic of ciguateric fish (0.1–0.5 nmol ciguatoxin-1/kg fish), signs in mice of intoxication by ciguatoxin (hypothermia to below 33°C as well as at least severe diarrhoea or lachrymation or hypersalivation) could be distinguished from the toxic reaction that follows administration of ciguatoxin-free ether extracts. Ciguatoxin recovery was similar for four variants of the ether–water partition, with the 2 M NaCl/ether partition extracting half the contaminants. The method described is selective for ciguatoxin and could be used to quantify natural levels ciguatoxin in the flesh of fish in the absence of a validated *in vitro* test.

CIGUATERA results from the consumption of fish contaminated by the ciguatoxin (CTX) class of lipid-soluble polyether toxins (MURATA *et al.*, 1990; LEWIS *et al.*, 1991; LEWIS and SELLIN, 1992). Most cases of ciguatera involve consumption of the flesh of ciguateric fish that contain the equivalent of 0.1–5 nmole ciguatoxin-1/kg (LEWIS, 1992; LEWIS and SELLIN, 1992). The mouse bioassay has been widely used to assess levels of CTX in crude lipid (ether) extracts of fish (YASUMOTO *et al.*, 1977; TOSTESON *et al.*, 1988; VERNOUX and TALHA, 1989; LEWIS *et al.*, 1992). Despite widespread use, the accuracy of this method has not been established. In this study we quantify the recovery of CTX from fish flesh spiked with purified CTX.

Flesh of a commercially purchased *Scomberomorus commersoni* (Spanish mackerel) and two *Plectropomus* spp. of the coral trout species complex (specimens A and B) were each consumed (250 g meals) by two volunteers. The absence of adverse effects confirmed that these fish were indeed non-ciguateric. Minced portions (100 g) of these fish were spiked with ~ 40 mouse units (one MU is the LD_{50} dose for a 20 g mouse) of partially purified CTX (i.p. $LD_{50} = 25\ \mu g/kg$). 40 MU of this CTX sample contained 180 (90% of total lethality), 90 and 40 ng of CTX-1, -2 and -3, respectively; relative levels typical of those found in the flesh and viscera of ciguateric fish (LEWIS *et al.*, 1991; LEWIS and SELLIN, 1992). Spiked and unspiked (control) flesh samples were stored at −20°C prior to standard extraction for ciguatoxins (LEWIS *et al.*, 1991, 1992; LEWIS and SELLIN, 1992). Each sample was thawed, cooked at 70°C for 30 min and cooled before extraction with acetone and partitioning into hexane, ethanol–water and ether extracts. The diethyl ether (ether) extract contained CTX in a mixture of lipids. These fractions were dried on a

TABLE 1. RECOVERY OF SPIKED CIGUATOXIN (CTX) FROM THE FLESH OF FISH†

Specimen	Sample #	Spiked CTX (MU)‡	Lipid extract (g)§	CTX extracted (MU)‡	Recovery (%)
S. commersoni	1	40 ± 2	0.28	28 ± 1**	70
	2	40 ± 2	0.29	29 ± 3*	71
	3	0	0.24	0	—
Plectropomus A	4	40 ± 2	0.28	23 ± 1**	58
	5	40 ± 2	0.27	21 ± 1**	53
	6	0	0.35	0	—
Plectropomus B	7	43 ± 5	0.07	33 ± 2	77
	8	43 ± 5	0.25	21 ± 1*	49
	9	0	0.15	0	—

† 100 g samples of flesh were extracted using a standard extraction procedure for CTX (LEWIS *et al.*, 1991, 1992). The fish flesh was confirmed non-ciguateric by human assay and was either spiked with CTX or left unspiked (putatively CTX-free control flesh samples).

‡ CTX was quantified (male mice, 19–21 g) in mouse units (MU) and signs of intoxication recorded.

§ The ether extract of each sample was freed of solvent and weighed prior to mouse bioassay. 20 mg of ether extract was injected per mouse except sample # 7, where 6 mg was administered.

MU data presented as means ± 1 S.E. ($n = 4$). *$P < 0.01$, **$P < 0.01$ indicate significant reduction in CTX extracted *vs* the quantity of CTX added as spiked CTX (two-tailed *t*-test).

rotavapor and finally freed of solvent under a stream of N_2 prior to quantification. The CTX (< 4 MU) present in ether extracts was quantified by mouse bioassay ($n = 2$–4 mice) using the dose *vs* time to death relationship, log (dose) = 2.3 log (1 + 1/*t*), where dose is in MU and time to death (*t*) is in hr for each mouse (LEWIS *et al.*, 1992). To ensure accurate quantification, dose *vs* time to death relationships for CTX may need to be established in each laboratory. All samples were suspended in 1% Tween 60, 0.9% saline prior to i.p. injection into 18–21 g Quackenbush mice (either sex) housed at 25°C (12 : 12 light–dark cycle). Food and water were provided *ad libitum*. Mice were injected mid-morning, since preliminary observations indicated that diarrhoea from CTX appeared more frequently with this timing.

For the above (standard) extraction procedure, all mice injected with 20 mg of ether extract from either the CTX spiked ($n = 37$) or unspiked fish (putatively CTX-free extracts, $n = 14$) displayed, to varying degrees, hypothermia and signs of laboured respiration and loss of activity. Fractions containing CTX additionally caused severe diarrhoea (97% of mice), hypersalivation (41%) and lachrymation (21%), whereas ether extracts from unspiked *Plectropomus* spp. additionally caused mild diarrhoea (50%) but no hypersalivation or lachrymation ($n = 10$) and the ether extract from unspiked *S. commersoni* caused no further signs of intoxication ($n = 4$). Only fractions containing ciguatoxin were lethal to mice (Table 1), with mice typically recovering from the effects of the CTX-free extracts (≤ 1.0 g/kg) within 1–3 hr of administration. The i.p. administration of CTX-free extracts at > 1 g/kg (not tested) may cause additional toxic effects including death. The OECD (1987) suggest an upper limit of 2 g/kg for assessing acute toxicity by the typically less sensitive oral route.

Using the mouse assay, 63% (range 49–77%) of spiked CTX was recovered with a standard extraction procedure (Table 1). To determine if lipids in ether extracts from fish flesh interfere in the mouse assay, CTX was added to 20 mg portions of ether extract from unspiked fish. The assayed level of CTX prior to addition of ether extract (1.9 ± 0.1 MU, $n = 4$) was not significantly altered ($P > 0.05$ two-tailed *t*-test) by the addition of ether

TABLE 2. COMPARISON OF FOUR LIQUID–LIQUID PARTITIONS FOR EXTRACTION OF 34 ± 1 MU OF SPIKED CIGUATOXIN (CTX)

Partition (a–d)†	Lipid extracted (mg)	CTX extracted (MU)	Recovery (%)	Diarrhoea	Rectal temperature (°C)‡ Control	60 min
spiked **a**	20	29 ± 1*	85	3/3	36.8 ± 0.3	31.9 ± 0.1**
unspiked **a**	27	0	—	0/3	36.7 ± 0.1	35.4 ± 1.2
spiked **b**	9	30 ± 7	88	3/3	36.8 ± 0.1	31.8 ± 0.6**
unspiked **b**	9	0	—	0/3	37.3 ± 0.3	35.2 ± 0.5*
spiked **c**	31	29 ± 8	85	3/3	37.0 ± 0.3	30.0 ± 1.7
unspiked **c**	34	0	—	1/3	37.2 ± 0.1	37.0 ± 0.1
spiked **d**	15	27 ± 3	79	3/3	36.6 ± 0.5	30.7 ± 0.8**
unspiked **d**	21	0	—	1/3	37.0 ± 0.2	37.0 ± 0.5

† **a** = 25% ethanol-water/ether (standard partition); **b** = 2 M NaCl/ether; **c** = water/ether; **d** = water/ethyl acetate. Each partition consisted of solvent extraction (55 ml, 3 ×) of the aqueous phase (55 ml of distilled water added). The hexane-insoluble material from an acetone extract of a coral trout was either spiked with 34 ± 1 MU of CTX ($n = 3$) or left unspiked (putatively CTX-free) prior to partitioning by methods **a–d**. Mice (either sex) were administered 10% of each extract and temperature measurements were taken 0, 10 and 30 min and 1, 2, 3, 4, 6, 24 and 48 hr after i.p. injection ($n = 3$).

‡ Rectal temperature measured with a probe thermometer (Comark, Type 1604) just prior to injection (control) and at 60 min after injection of mice.

Temperature and mouse unit (MU) data presented as means ± 1 S.E. ($n = 3$). *$P < 0.01$, **$P < 0.01$ indicate significant hypothermia (paired t-test) or significant reduction in CTX after extraction (two-tailed t-test).

extracts of samples #3, 6 and 9 in Table 1 (1.7, $n = 2$; 2.2, $n = 2$; and 2.0 ± 0.2 MU, $n = 4$, respectively). The addition of ether extracts also did not alter the rate or extent of hypothermia that developed in mice following i.p. CTX (data not shown). These results indicate that the mouse bioassay is selective for CTX in up to 20 mg of ether extract. With appropriate adjustment for incomplete recovery, this method can be used to quantify CTX in fish flesh with an accuracy of within ±20%.

Several different liquid–liquid partitions have been employed to separate CTX from aqueous soluble material (BANNER *et al.*, 1960, 1963; LEWIS and ENDEAN, 1984; NUKINA *et al.*, 1984; VERNOUX and TALHA, 1989). To find the most efficient of these partitions, flesh from a third non-ciguateric coral trout was extracted with acetone and the material, after removal of the hexane soluble components, was separated into four portions (each equivalent to 50 g of raw flesh). To each portion was added 34 MU of CTX and the mixture dried prior to liquid–liquid partitioning by methods **a–d** (Table 2). Four unspiked (control) portions were similarly extracted. Similar quantities of CTX (79–88%) were extracted by each partition; however, the 2 M NaCl/ether partition extracted half the contaminants (Table 2) and this partition could be used to improve assay sensitivity. For each partition, the spiked and unspiked samples induced signs of loss of activity and laboured respiration in at least two of three mice. However, mice receiving CTX displayed severe diarrhoea, whereas no more than one of three mice receiving unspiked extracts displayed diarrhoea (Table 2), which was always mild. Significant hypothermia developed in mice within 1 hr of administration of extracts containing CTX (Table 2; LEWIS *et al.*, 1993), but in 1–3 hr of administration of CTX-free extracts ($P < 0.05$, paired t-test). Maximum hypothermia following injection of CTX-free extracts from partitions **a–d** ($\bar{x} \pm$ S.E. values of 34.4 ± 0.8, 35.4 ± 1.2, 34.4 ± 0.6 and 33.6 ± 1.6°C, respectively) was less ($P < 0.05$, two-tailed t-tests) than maximum hypothermia following CTX.

We have validated a mouse bioassay for the quantification of CTX in up to 20 mg of lipid extract from fish flesh using a small number of mice. This assay revealed that

63 ± 14% of spiked CTX was recovered with a standard extraction procedure. For ether extracts containing less than a minimum lethal dose of CTX (0.4–1.0 MU) in a 20 mg portion, bioassay signs selective for the presence of CTX in fish flesh include rectal temperatures below 33°C as well as at least severe diarrhoea or hypersalivation or lachrymation (LEWIS *et al.*, 1993). Using these criteria, 71% of fish confirmed to be implicated in cases of ciguatera in Queensland ($n = 34$) contained levels of CTX in standard extracts that were detectable by mouse bioassay (unpublished result). Identification of CTX-1, -2 and -3 as the major toxins from many of these naturally toxic flesh samples confirmed that CTX was indeed present in these extracts (LEWIS and SELLIN, 1992). The flesh samples from the remaining 29% of ciguateric fish apparently contained levels of CTX that were below the limit of detection for this method (~ 0.5 nmole CTX-1/kg flesh). The method described is selective for ciguatoxin and can be used to quantify natural levels of ciguatoxin in the flesh of fish in the absence of a validated *in vitro* test.

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Appendix X

Socioeconomic impacts and management of ciguatera in the Pacific

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Key-words: Ciguatera, Socioeconomical impacts, Fisheries, Management Pacific.

Mots-clés : Ciguatera, Impacts socio-économiques, Pêches, Prise en charge, Pacifique.

IMPACTS SOCIO-ÉCONOMIQUES

Exposé introductif

SOCIOECONOMIC IMPACTS AND MANAGEMENT CIGUATERA IN THE PACIFIC

By Richard J. LEWIS (*),

Impacts socio-économiques et gestion de la ciguatera dans le Pacifique.

Résumé : *Les ressources de la pêche lagonaire sont importantes pour la santé et la culture de la plupart des habitants des îles du Pacifique. Les poissons ciguatériques sont la cause d'une série de désordres, parfois graves, intestinaux, neurologiques et cardio-vasculaires. De ce fait, la ciguatera constitue un frein à l'exploitation de ressources qui seraient exploitables. Pour beaucoup de malades, les symptômes observés pendant la phase chronique de l'intoxication ciguatérique (et pouvant durer des semaines, des mois et parfois des années), sont exacerbés lors de la consommation de certains aliments, en particulier de poissons non toxiques. Après chaque poussée, les patients et les membres de leur entourage socio-professionnel perçoivent un risque accru à la consommation de poissons lagonaires. L'impact de la ciguatera est plus important dans les atolls où les poissons sont la première source en protéine. L'impact dans les îles hautes est lui aussi important. L'évaluation de l'impact socio-économique de la ciguatera et de sa prise en charge dans les pays insulaires du Pacifique nécessite la quantification régulière :*

— de l'incidence réelle de la ciguatera;
— des mesures qu'emploient les différentes communautés pour éviter le risque ciguatérique;
— des conséquences néfastes sur la santé, le travail, le commerce et le tourisme.

Depuis 15 ans, à partir des données du South Pacific Epidemiological and Health Information Service (SPEHIS) jusqu'en 1990, certains pays ont noté une diminution de la ciguatera (Nouvelle-Calédonie, Iles Marshall), d'autres pays ont noté une augmentation (Kiribati, Tuvalu, Polynésie française), alors que d'autres encore ont remarqué une augmentation suivie d'une diminution de la ciguatera (Tokelau, Samoa Américaines, Samoa Occidentales, Fidji et Vanuatu). Il est possible qu'il existe des tendances saisonnières de l'incidence dans certains pays comme Fidji.
La prise en charge de la maladie comporte actuellement trois étapes :

— le traitement par le Mannitol i.v.;
— la diffusion de l'information sur les zones à risque de ciguatera de façon à permettre aux communautés concernées de les éviter;
— la modification de la pression écologique sur les récifs, liée à l'augmentation de la population, à la pollution et au développement.

Dans le futur, de nouvelles stratégies de lutte pourraient inclure un programme de détection des poissons ciguatériques et l'immunisation des communautés à risque.

Summary: *The inshore fisheries resource is important to the health and culture of many of the inhabitants of Pacific Island countries (PIC). Ciguateric fishes (mainly demersal reef fishes) cause a range of distressing and often debilitating gastrointestinal neurological and cardiovascular disturbances. Consequently, ciguatera limits the utilisation of this otherwise over-exploited resource. For many victims, the symptoms suffered during the chronic phase of ciguatera (lasting weeks, months and occasionally years) are exacerbated upon consumption of certain foods, particularly non-toxic fishes. After each outbreak, victims and members of their social-network experience a transient increase in perception of the risk of eating reef fish. The impact of ciguatera is greatest in atoll island countries where fish is the primary source of protein (it also has a major impact and to facilitate the management of ciguatera in PIC, regular information is required that quantifies:* (i) *the true incidence of ciguatera;* (ii) *the extent and way in which different communities avoid ciguatera; and* (iii) *the adverse impact ciguatera has on health, the workforce, trade and tourism. Over the last 15 years (based*

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on SPEHIS data to 1990), some countries recorded a decrease in the ciguatera problem (New Caledonia, Marshall Is.), other countries an increase (Kiribati, Tuvalu, French Polynesia), while still other countries recorded an increase followed by a decrease in ciguatera (Tokelau, American Samoa, Western Samoa, Fiji and Vanuatu). There may also be seasonal trends in the incidence of ciguatera in some countries e.g. Fiji. Management options presently implementable in PIC include: (i) *treatment with i.v. mannitol;* (ii) *provision of timely advise on the location and status of ciguatera « hot spots » in each country that would allow affected communities to react objectively to the risk posed by ciguatera; and* (iii) *modification of human behaviour and aspirations to reduce the impact of increasing population, pollution and development (e.g. causeways) pressures. Future management strategies may include:* (i) *the detection of ciguateric fishes prior to consumption; and* (ii) *the immunisation of communities most at risk.*

INTRODUCTION

Ciguatera is a disease caused by the consumption of the flesh and less frequently the viscera of fishes contaminated with ciguatoxins. The majority of ciguateric fishes are captured in the inshore fisheries associated with coral reefs. Three ciguatoxins (ciguatoxin-1, -2 and -3) have been isolated from the flesh and viscera of ciguateric fishes (1, 2). The precursors of these ciguatoxins are produced by certain strains of the benthic dinoflagellate, *Gambierdiscus toxicus* (3-6). *G. toxicus* is common on macroalgae on reefs throughout tropical and sub-tropical waters in areas typified by high salinity and with the exception of the reef itself, these areas have low productivity.

Ciguatera is prevalent on islands in the Pacific and Indian Oceans and in the Caribbean Sea (7, 8). The early report of BANNER and HELFRICH (8) shows that ciguatera has long been widespread in the Pacific, affecting many high island (e.g. Fiji) and atoll island countries (e.g. Kiribati). Victims of ciguatera suffer a range of distressing and often debilitating gastrointestinal, neurological and cardiovascular disturbances. The disease is rarely fatal, possibly because fish succumb to the lethal effects of high concentrations of the ciguatoxins (9). The signs and symptoms of ciguatera have been reported for Australia (10, 11) and for several Pacific Island countries (PIC) including French Polynesia (12, 13), Hawaii (14, 15), New Caledonia (16) and Fiji (17, 18).

The impact of ciguatera in several PIC has been studied by Nancy LEWIS (19) and most recently reviewed by her at the second International Conference on Ciguatera in 1985 (20). In the present review, the socioeconomic impacts of ciguatera are reassessed for PIC and management options to reduce the impact of ciguatera are discussed.

PACIFIC ISLAND COUNTRIES (PIC)

PIC are composed of atoll and high islands and include western-style urban areas (especially cities of high islands) and rural areas. These countries are subject to the stresses of increasing population, pollution and westernisation. Throughout PIC there is a heavy dependence on the inshore fishery resource

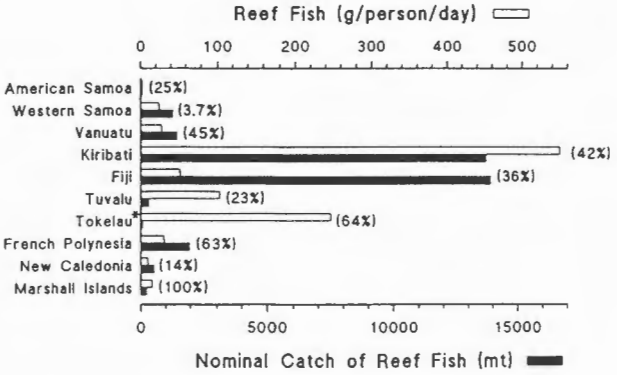

Fig. 1. — Reef fish catch in metric tons (mt) for selected Pacific Island countries. Data from FAO (47) or from Zann and Aleta (48) for Tokelau (*). For each country, catch is also expressed in g/person/day, using SPEHIS, 1987 (25) population estimates. Percentage contribution of reef fish to total fish catch are indicated in parenthesis.

(IFR) to provide demersal reef fish for food. JOHANNES (21) has suggested that the IFR is of greater importance per capita to PIC than in any other region of the world. The IFR provides essential dietary protein and animal fats, and is considered to be an important source of foreign exchange (21). The importance of the IFR to a community is inversely proportional to its access to alternative food, consequently the IFR is more important to rural than to urban communities and to atoll than to high island communities. Figure 1 shows for selected PIC that more demersal reef fish are caught per person in the less developed atoll island countries (Kiribati, Tokelau, Tuvalu) than in high island countries (Fiji, Vanuatu, Western and American Samoa, New Caledonia). These data indicate that reef fish contribute significantly to the protein needs of people, particularly those of the poorer atoll island countries. The low figure for the Marshall Islands may reflect access to subsidised imported foods, whereas the low figure for French Polynesia is surprising and may reflect under-reporting. Reflecting its importance, the IFR is controlled through a system of traditional rights which define community access and provide a mechanism for resource conservation, *i.e.* each community benefits from, and takes responsibility for its fishing area (21, 22). Increasing westernisation (altered values,

new technology, capitalism, etc.) is eroding these customary rights (22).

The small size of the IFR makes it highly vulnerable to the effects of overfishing (artisanal and commercial) and pollution (21, 23). In some areas the inshore fisheries resource can no longer fully support local subsistence needs (21). Ciguatera limits the value of the IFR, and has hindered the colonisation of some islands, e.g. Sydney Island in the Phoenix Islands, Kiribati (24).

CIGUATERA IN PACIFIC ISLAND COUNTRIES (PIC)

The incidence of ciguatera in PIC is estimated from reports submitted to the South Pacific Epidemiological and Health Information Service (SPEHIS) (25). These reports include cases of ciguatera as well as other forms of marine food poisoning (scombroid poisoning, clupeotoxism, mullet poisoning, puffer fish poisoning and invertebrate intoxications). The contribution of ciguatera to these reports and the extent of underreporting are not known and are likely to vary between countries in the region. Previous studies indicate that ciguatera comprises 96 % of fish poisoning cases in Fiji (26), 37 % of fish poisoning cases in American Samoa (27), 51 % of fish poisonings in Hawaii (14) and the majority of the cases of fish poisoning in the Kiribati (24, 28) and the Marshall Islands (29). A recent household survey of the inshore resources of Upolu, Western Samoa (1980-1990) (30) indicates that approximately 49 cases of confirmed ciguatera per 10,000 population occurred each year (assuming 50 % of household members were affected once in this period), *i.e.* < 10 % of the ciguatera cases were reported to SPEHIS in this period (see fig. 2). Similar levels of under-reporting are likely for other PIC and Australia. This review utilises the SPEHIS data on fish poisoning to indicate the incidence of ciguatera in the Pacific (25).

Ciguatera is prevalent throughout PIC with the exception of the Solomon Islands and Pitcairn Island (fig. 2). A range of inshore fish species are typically involved in ciguatera across the Pacific (7, 20), including the Lutjanids, the Sphyraenids and the Epinephelids in the central Pacific (17, 24, 26-29) with the exception of New Caledonia where Serranids, Lutjanids, Lethrinids, Scombrids and Scarids predominate (16). Serranids, Scarids, Acanthurids, Lethrinids and Lutjanids predominate in the eastern Pacific (French Polynesia) (31). Interestingly, a comparison of fish most often responsible for ciguatera in the different archipelagoes of French Polynesia revealed further differences in the species involved (31). Many of these species are considered « fatty » and as a consequence are highly rated as food in PIC, particularly the larger individuals of these species (24).

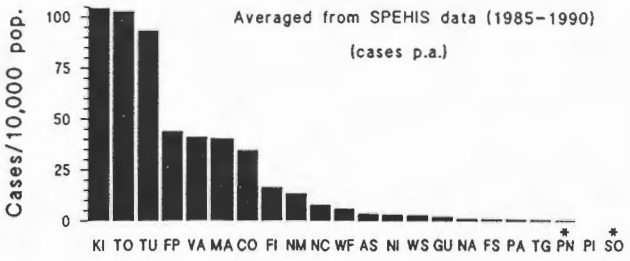

Fig. 2. — Incidence of ciguatera in Pacific Island countries. Cases per 10,000 population are indicated for Kiribati (KI), Tokelau (TO), Tuvalu (TU), French Polynesia (FP), Vanuatu (VA), Marshall Islands (MA), Cook Islands (CO), Fiji (FI), Northern Marianas (NM), New Caledonia (NC), Wallis and Futuna (WF), American Samoa (AS), Niue (NI), Western Samoa (WS), Guam (GU), Nauru (NA), Federated States of Micronesia (FS), Palau (PA), Tonga (TG), Papua New Guinea (PN), Pitcairn (PI) and Solomon Islands (SO). Asterisks indicate incomplete reporting to SPEHIS from these countries.

In Australia, pelagic fishes as *Scomberomorus commersoni* and Sphyraenids as well as the Serranids (particularly *Plectropomus* spp.) are most often involved (10, 11). Pelagic fishes (the Carangids and *Seriola dumerili*) are also responsible for most cases of ciguatera in Hawaii (15). Throughout the Pacific moray eels (Muraenidae) are often highly toxic and as a consequence are usually actively avoided in ciguatera-prone areas.

The highest average incidence of ciguatera (1985-1990) is approximately 100 cases/10,000 population per annum (p.a.) in several atoll island countries (Kiribati, Tokelau, Tuvalu) (fig. 2). The average incidence of ciguatera is less than half levels in French Polynesia, Vanuatu, Marshall Islands and the Cook Islands (fig. 2). The remaining 13 countries reported < 15 cases per 10,000 p.a. Over the same period, the average incidence of ciguatera in Queensland (population 2.9 million) is 0.16 cases per 10,000 p.a., a level similar to that reported for Tonga. The apparent absence of ciguatera in the Solomon Islands (fig. 2) (8) requires verification.

Figure 3 illustrates for selected PIC the history of ciguatera from 1973-1990. Ciguatera has increased over this period in Kiribati and Tuvalu, to reach 202 cases/10,000 p.a. in 1990 and 320 cases/10,000 in the first 9 months of 1991, respectively. Several countries (New Caledonia, Tokelau, American and Western Samoa) have reported dramatic increases followed by dramatic declines in the annual incidence of ciguatera. The highest yearly incidence of ciguatera in PIC was 650 cases/10,000 p.a. for Tokelau in 1984. French Polynesia experienced a dramatic increase in ciguatera between 1960 and 1973 (31) which has slowed since 1973 (fig. 3). The incidence of ciguatera in the Marshall Islands has apparently decreased since 1973, perhaps reflecting a reduced dependence on reef fish. The apparent dramatic increase in ciguatera in Fiji and Vanuatu in 1985 may

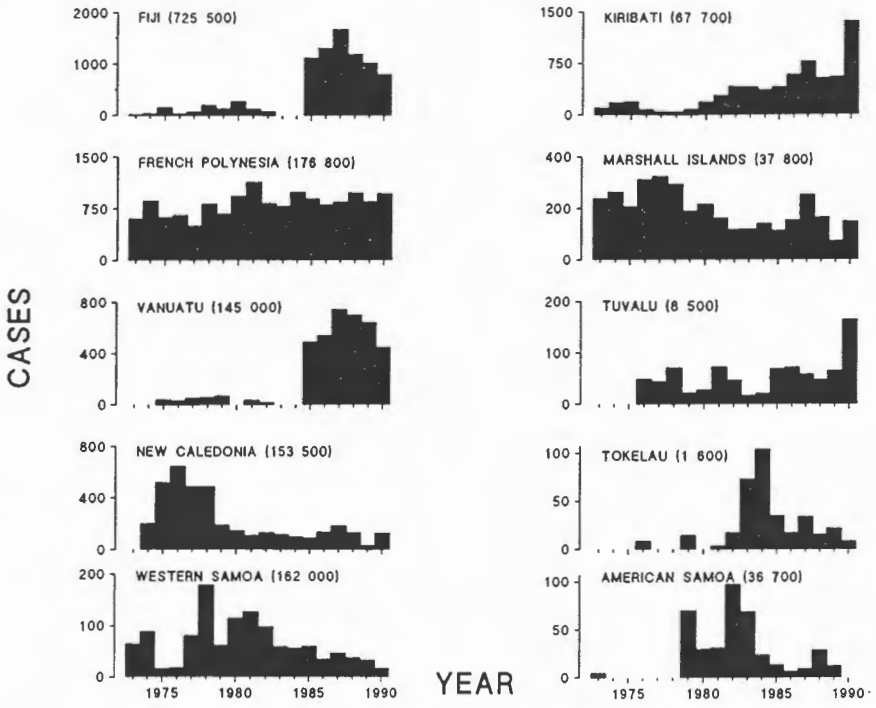

Fig. 3. — Annual cases of ciguatera for selected Pacific Island countries. Data from SPEHIS reports (25). Population in each country indicated in parenthesis (SPEHIS, 1987). Prior to 1982, data for the Marshall Islands included ciguatera from the Federated States of Micronesia, the Northern Marianas and Palau.

represent an artefact of improved reporting to SPEHIS. Early reports to SPEHIS (1981-1982) of ciguatera from Fiji are significantly less than reported by NARAYAN (18). The history of ciguatera shown in fig. 3 reflect the patterns of evolution of ciguatera described by HELFRICH and BANNER (32).

Unfortunately, SPEHIS does not include data on the within-country distribution of ciguatera. However, the distribution of ciguatera has been studied in detail throughout French Polynesia by BAGNIS since 1960 (31) and short-term studies on the distribution of ciguatera have been conducted in New Caledonia (16), Gilbert Islands, Kiribati (24, 28), Fiji (26) and American Samoa (30). Ciguatera is prevalent throughout these islands, usually at well defined locations. In American Samoa, ZANN (30) found that 18 of the 22 regions studied in Upolu were affected by ciguatera fishes, with 2.5 % to 38 % of households experiencing ciguatera over the last decade (1980-1990). Much local knowledge on the location of toxic areas in PIC remains undocumented. The distribution of ciguatera has also been studied in Australia (3, 11) and Hawaii (14, 15).

It is conceivable that global climatic changes may influence the incidence of ciguatera. However, the timing of the major upsurges in ciguatera reported by PIC are unrelated in time (fig. 3). A minor upsurge in incidence is noted in 1987 for five of 10 countries analysed (Fiji, Vanuatu, New Caledonia, Marshall Islands and Kiribati), that may have been driven by large scale climatic events such as the « El Niño » which contribute to low rainfall in the Pacific basin. El Niño events occurred in 1972, 1977, 1982, 1987 and 1991. Seasonal factors are also likely to

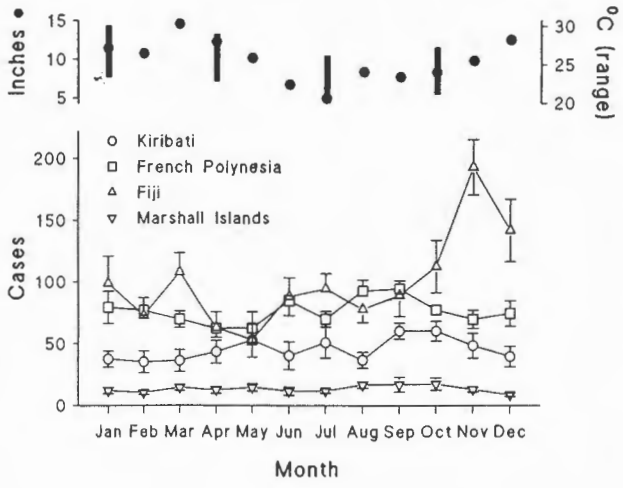

Fig. 4. — Seasonal incidence of ciguatera. Averaged monthly data (± 1 SE) from SPEHIS reports (25) for Kiribati (1984-1989), French Polynesia (1984-1990), Fiji (1985-1990) and Marshall Islands (1984-1990) are indicated. These countries were chosen as each had a relatively constant annual incidence of ciguatera over at least 6 years. Temperature range and average rainfall are indicated for Fiji (49).

influence the incidence of ciguatera. Analysing the monthly reports of ciguatera for Kiribati, French Polynesia, Fiji and Marshall Islands, revealed a seasonal change in ciguatera incidence only for Fiji (fig. 4). The incidence increased in spring in Fiji and reached a maximum in November. This pattern may indicate that the fluxes of ciguatoxin into and out of the marine food web (34) can be rapid. The pre-

cise lag-time between an increased input of ciguatoxins and their precursors and subsequent increase in the incidence of ciguatera has not been established. However, it is possible that the winter « dry » period and/or increasing temperature are involved (fig. 4). A seasonal increase in spring has been indicated previously in Fiji (17), New Caledonia (16) and American Samoa (27). It is noteworthy that only high island countries have been reported to show any seasonal pattern. The influence of terrestrial run-off from high islands may be involved. Seasonal increases in the toxicity of barracuda in winter-spring and summer-autumn has been observed in southwest Puerto Rico (33).

An upsurge in ciguatera incidence is often followed by a similarly rapid decline (see fig. 3), suggesting in these instances that ciguatoxins enter the food chain as a pulsed event. For $\log_2$ (cases) *vs* year, a significant ($P < 0.05$) linear relationship was obtained for Tokelau (1984-1990, $r^2 = 0.61$), American Samoa (1982-1987, $r^2 = 0.89$), Western Samoa (1984-1990, $r^2 = 0.72$) and New Caledonia (1976-1985, $r^2 = 0.86$). The slope of these regressions reveals that the time for a 50 % decline in the incidence of ciguatera was 164, 299, 91 and 124 days for Tokelau, American Samoa, Western Samoa and New Caledonia, respectively. These values are similar to the half-life for the loss of ciguatoxin (214 days) in a population of moray eels (34).

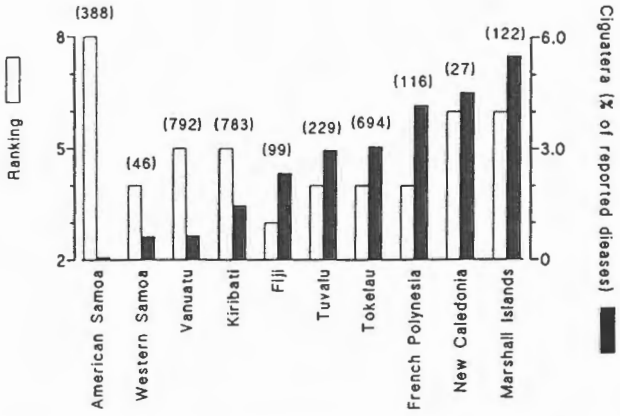

Fig. 5. — Contribution of ciguatera relative to total disease in PIC. Ciguatera cases (SPEHIS, 1987) (25) (i) *ranked in importance relative to other reported diseases and* (ii) *expressed as the percentage of all cases of disease reported to SPEHIS (1987) (25). Total reported disease per 1,000 population (SPEHIS, 1987) are indicated in parenthesis for each country. Countries were those selected in fig. 3.*

ADVERSE IMPACTS OF CIGUATERA

Ciguatera directly impacts on the health of inhabitants adjacent to coral reefs. Nowhere is this impact greater than on atoll island communities in the Pacific, particularly those communities whose traditional fishing areas are affected by ciguateric fishes. Impacts on health include the direct effects of ciguatera which can last for many weeks. Compared with other diseases reported to SPEHIS (for selected PIC), ciguatera ranks highest in Fiji (3rd) and lowest in American samoa (8th) (fig. 5). Diseases typically reported more frequently than ciguatera include conjunctivitis, diarrhoea (presumed infectious) and influenza (25). Ciguatera may exacerbate the effects of such diseases. Expressing the contribution of ciguatera relative to total disease reported in these countries further indicates that ciguatera is a major health problem for PIC. However, Pacific islander concerns over ciguatera are likely to be less than for other diseases because these people often have a developed system of traditional beliefs which act to reduce their perception of ciguatera (discussed further below).

Ciguatera also has indirect effects on health through avoidance of reef fish which would otherwise provide an important source of protein and can debilitate sections of the work-force. This latter effect would reduce the ability of subsistence communities to provide food, especially the poorer atoll islands. Ciguatera can affect trade (20) and may result in bans on the importation of reel fish considered suspect. Many countries are likely to be less accepting of ciguatera than are PIC. Ciguatera also has the potential to damage tourism. Generally tourists are not informed of the risk of ciguatera in PIC. While well recognised, the adverse effects of ciguatera are presently difficult to quantify (20). Accurate information on (*i*) the true incidence of ciguatera in PIC (*ii*) the extent and way in which different communities avoid ciguatera (*iii*) the extent ciguatera impacts on the health, work-force, trade and tourism is required before the socioeconomic impacts of ciguatera can be more accurately quantified. Such information will be invaluable if ciguatera is to be managed appropriately.

The perception of the risk of ciguatera can be influenced by traditional beliefs. Magic, folk remedies and traditional tests have all been employed by Pacific islanders to reduce the concerns and possibly the disconfort associated with the consumption of potentially ciguateric fish, e.g. magic is believed to protect Abaiang (Kiribati) from ciguatera (24, 28). The extent ciguatera is avoided relates inversely to the reliance on potentially ciguateric fishes for food. Culture (values, lifestyle, economy and legalities) and community knowledge of the history of ciguatera can also influence the perceptions (sensitivity to) of ciguatera. After each outbreak, victims and members of their social network experience a transient increase in the perception of the risk of eating potentially ciguateric fishes. A more extreme example of this situation is Australia, where outbreaks of ciguatera are documented with front-page headlines and consumption of fish can drop considerably for many

weeks after such events (unpublished observation). The large reaction to ciguatera in Australia reflects a lack of dependence on fish (one of the prime reasons for eating fish in Australia is because it is believed to be a « healthy » food) and the limited understanding of ciguatera in the community.

HUMAN ACTIVITIES AND THE SPREAD OF CIGUATERA

Human activities (anthropogenic effects) are increasing in PIC as a consequence of increasing population in the region. Anthropogenic effects on coral reefs have been recently reviewed by HATCHER *et al.* (35). Synergism among anthropogenic effects and natural stresses (e.g. u.v. radiation), and the potential for such effects to shift the balance toward algal dominated reef communities are discussed in their review. Such a shift may favour the proliferation of *G. toxicus*. Human activities have often been associated with upsurges in ciguatera incidence (24, 28, 31, 36, 37). However, the key factor(s) involved have only been speculated upon (3, 5, 24, 36). Table I summarises the human activities that may increase the incidence of ciguatera. One factor, groundwater pollution, is likely to occur in populated regions of atoll island countries. Where groundwater connects with the sea there is the potential for it to influence growth and toxicity of *G. toxicus* and consequently alter the incidence of ciguatera. The high incidence of ciguatera in south Tarawa and western Marakei (28) may reflect the influence of polluted groundwater in these more populated areas. Groundwater discharge occurs in shallow waters and can deliver substantial nutrients (nitrate and silicate) to coastal waters (38, 39), including coral reef lagoon waters (40, 41). The influence of groundwater discharge on the growth and toxicity of *G. toxicus* has not been studied.

Table I. — Human activities that may increase the risk of ciguatera.

Pollution of groundwater and seawater on atoll islands (human waste)
Pollution of seawater on high islands (human and industrial wastes, farming, land cleaning)
Reef degradation (dredging, shipgroundings, trampling, anchors, removal, blasting, construction)
Translocation of causative organism (rafting, ships hulls, ballast water)

It is possible that the incidence of ciguatera will increase in the Pacific, unless the impacts of population expansion in PIC are minimised. Because of potential to blame « outside » influences (e.g. shipwrecks, causeways) for existing ciguatera problems, it is appropriate that baseline studies be conducted prior to, as well as after any development likely to damage coral reef. Monitoring the influence of human activity on the frequency of ciguateric fishes in that area may provide the clues to unravel the factor(s) contributing to upsurges in ciguatera.

MANAGEMENT OF CIGUATERA

Management options to reduce the impact of ciguatera that might be implemented in PIC include: (*i*) avoidance of reef fish, (*ii*) detection, (*iii*) treatment, (*iv*) modification of human behaviour and (*v*) immunisation. The simplest way of avoiding ciguatera is to avoid eating fish known to be ciguatoxic. However, the need for protein and a craving for animal fat (24) make this an impractical solution, unless alternative foods are available. An effective compromise may be for a community to mix several fish so that the quantity of any one fish in a meal is small (*i.e.* < 100 g). Bans on the sale of fish with a reputation for causing ciguatera have been employed with apparent success in American Samoa (27), Australia (11), French Polynesia (20) and Fiji (17). The low reported incidence of ciguatera from moray eel, arguably the most toxic fish in the Pacific (1, 6, 34), indicates that this species is avoided in ciguatera-endemic areas without the need for it to be banned. In areas with a reputation for harbouring toxic fish, passive avoidance often extends to a wider range of species. However, the temptation to eat certain high risk species can overcome local knowledge, e.g. the consumption of the livers of toxic *L. bohar* on Jaluit, Marshall Islands (unpublished observation).

The detection of ciguateric fishes prior to consumption offers possibly the most practical means of reducing the incidence of ciguatera. The test must be selective for toxins involved in human poisoning. In the flesh of ciguateric fish, three closely related toxins (ciguatoxin-1, -2 and -3) have been isolated (1, 2). [The role in ciguatera played by maitotoxin and other polyether toxins such as okadaic acid remains unsubstantiated.] The test must be sufficiently sensitive to detect 0.1 nM ciguatoxin-1 per kg fish flesh (9) and be correspondingly less sensitive at detecting less orally potent compounds. To be useful in PIC, the test must be robust, cost effective and easily implemented at the community level. Antibody-based tests have the potential to satisfy the above criteria and several groups are currently in the process of developing such tests for ciguateric fishes. Research on the detection of ciguatoxins using fluorescence HPLC is also being undertaken. Traditional tests for ciguatera (silver coins, copper wire, flies, ants) have proved ineffective (42). An adult family member or a cat are presently the most reliable tests available to

Pacific islanders. Such testing may be employed several times a year to reassess the status of ciguatera in an area (unpublished observation), and may be used to protect children from ciguatera (24).

Since it is not possible to easily avoid ciguatera in PIC, the implementation of the mannitol treatment for ciguatera could play an important role in reducing its impact. This treatment originated in Majuro, Marshall Islands (43) (Where it now has wide acceptance) and has proven effective upon independent assessment (44). However, it is not widely used in other PIC. Treatment involves the intravenous infusion (over 30 min) of a 20 % mannitol solution administered at 1 g per kg body weight (*i.e.* 5 ml/kg) (43, 44). Treatment should commence only after ciguatera is diagnosed and adequate hydration is established. Best results are achieved when mannitol is given early in the acute phase of ciguatera (unpublished observation). The response to mannitol can often be dramatic, particularly when used to treat the more severe cases of ciguatera. A reduction in ciguatoxin-induced oedema of Schwann cells surrounding peripheral nerves may underlie the beneficial effects of the therapy (44). Traditional remedies and emetics are widely used in PIC (18, 24, 45, 46). Screening traditional remedies with a novel mouse bioassay has found that an extract from the leaves of *Argusia argenta* can reduce the effects of ciguatoxin (45). Traditional remedies may provide the first proven orally active treatment for ciguatera. Such a treatment could be expected to be widely accepted in PIC.

As discussed earlier, many human activities may contribute to increasing the incidence of ciguatera. Modifications to human behaviour and aspirations are required if the effects of increasing population, pollution and development are to be stemmed. However, further research is required to determine which key factor, or factors, are involved in increasing the quantity of ciguatoxins entering the marine food web. A final solution to ciguatera may eventually come through immunisation, particularly for communities most at risk from ciguatera. Immunisation requires the development of suitable immunogens. These may be developed from molecular mimics of ciguatoxin or from anti-idiotypic antibodies to ciguatoxin. Unlike ciguatoxin, these compounds should be non-toxic and highly immunogenic. Given the recent rapid progress in our understanding of ciguatera and the research programs now in place, it is likely that the adverse impacts of ciguatera can now start to be lessened in PIC.

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Appendix XI

Ciguatera: ecological, clinical, and socioeconomic perspectives

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Ciguatera: Ecological, Clinical, and Socioeconomic Perspectives

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ABSTRACT: Ciguatera fish poisoning, found throughout the world in warm waters, is the most common type of marine biotoxin ingestion. A polymorphous disease caused by toxins produced by coral reef dinoflagellate(s) and which concentrate up the food chain, ciguatera poses important health, nutritional, economic, and social problems for inhabitants of endemic areas. Despite considerable recent study and progress, the ecology and pathophysiology of the disease remain relatively little understood. Areas in which progress has been made include identification of the causative organisms and toxins and, to a lesser extent, treatment of affected persons. It has become clear that a variety of disturbances of the ecology of coral reefs may be associated with outbreaks of ciguatera. This paper reviews broadly the current knowledge of ciguatera, particularly the ecological, clinical, and socioeconomic aspects.

KEY WORDS: ciguatera, dinoflagellate plankton, ciguatoxins, sensitization, mannitol, fish, fisheries, gambier toxins, coral reefs.

I. INTRODUCTION

Ciguatera is the disease caused by the consumption of the flesh (muscle), and less frequently the viscera, of fishes contaminated with ciguatoxins. Most ciguateric fishes are captured from inshore fisheries associated with coral reefs. Ciguatera is a circumtropical disease, likely to afflict in excess of 25,000 persons annually. Its greatest impact is on the inhabitants of atoll island countries of the Pacific basin.[1] Ciguatera is rarely fatal, possibly because fish succumb to the

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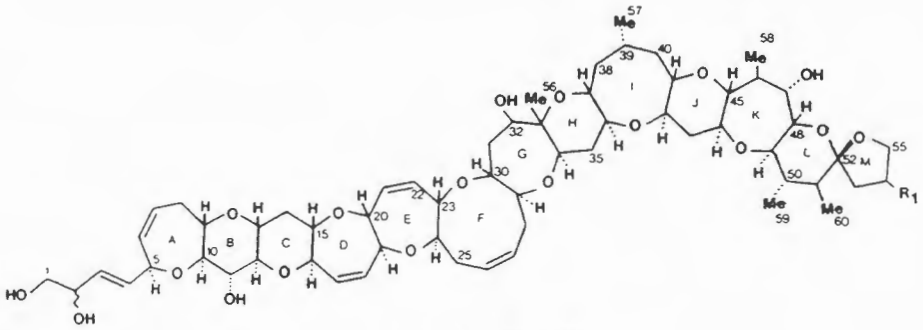
lethal effects of ciguatoxin before concentrations likely to be lethal for humans can be accumulated.[2] It is not surprising that there is an absence of reported fish kills attributable to the ciguatoxins, given that most ciguateric fish are of low-to-moderate toxicity and that ciguatoxin-affected fish may be preferentially preyed upon.

Ciguatera has been the subject of several recent reviews, including those of Gillespie et al.,[3] Lewis,[4] Hokama,[5,7] Vernoux,[6] Juranovic and Park,[8] Russell and Egen,[9] and Lewis.[1] The present review focuses on clinical aspects of ciguatera, as well as its socioeconomic impacts and the recent advances in our understanding of the origin of ciguatera.

II. ORIGIN OF CIGUATERA

A. The Toxins Involved

Three ciguatoxins (CTX-1, CTX-2, CTX-3) appear to be the major toxins responsible for ciguatera.[10–13] They are heat-stable, lipid-soluble polyether compounds of around 1100 mol wt (Figure 1). These toxins have been found in the flesh and viscera of fishes. CTX-2 was recently found to be a diastereomer of CTX-3 (Figure 1).[13] CTX-1, typically the most abundant of these, also is the most potent, with an LD_{50} intraperitoneally to mice of 0.25 μg/kg.[11] Each ciguatoxin acts through binding to site 5 on voltage-dependent sodium channels in the cell membranes of excitable tissues (nerve and muscle), a site also targeted by the brevetoxins,[11] which also cause neurotoxic shellfish poisoning. The result



CTX - 1 R_1 = OH

CTX - 2 R_1 = H

CTX - 3 R_1 = H

FIGURE 1. Structures for three major ciguatoxins from fish. Structures for CTX-1,[10] CTX-2,[11,13] and CTX-3[11] have been proposed on the basis of NMR and mass spectral studies of moray eel ciguatoxins. CTX-2 is a diastereomer of CTX-3, differing only in stereochemistry at carbon 52. This figure shows the stereochemistry at carbon 52 for CTX-1 and CTX-3.

of this binding is that normally closed sodium channels are opened, allowing uncontrolled influx of sodium ions into cells that results in membrane depolarization. A comparison of pharmacological activity revealed that each ciguatoxin possesses a similar ability to activate voltage-dependent sodium channels, although the affinity of these ciguatoxins for neuronal vs. myocardial sodium channels differed.[14]

B. The Dinoflagellates Involved

Many marine biotoxins involved in disease are produced by unicellular algae, particularly dinoflagellates. Working in a ciguatera-endemic area, a Japanese-French collaboration was the first to implicate a new species of dinoflagellate,[15] *Gambierdiscus toxicus*,[16] as the origin of ciguatera (Figure 2). This organism is chiefly benthic (bottom-living), attaching to a variety of macroalgae and turf algae colonizing coral reef surfaces. A lipid-soluble extract from coral reef biodetritus containing a bloom of *G. toxicus* yielded a toxin that was suggested to be identical to ciguatoxin.[15] The structural similarity of CTX-1 and a less polar ciguatoxin analog, gambiertoxin-4b (GTX-4b), isolated from wild *G. toxicus*

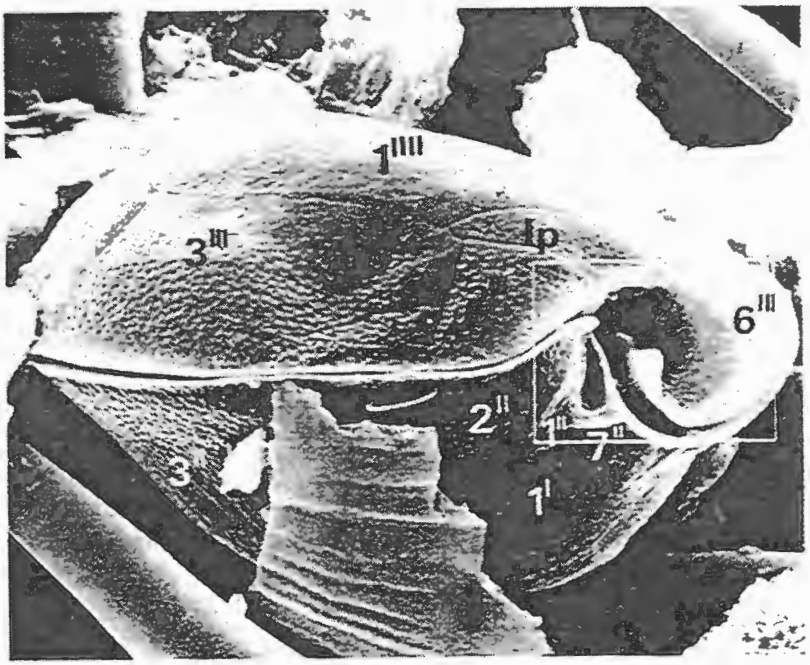


FIGURE 2. A scanning electron micrograph of *Gambierdiscus toxicus*. The ventral region with hypotheca uppermost is shown. The boxed region indicates the sulcus of *G. toxicus*. The thecal plates are labeled according to Adachi and Fukuyo.[16] (Diameter ~0.08 mm.) (Photograph by R. Lewis from a sample provided by N. Gillespie.)

provided further evidence for the involvement of *G. toxicus* in ciguatera. It appeared that GTX-4b was a precursor of CTX-1 that was bioconverted to CTX-1, presumably in fish.[10,11] CTX-3, an intermediate in the bioconversion of GTX-4b to CTX-1, has now been identified in the flesh and viscera of fish.[11,12] The precursor for CTX-2 remains to be structurally characterized.[13]

Further confirmation that *G. toxicus* is the origin of ciguatera was achieved when gambiertoxins were identified from laboratory culture of *G. toxicus*.[17,18] These studies also showed that a majority of the *G. toxicus* strains (11 of 13 tested) were not capable of producing any detectable gambiertoxin, but that each strain produced large amounts of a more polar toxin, maitotoxin. Evidence for the involvement in ciguatera of maitotoxin or other toxins from benthic dinoflagellates is scant.

C. Fish Involved

A range of inshore fish species are typically involved in ciguatera. Fishes more commonly implicated in ciguatera include the Acanthurids (surgeon fishes), Lethrinids (emperors are large-eyed breams), Lutjanids (snappers), Sphyraenids (barracudas), Serranids (rockcods and groupers), Scombrids (mackerels), and Scarid (parrot fishes).[4,19–27] Many of these fish are considered ''fatty'' and as a consequence are highly rated as food in Pacific island countries, particularly the larger individuals of these species.[22] In Australia, pelagic (open sea) fishes such as the Spanish mackerel (*Scomberomorus commersoni*) and Sphyraenids as well as the Serranids (particularly the coral trout *Plectropomus* spp.) are most often involved.[3,28] Pelagic fishes also are responsible for most cases of ciguatera in Hawaii[29] and many cases in the Carribean.[30] Throughout the Pacific, moray eels (Muraenidae) are often highly toxic and as a consequence are usually actively avoided in ciguatera-prone areas.

D. Factors Contributing to Outbreaks of Ciguatera

The incidence of ciguatera in ciguatera-endemic areas is often not static, and may increase dramatically over a short period of time (Figure 3). Such increases may be followed by a rapid, often exponential, decline in incidence.[1]

The precise factors contributing to these changes in the risk of ciguatera have not been elucidated. Global climatic changes, e.g., El Niño events, appear to have no more than a minor influence.[1] Anthropogenic factors may contribute to an increase in the risk of ciguatera.[1] A list of general factors that may be involved is given in Table 1. However, the precise mechanisms involved have not been elucidated. Presumably, the factors contributing to such increases in ciguatera incidence act to accelerate the growth of and/or gambiertoxin production by strains of *G. toxicus* genetically capable of producing gambiertoxins.[17] The lack of

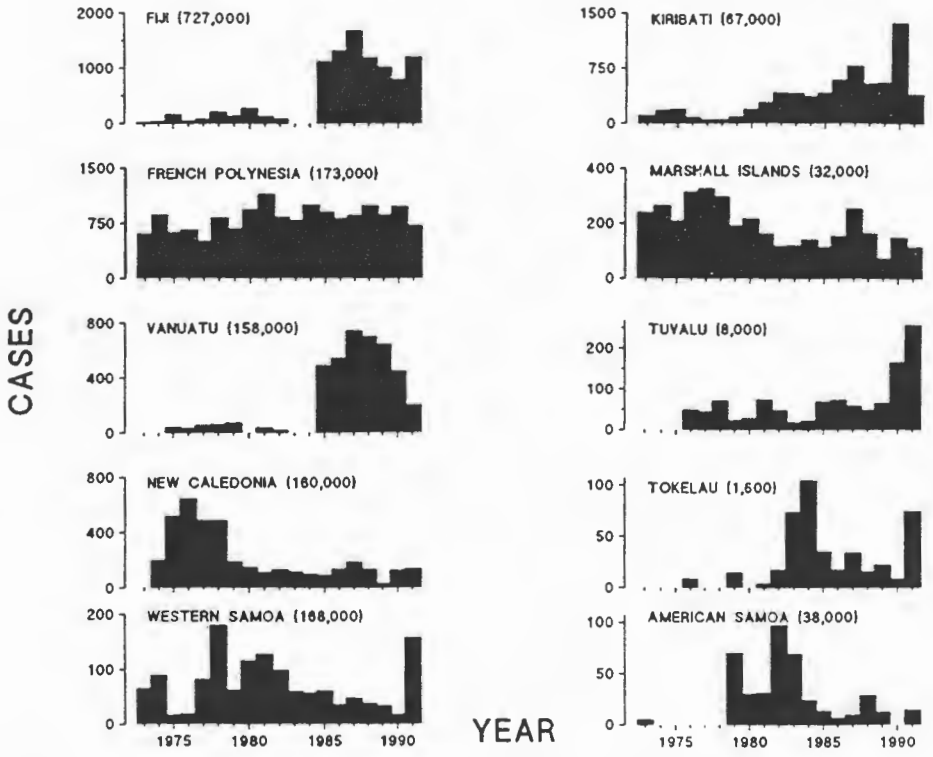


FIGURE 3. Annual cases of ciguatera for selected Pacific Island countries, 1973–1991. Data are from reports of fish poisoning to SPEHIS.[70] The 1988 population estimates for each country are indicated in parenthesis.[71] Prior to 1982, data for the Marshall Islands also included data from the Federated States of Micronesia, the Northern Marianas, and Palau.

TABLE 1
Human Activities That May Increase the Risk of Ciguatera

Pollution of groundwater and seawater on atoll islands (human wastes)
Pollution of seawater on high islands (human and industrial wastes, farming, land clearing)
Reef degradation (dredging, shipgroundings, trampling, anchors, removal, blasting, construction)
Translocation of causative organism (rafting, ships hulls, ballast water)

understanding of the extent of the time-lag between the proliferation of gambiertoxins in the benthos and an upsurge of ciguatera hinders attempts to elucidate the factors responsible for these upsurges.

III. CLINICAL FEATURES

A. Clinical Manifestations

The clinical manifestations of ciguatera are protean, and in areas where the disease is not endemic, the diagnosis is often not considered by physicians unfamiliar with ciguatera. In many parts of the world, increasing international travel and the 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 that the diagnosis is considered in those presenting with illness developing soon after eating fish.

Ciguatera is a polymorphous disease, resulting in variable combinations of gastrointestinal, neurological, general, and cardiovascular manifestations. Symptoms usually develop 1 to 6 h after ingestion of toxic fish — in about 90% of cases within 12 h,[3,31] but in a few after more than 24 h.[32–34] Gut involvement usually consists of an acute self-limiting syndrome akin to gastroenteritis, which may be severe, but generally lasts less than 24 to 36 h.[3,31,35,36] 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 may be compounded by myocardial depression and disturbed vasomotor regulation (including deranged blood pressure control). Neuromuscular disturbances are most commonly sensory, but also may be motor. Although neurological dysfunction is typically suggestive of primary 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[37] or occasionally unilateral.[38] Manifestations may include coma, seizures, ataxia (disordered coordination 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, which produces a distressing burning sensation.[3] 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 most common neurological signs. A fizzy, metallic alteration of taste sensation may occur. Muscle weakness — most commonly distal or generalized, occasionally asymmetrical — may occur, sometimes involving 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.[39]

General (nonlocalizing) and additional symptoms are common, and 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 (localized or generalized), dental pain, a sensation of looseness of the teeth, and dysuria (painful urination) also may occur. A variety of skin rashes, most commonly maculopapular, may occur, often with desquamation (peeling) during the healing phase.

Bradydysrhythmias (cardiac rhythm disturbance with slow pulse rate) and atrioventricular heart block, myocardial depression, and loss of vasomotor regulation with hypotension, often postural, may occur in the early phase and tend to resolve more quickly than the general and neurological symptoms. Autonomic dysfunction also may be manifested by sweating, lacrimation (excessive tears), salivation, and internal ophthalmoplegia (paralysis of ocular accommodation and pupillary responses).

Symptoms often fluctuate considerably from day to day and at different times of day. The time course is generally one of improvement over days to weeks, but it is not uncommon for symptoms to persist for months or, rarely, years. Consumption of alcohol commonly exacerbates symptoms.[3,31] Death is rare (of the order of 0.1% of recorded cases).[3,8,31–33] Illness patterns and severity vary considerably, even among individuals poisoned by the same fish. Reliable diagnosis is difficult because of this and the clinical nature of the diagnosis — there is no specific human diagnostic test for ciguatera. Diagnosis is especially difficult when only one person presents with few symptoms. Nerve conduction studies may be helpful, and demonstration of toxin in any remaining fish samples, while very useful, is often not possible. The most commonly used clinical criteria for the diagnosis of ciguatera are the combination of compatible gastrointestinal and neurological symptoms following the ingestion of potentially toxic fish. This combination, however, occurred in only 25 of 53 (55%) patients in one well-documented common source outbreak,[36] and 52 of 57 (91%) patients in another common source outbreak.[35]

B. Person-to-Person Transmission

Although the vast majority of ciguatera cases is caused by ingestion of toxic fish, various forms of person-to-person transmission have been described, indicative of the persistent and lipid-soluble nature of ciguatoxins. These include

- Transmission via breast milk to breastfed infants[33,40,41] (although hyperesthesia of the nipples of a lactating mother may interfere with breast feeding[42])
- Transmission transplacentally, with transient neurological manifestations in the newborn following maternal illness near term[42]
- Apparent sexual transmission from female to male (penile pain after intercourse in the male partner of an affected woman)[43] and vice versa (pelvic and vaginal pain after intercourse in the female partners of affected men)[44]

C. Sensitization and Recurrent Attacks

These are two of the most enigmatic aspects of ciguatera, and increase the morbidity of ciguatera as well as its social and economic effects. Not only does immunity not follow an attack of ciguatera, but there is good evidence from a variety of locations that second and subsequent attacks tend to be more severe than first attacks.[32]

Also well-documented is the phenomenon of sensitization, where persons who have previously had ciguatera may suffer a recurrence of typical ciguatera symptoms after eating fish that do not cause symptoms in other persons.[34] Consumption of alcohol or chicken may have the same effect.[3,31] Such sensitization can occur many months or even years after an attack of ciguatera.

Both of these factors are most troublesome in areas where people depend heavily on fish as their major dietary source of protein.

The basis for sensitization and recurrent attacks tending to increase in severity is not known, but is presumably 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 sensitization following ciguatera.[45]

IV. PATHOLOGY AND PATHOPHYSIOLOGY

Human pathological studies of ciguatera are few. In 1983, Nakano[39] reported high blood levels of creatine phosphokinase (CPK, a muscle enzyme) in seven men affected with typical ciguatera symptoms during an outbreak on Midway Island in the Central Pacific. The CPK level, initially >1000 IU/l (normal <200 IU/l) in each case, returned to normal within 10 days. Although motor and sensory nerve conduction velocities remained normal in these patients, 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 two patients. Muscle biopsies from three patients showed muscle fiber splitting, degeneration, and necrosis, with subsarcolemmal tubular aggregates and small lipid vacuoles. A near-fatal case in Hawaii was associated with prominent generalized muscle spasm and high blood levels of CPK (41,000 IU/l, reference range 45 to 35) and other muscle enzymes.[46] Palytoxin present in the smoked mackerel (which originated in the Philippines) was thought to be responsible for these effects. Similar cases also have been described following parrot fish ingestion in Japan.[47] A possible association between polymyositis (a chronic inflammatory disease of muscle) and ciguatera occurring some years previously has been suggested[48] but remains speculative.

The major morbidity of ciguatera, however, is probably attributable to its effects on peripheral nerves. In 1978, Ayyar and Mullaby[49] reported slowed sensory conduction velocities without a decrease in sensory nerve action potential

amplitude in affected patients. Other studies[50–52] have documented various findings: 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 predominantly demyelinating rather than axonal in type (damaging the myelin sheaths of nerves, which are part of Schwann cells, rather than the nerve fibers themselves).

The only published report of nerve biopsy findings in ciguatera is by Allsop and colleagues,[50] who found striking edema of vacuoles in Schwann cell cytoplasm adaxonally (immediately abutting axons), with axonal compression and vesicular degeneration of myelin.

Some reports[39] describe diffuse slowing on electroencephalography, elevated cerebrospinal fluid pressures, and abnormal brainstem auditory evoked responses in patients with ciguatera, although these are not commonly reported or specific findings.

One interesting finding in rats is that a blood ethanol (alcohol) level of 0.05% was found to significantly prolong the abnormal supernormal response observed in ciguatoxin-treated rats.[52] The mechanism of this potentiation, which is consistent with clinical experience in humans, is yet to be elucidated. The nature of the human immune response to ciguatera is essentially unknown.

V. TREATMENT

Despite impressive recent advances in understanding the nature and pharmacology of ciguatera toxins, this has yet to translate into major 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 forms of treatment have been tried and although some important uncontrolled observations have been reported, particularly in relation to mannitol, no double-blind controlled clinical trial results are available for any treatment modality.

Supportive and symptomatic therapy may include analgesia, fluid and electrolyte replacement, airway protection and ventilatory support, circulatory support (may include positive inotropic agents), management of dysrhythmias (most commonly bradycardias and atrioventricular block), general care of the unconscious patient, antihistamines, and cool showers for pruritis, hypnotics, etc. In French Polynesia, standard[53] (but unproven[54]) therapy for hospitalized 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.[22,34,55–57] Screening of traditional plant remedies with a novel mouse bioassay has found that an extract from the leaves of *Argusia argenta* can reduce the effects of ciguatoxin.[55] No scientific studies on the efficacy and safety in humans of any of these traditional remedies are available.

Occasional success has been reported with low-dose amitriptyline, a tricyclic antidepressant, particularly for chronic paraesthesia and other neurological symptoms.[58–60] Recently, fluoxetine (a newer antidepressant drug that 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 9 months.[61] Nifedipine (a calcium channel blocker)[60] and tocainide (a lignocaine-like local anesthetic agent)[62,63] 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.[64] 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. Two patients in coma and one in shock responded within minutes, with full and rapid recovery, hitherto virtually unknown in severe ciguatera (recovery typically takes at least 1 and, more usually, 2 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[65–67] confirm that in some patients (including young children)[68] 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 h of symptoms onset) would appear most likely to benefit from mannitol. The mechanism of action of mannitol in ciguatera is unclear — possibilities suggested[65] 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.[69]

The first controlled trial of mannitol in ciguatera was reported recently by Bagnis et al.[53] Thirty-four patients were treated with mannitol, 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 h 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 h 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 h despite mannitol infusion. Further studies of mannitol treatment are underway in Fiji and the Marshall Islands. 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 is evident when it occurs, the

administration of mannitol would seem justified in patients whose illness is moderate or severe, and particularly those who present during the acute phase of the illness, typically within 24 h of the onset of symptoms. A dose of 1 g mannitol per kg body weight, as a 20% solution, infused over about 30 min has been most commonly used.[64,65] The clinical impression is that half this dose, infused over 60 min, appears to be less effective.[65] No adverse experiences have been reported with use of mannitol in ciguatera, but care should be taken to ensure 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, however, on the availability of medical treatments, particularly one requiring careful supervision and intravenous infusion. A safe orally active therapy requiring minimal supervision would be a major breakthrough.

All patients suffering from ciguatera should be advised to avoid fish and alcohol for at least 3 months, and to subsequently reintroduce them into their diet extremely cautiously, recognizing that ingestion of either may precipitate a relapse of symptoms. Many sufferers of ciguatera, particularly those in Western cultures, lose all inclination to again eat reef fish.

VI. SOCIOECONOMIC IMPACTS OF CIGUATERA

A. Incidence of Ciguatera

Ciguatera is prevalent in many countries that harvest fish from coral reef waters. The most comprehensive regional database on ciguatera is that compiled by the South Pacific Epidemiological and Health Information Service (SPEHIS) for countries in the South Pacific Commission.[70] These reports include cases of ciguatera, as well as other forms of marine food poisoning (scombroid poisoning, clupeotoxism, mullet poisoning, puffer fish poisoning, and invertebrate intoxications). However, ciguatera typically dominates as a cause of fish poisoning in the Pacific region.[1] Ciguatera is reportedly prevalent throughout Pacific island countries with the exception of the Solomon Islands and Pitcairn Island (Figure 3).[70]

Ciguatera is invariably an underreported disease. In Australia, it is estimated that as few as 20% of cases are reported. Less than 10% of ciguatera cases of Western Samoa are reported to SPEHIS.[1] Similar levels of underreporting are likely for other countries. Underreporting may vary within and between countries, as well as over time.

For countries of the Pacific, the highest average incidence of reported ciguatera was approximately 100 cases per 10,000 population per annum (p.a.) in several atoll island countries (Kiribati, Tokelau, Tuvalu) (Figure 4).[70] 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 per 10,000 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.

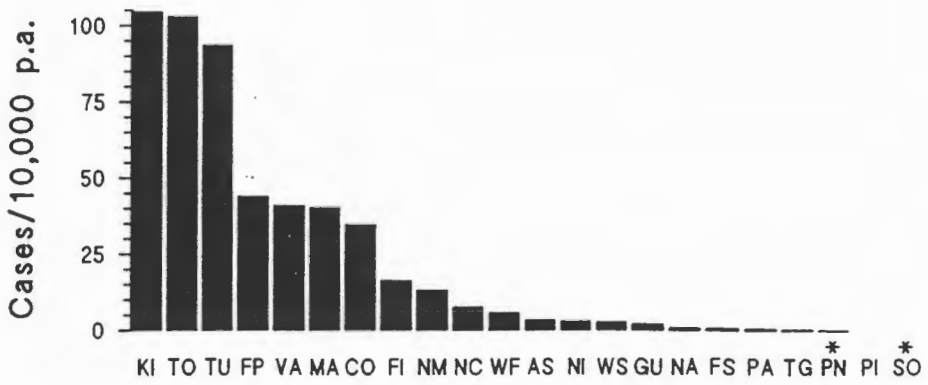


FIGURE 4. Incidence of ciguatera in Pacific Island countries. Cases per 10,000 population are indicated for Kiribati (KI), Tokelau (TO), Tuvalu (TU), French Polynesia (FP), Vanuatu (VA), Marshall Islands (MA), Cook Islands (CO), Fiji (FI), Northern Marianas (NM), New Caledonia (NC), Wallis and Fortunas (WF), American Samoa (AS), Niue (NI), Western Samoa (WS), Guam (GU), Nauru (NA), Federated States of Micronesia (FS), Palau (PA), Tonga (TG), Papua New Guinea (PN), Pitcairn (PI), and Solomon Islands (SO). Data are given per annum (p.a.) and were averaged from SPEHIS data[70] covering the period 1985–1990. Asterisks indicate incomplete reporting to SPEHIS from these countries.

B. Adverse Impacts of Ciguatera

Ciguatera impacts directly on the health of inhabitants adjacent to coral reefs. Throughout Pacific Island countries, there is a heavy dependence on the inshore fishery resource for demersal (bottom-associated) reef fish, which provide essential dietary protein and satisfy a desire for animal fats. Johannes[72] has 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 island countries of the Pacific where intake of reef fish is often >100 g per person per day.[1] Ciguatera also is important in relative terms because it is one of the more common of the diseases reported to SPEHIS.[1]

In addition to the direct effects of ciguatera on public health, ciguatera also 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 in the poorer atoll islands of the Pacific, also may be impaired through the effects of ciguatera.[4] Ciguatera also may have direct economic effects, reducing trade opportunities in potentially ciguatera fishes[4] and has the potential to damage tourism.[4] Concerns over ciguatera are likely to be reduced in countries where alternative dietary protein sources to locally caught fish are costly and few, and where a system of traditional beliefs act to reduce the perception of the adverse impacts of ciguatera.[1] People in larger and developed countries (e.g., Australia) are likely to be less accepting of ciguatera than are people in many of the Pacific Island countries.[1]

The need to avoid fish after an outbreak of ciguatera may exacerbate undernutrition, especially among children.[73] Fear of poisoning accentuates any depen-

dence on imported food, so-called "dietary colonialism." In many Pacific locations, as much as 90% of fish eaten comes out of a can.[4] An increased intake of imported food is often associated with a higher salt, fat, and refined carbohydrate diet that may contribute to a rising prevalence of chronic diseases such as diabetes,[74] gout,[75] hypertension,[76] and atherosclerotic vascular disease[77] in a variety of indigenous Pacific populations.

The adverse effects of ciguatera are presently difficult to quantify.[1,4] Accurate information on (1) the true incidence of ciguatera in Pacific Island countries; (2) the extent and way in which different communities avoid ciguatera; and (3) the extent ciguatera impacts on health, the work-force, trade, and tourism is required before the socioeconomic impacts of ciguatera can be more accurately quantified and more appropriately managed.

VII. PREVENTION

A comprehensive approach to the prevention of ciguatera involves approaches at the individual, community, and population levels. Key elements of such an approach are outlined below.

A. Individual Level

There are various measures that individuals can adopt to reduce their risk of contracting ciguatera:

- Avoidance of warm water reef fish, particularly those with a known propensity to carry ciguatoxins, and avoidance of certain pelagic fish that feed on them (e.g., barracuda and mackerel spp.), especially in areas with a history of ciguatera.
- Avoidance of all fish from locations that are a known recent or current source of toxic fish. Reference to local knowledge and experience is valuable in relation to both the above measures.
- Complete avoidance of moray eels, which are commonly highly toxic,[10,11,78] except when this species is captured in areas with no history of ciguatera.
- Avoidance of large fish from the top of the food chain (carnivores) may reduce, but does not eliminate, the risk of contracting ciguatera. Carnivorous fish are more likely to be toxic that herbivorous fish because ciguatoxins tend to be concentrated as they pass up the food chain, and larger fish (particularly more than 2.5 kg in weight) are more likely to be toxic than smaller fish.[79]
- Avoidance of the head, roe, and viscera of potentially toxic fish. Concentrations of ciguatoxins in fish livers may be up to 50-fold higher than those in muscle.[80]
- Eating a small portion (<50 to 100 g) from any one fish at the first sitting.[1]

- Feeding a large fish flesh meal to a cat that is observed for at least 6 h prior to human consumption of portions of the same fish.[1,22]
- Washing of 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.[8] This has not, however, been demonstrated to be useful.

In endemic areas, the optimal method for protecting individuals from ciguatera would most likely be immunization. Unfortunately, the immunology of ciguatera is hardly understood and prospects for an effective vaccine are distant.

B. Public Health Measures

These include

- Education of fisherpeople and the public about the risk of ciguatera and how this risk can be reduced.[81]
- Closure of highly toxic areas for fishing.[81]
- Bans on the sale of high risk fish from known toxic locations. Such bans have been used in American Samoa,[24] Queensland,[28] French Polynesia,[4] Fiji,[21] Hawaii,[81,82] and Miami,[83] apparently with some success, but with attendant economic loss.
- The 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 n*M* CTX-1 per kilogram of fish flesh.[2] To be used effectively at the community level, they should be robust, temperature-insensitive, reliable, inexpensive, and simple to use.

Hokama has pioneered studies directed toward the development of such a test to detect ciguateric fish.[7] A radioimmunoassay (RIA) developed by Hokama and co-workers in Hawaii and subsequently modified to a simpler enzyme immunoassay (EIA)[84] has been further simplified to a "stick" test, which has been used to screen fish caught in Hawaii.[85] All of 57 fish, provided by the Hawaiian Department of Health in 1987–1989 and implicated in cases of ciguatera, tested positive on a stick enzyme immunoassay (S-EIA) using a monoclonal antibody against ciguatoxin (MAb-CTX).[85] 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.[85] However, a high proportion, 1195 of 2190 (55%), of randomly tested fish of 19 different, potentially ciguatoxic, species tested borderline or positive,[85] suggesting a high rate of false-positive tests. The false-negative rate, however, being of more immediate importance, would appear to be acceptably low.

Although the test has problems of specificity, e.g., 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,[85] it holds promise as a practical measure in ciguatera control, particularly for large fish handled commercially. Several groups are currently 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 the detection of ciguatoxins using fluorescence high pressure liquid chromatography (HPLC) also is being undertaken. HPLC-based assays, perhaps linked to fluorescence or mass spectral detectors, have the potential to provide a means of confirming the presence of 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.[22]

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.[81] 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), also could make an important contribution to our understanding of the genesis of ciguatera. Such monitoring should be initiated with baseline studies prior to major development likely to damage or alter a coral reef. There is widespread concern, particularly in the Pacific, that population expansion and economic development may increase the per capita incidence of ciguatera.[1,4] The possible effects of global warming, stratospheric ozone depletion, and other global environmental changes on ciguatera are unknown and provide additional justification of long-term environmental monitoring.

Restriction of human activities likely to be associated with coral reef damage also may be beneficial. In some areas in the Pacific, such as the Line Islands,[86] Gilbert Islands,[22] and the islands of Hao, Moruroa, and Mangareva in French Polynesia,[87,88] military-related dumping of material on reefs, construction activities, and nuclear test explosions have been clearly associated with outbreaks of ciguatera. Similarly, outbreaks have followed shipwrecks, shore modification, and other construction activities in the Marquesas islands[89] and Hawaii.[82,90] There is scope for avoiding or modifying such environmentally damaging activities in fragile coral reef environments, with additional benefits unrelated to ciguatera. These include scenic and cultural values, habitat and biodiversity preservation, and reduction in military expenditures and attendant risks of conflict and war.

VIII. FUTURE DEVELOPMENTS

Important areas for progress in understanding and control of ciguatera include

- An understanding of key environmental factors involved in upsurges of ciguatera and how *G. toxicus* responds to these influences.
- Better tests for ciguatoxins. Antibodies that are more selective and have higher affinity for the ciguatoxins than those currently in use are needed.
- An understanding of the human immune response to ciguatera and the pathophysiological mechanisms underlying the phenomenon of sensitization.
- Treatment for ciguatera that is simple to administer (preferably orally), inexpensive, and is demonstrated to be effective and safe.

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Appendix XII

Trip report

CAN FISH BE EATEN AFTER BEING POISONED?

People who have been poisoned should avoid eating warm ocean fish for 6 to 12 months. Alcohol should be avoided for about 3 months because it can cause symptoms to recur. Prawns, crabs, cold water fish and estuarine fish such as dory, garfish, mullet, whiting, black bream and flathead should not cause problems. Nevertheless, only small portions of seafood should be eaten initially. If on eating fish mild symptoms recur (including tingling sensations), people should refrain from eating that type of fish for several more months.

CIGUATERA SURVEY

Little is known about the distribution of ciguatera poisoning in Queensland. It is therefore important that information be obtained on the type of fish involved and where the toxic ones are caught. Persons having experienced any of the symptoms described in this leaflet, should contact one of the below, who will arrange an interview with the Health Inspector.

Chief Inspector of Foods
Telephone (07) 234 0952
or
State Health Offices at –

Bundaberg	(071) 73 8131
Cairns	(070) 51 2188
Mackay	(079) 51 8842
Sunshine Coast	(071) 43 7144
Gold Coast	(075) 56 1655
Rockhampton	(079) 22 1872
Toowoomba	(076) 38 1500
Townsville	(077) 27 0203

FURTHER INFORMATION ON CIGUATERA

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CIGUATERA POISONING

QFMA

QLD STATE HEALTH DEPT

Queensland Department of Primary Industries

INFORMATION AND TREATMENT

WHAT IS CIGUATERA?

Ciguatera is an uncommon form of food poisoning in Australia. It is caused by eating ciguatera carrying fish that live in warm ocean waters. Ciguatoxin is produced by a tiny organism called a dinoflagellate which is usually attached to algae growing in reef areas. The toxin is transferred to plant-eating fish and then to larger predatory fish where it accumulates. Cooking does not inactivate the toxin.

WHAT ARE THE SYMPTOMS?

Symptoms commence 1 to 24 hours after fish are eaten depending on the toxicity of the fish, the amount eaten and the susceptibility of the consumer.

Symptoms may include:

- tingling and numbness often in fingers and toes but also around the lips, tongue, mouth and throat;
- burning sensation or skin pain on contact with cold water;
- joint and muscle pains with weakness and/or cramps;
- nausea, vomiting, diarrhoea and/or abdominal cramps;
- muscular weakness, headache, fatigue, fainting;
- exteme itchiness, often worsened by drinking alcohol; and
- in severe cases, difficulty in breathing.

Medical examination should exclude other conditions such as botulism.

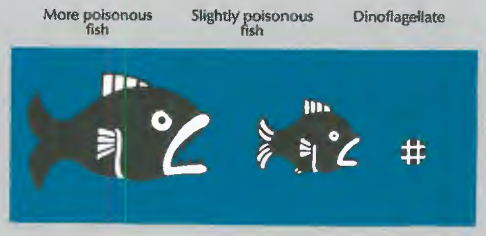
WHAT TYPE OF FISH CAUSES POISONING?

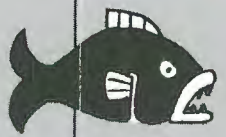
Any fish that inhabits warm sea waters is a potential ciguatera carrier. Ciguatera poisoning occurs sporadically and unpredictably. However, there are certain fish caught in warm ocean waters such as the **moray eel, chinaman, red bass** and **paddletail** species that are reputed to be regular ciguatera carriers and because of this are not accepted for sale by marketing authorities. Because these fish are not marketed they cause few problems. Problems are more often encountered with **coral trout, Spanish mackerel, reef cod, barracuda, emperor, groper** and **surgeon fish. Trevally** and **kingfish** can also cause problems.

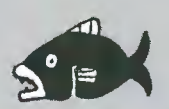
Ciguatera victims are often anglers and their families who have feasted upon a particularly large 'catch'. The toxic fish is often one of the larger of the batch and is usually a predatory species.

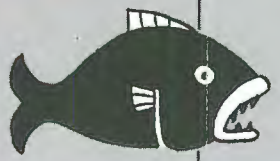
The presence of toxin, even in high concentration, does not alter the appearance, smell or taste of the fish. Cooking or freezing does not destroy the toxin and no known culinary method removes it.

CIGUATERA FOOD CHAIN









PREVENTION

As no simple way exists for the detection of ciguatoxin, caution should be observed in the taking and consumption of fish.

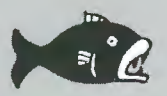
At present, the only way to minimise the risk of poisoning is to observe the precautions below.

- Fish species that are locally implicated in poisonings should be avoided.
- Moray eel, red bass, chinaman and paddletail should not be eaten.
- Large warm ocean water fish should be treated with suspicion and avoided if possible.
- No more than 250 grams of flesh from any warm ocean water fish should be eaten at the first sitting.
- Under no circumstances should the head, roe, liver or other viscera be eaten as toxin concentrates in these parts.
- If symptoms of poisoning such as tingling or numbness develop no more of that particular fish should be eaten. The remainder of the fish including the viscera should be retained for toxicity testing.

TREATMENT

People who develop symptoms of ciguatera poisoning should seek immediate medical advice. This is important because an intravenous infusion of mannitol may give significant relief (in severe cases) if commenced promptly. Treatment details are available from the Poisons Information Centre (07) 253 8233.

Patients need not be alarmed about long term health effects. Most symptoms subside within 2 weeks with the worst disappearing in days. Occasionally some symptoms may persist for a few months or longer.



Appendix XIV

Original grant application

FISHING INDUSTRY RESEARCH TRUST ACCOUNT

APPLICATION FOR GRANT 1988/89

1. TITLE OF PROPOSAL

Experimental and clinical studies of mannitol in the treatment of ciguatera.

2. NAME OF APPLICANTS

(i) Queensland Department of Primary Industries (QDPI)
(ii) University of Queensland (UQ)

3. DIVISION, DEPARTMENT OR SECTION

(i) Division of Dairying and Fisheries, Fisheries Research Branch (QDPI)
(ii) Department of Child Health (UQ)

4. PROPOSAL

It is proposed to assess the clinical efficacy of mannitol in the treatment of ciguatera in Australia and to determine experimentally the mechanism(s) underlying the interaction between ciguatoxin (CTX) and mannitol. Encouraging preliminary results have indicated that mannitol infusion may provide unique benefit to sufferers of ciguatera. This project will involve: (i) the treatment of ciguatera sufferers with mannitol and assessment of the extent and duration of beneficial effects; (ii) the development of an in vivo model of ciguatera (e.g., mouse) able to quantify the protective effects of mannitol and other potential therapuetic agents; (iii) the use of in vitro models of ciguatera (i.e., isolated guinea-pig atria and ileum) to determine the mechanism(s) by which mannitol and related compounds interact with CTX.

This research has the potential of outlining, for the first time, a rational basis on which to provide permanent relief to ciguatera sufferers.

5. NAME OF PERSONS RESPONSIBLE FOR PROGRAMME, TELEPHONE, FACSIMILE AND TELEX NUMBERS

Mr R.G. Pearson, Director, Fisheries Research Branch, QDPI
GPO Box 46, Brisbane, Q 4001
Phone: (07) 224 6346; Fax: (07) 221 2490; Telex: 41620

6. QUALIFICATIONS OF PERSONNEL TO BE EMPLOYED ON PROGRAMME

Project Leader

Dr R.J. Lewis, BSc, PhD, Fisheries Biologist, Fisheries Research Branch (QDPI), is responsible for co-ordinating the various aspects of the programme. Dr Lewis has been active in the ciguatera field for a number of years and has completed pharmacological, epidemiological, chemical, immunological and ecological studies on ciguatera in Australia and overseas.

Associate Investigators

Professor J.H. Pearn, BSc, MD, PhD, Professor and Head of Department of Child Health, Royal Children's Hospital, University of Queensland. Professor Pearn is a researcher and paediatrician of international standing with extensive experience of poisonings including ciguatera. His knowledge of the ciguatera syndrome and therapuetic procedures and his ability to gain the co-operation of medical practitioners along the coast of Queensland are valuable additions to this project.

Dr N.C. Gillespie, BSc, PhD, Assistant Director, Fisheries Research Branch (QDPI) has been active in ciguatera research for a number of years and has completed epidemiological and ecological studies on ciguatera in Australia. His understanding of the ciguatera syndrome, as it presents in sufferers, will be invaluable in our interpretation of the clinical trial of mannitol.

Mr A. Wong Hoy, BSc, is due to complete a PhD study (May 1988) on the pharmacology of marine toxins at the University of Queensland. He will be employed on the project.

7. OBJECTIVES

i) Clinically assess the efficacy of mannitol in the treatment of ciguatera with the aim of providing an effective treatment for ciguatera;

ii) Develop *in vitro* and *in vivo* models of ciguatera able to assess the protective effects of mannitol with the aim of optimising the use of mannitol in the treatment of ciguatera;

iii) Determine the mechanism(s) underlying the protective effects of mannitol against ciguatera;

iv) Use mannitol as a "lead" compound in the search for other potential therapeutic agents with the aim of further improving the treatment for ciguatera.

8. JUSTIFICATION, INCLUDING PRACTICAL APPLICATION

Ciguatera (fish poisoning) is a significant cause of acute and chronic morbidity in Australia. A recent study from our laboratory has identified in excess of 500 cases of ciguatera [1] which form the basis of a computer data base on ciguatera. The incidence of ciguatera in Queensland is at least 100 per year over the last 20

years [1]. Ciguatera results from the consumption of otherwise edible fish (e.g., coral trout and Spanish mackerel) that have accumulated ciguatoxin through the marine food chain [2]. Humans and animals are poisoned as the final link of this food chain.

At this stage of knowledge no treatment for the distressing condition of ciguatera is available. In the last 3 years trials of amitriptyline, and other supportive therapy, have NOT materially altered the clinical course of the disease [3,4]. Symptoms include prostration, vomiting, diarrhoea and neurological abnormalities including hyperaesthesia and dysaesthesia [1,5]. For the majority of victims, symptoms are usually subacute or chronic. A significant proportion of human victims in Australia remain incapacitated for months as opposed to the normal course of the disease which is for several weeks [1]. The mortality rate of ciguatera is low [5]. Ciguatera is now the greatest single factor affecting the marketing of tropical fishes and victims may successfully seek compensation through legal channels if the patient presents with long term effects. Such a legal challenge is at the moment in progress and involves approximately 40 Sydney residents poisoned by Spanish mackerel.

A recent unpublished report from the Marshall Islands [8] indicates dramatic improvement of ciguatera patients following i.v. infusion of an unspecified dose of mannitol. Telephone conversation with one of the doctors involved (Dr L. Jain) revealed mannitol was first used by chance and that it apparently gives complete, permanent and dramatic relief from ciguatera. This series of cases, involving the treatment of 11 cases of ciguatera by mannitol, comprises the first report of any therapy reversing the signs and symptoms of ciguatera in humans. As such it requires clinical and experimental investigation: and after years of therapeutic inadequacy the potential exists for treating in the near future victims of this common and non-trivial intoxication. No deleterious side-effects of the mannitol therapy have occurred.

We have now commenced the clinical trial of mannitol in Australia. Four patients suffering ciguatera have agreed to be treated, with some patients experiencing improvement. No adverse effects from the treatment were reported. These preliminary results indicate that the timing of treatment following poisoning may be critical to the outcome and that two infusions or a higher dose of mannitol may be required to obtain satisfactory recovery in some cases of ciguatera. These results allow us to be confident of developing an effective cure for ciguatera.

Ciguatoxin (CTX), the toxin responsible for ciguatera [9], is believed to be produced by a dinoflagellate and has a molecular weight of 1112 daltons and an LD_{50} i.p. in mice of 0.45 ug/kg [10]. The chemistry of CTX is poorly understood. Ciguatoxin's mode of action is to increase the permeability of Na channels [11,12,13] by binding to a unique site on the sodium channel [12]. CTX increases Na channel permeability by shifting in the hyperpolarising direction the voltage dependence of activation [13]. This effect causes spontaneous firing of adrenergic [14] and cholinergic nerves [15] followed by suppression of nerve conduction [15]. Neural block of motor nerves by CTX is seen without preceding neural stimulation [16]. CTX causes direct and indirect positive inotropy [11] and

direct negative inotropy in cardiac preparations [17,18]. Death is from respiratory failure [19] but in artificially respired animals cardiac failure can be the cause of death [20]. Lewis and Endean in a previous FIRTA funded project on ciguatera revealed that ciguatoxin in Australian fishes is similar to, if not identical with ciguatoxin present in fishes of the central Pacific in chemistry and pharmacology [11, 15, 16, 21, 23].

Several hypotheses that could underly an interaction between CTX and mannitol in vivo include (1) mannitol competes for an overlapping site with CTX, thereby displacing CTX; (2) mannitol reduces CTX binding by an allosteric mechanism, thereby reducing the affinity of the binding site for CTX; (3) mannitol changes the 3-D structure of CTX, thereby reducing the affinity of CTX for its binding site; and (4) mannitol forms a stable complex with CTX, thereby increasing the rate of efflux of CTX. The research plan following is designed to determine the efficacy of mannitol in the treatment of ciguatera in clinical trials. The experimental outline described is designed to gain an understanding of the mechanism of the mannitol-CTX interaction in vivo. In addition, using mannitol as a "lead" compound will allow the possibility of finding additional compounds able to treat ciguatera.

Preliminary results have indicated that mannitol can displace ciguatoxin from its binding site on the guinea-pig atria. This encouraging result indicates that mannitol is the first compound with this action and is strong support for the clinical findings and supports the choice of the guinea-pig atria as a model of ciguatera.

Potential significance

No effective treatment of ciguatera is available at this stage of knowledge: management (today) consists of non-specific and supportive measures only. Clinical studies using mannitol in the treatment of ciguatera will, if successful, allow a rational basis for the treatment of ciguatera to be established for the first time. This will have obvious benefits to the fish-eating public as well as to the tourist, fishing and related industries. Experimental studies will provide details of the CTX-mannitol interaction. This information will provide the basis for further improvement in the treatment of ciguatera. This study will more precisely define the CTX binding site associated with the Na channel, information adding to the structure-function jigsaw of the Na channel. Using mannitol as a "lead" compound, a class of compounds antagonistic to the binding of CTX may be forthcoming from research extended along the lines outlined. Trials of these compounds may reveal additional compounds able to treat ciguatera.

In addition, a permanent cure for ciguatera will overcome the threat of legal action that hangs over suppliers of ciguatoxic fish. If a successful legal challenge occurs, insurance companies are likely to think twice about insuring suppliers of potentially toxic fish: including the coral trout, Spanish mackerel, red emperor etc. (see ref. 1).

Two mice will be used to quantify purified CTX.

The interaction between CTX and mannitol will be assessed by

(a) determining the ability of 0.5 g/kg mannitol i.v. (via tail vein) to reverse the effects of 1.5 M.U. of CTX i.p. when injected at the calculated $t_{0.5}$ (n = 2);
(b) determining the effect of dose of mannitol and timing of mannitol injection on the reversal of effects of 1.5 M.U. of CTX (n = 12);
(c) determine if oral or i.p. mannitol (at maximum effective dose found i.v.) is effective against 1.5 M.U. of CTX (n = 4);
(d) Body temperature and respiration rate will be monitored during the experiment and alterations in symptomology and weight gain documented;
(e) If symptoms persist 6 hr after the administration of the last substance (mannitol or CTX) the experiment will be terminated. If mice recover they will be observed for 24 hr before terminating the experiment with a phenobarbital overdose. The maximal dose of mannitol to be used i.v. will be 2.0 g/kg;
(f) Brevetoxin, another dinoflagellate toxin involved in neurologic shellfish poisoning, also binds to a unique site on the sodium channel [26] and has pharmacological features in common with that of CTX [1]. We propose to determine if mannitol is able to relieve the effects of 1.5 M.U. of brevetoxin (BTX) in mice. This will give evidence as to whether BTX binds to the same site on the Na channel as CTX (n = 2);
(g) Appropriate control experiments will be conducted (n = 12);
(h) Results from this line of research may have an influence on the protocol for ciguatera finally decided upon. When triteated CTX is available the mouse can be used to monitor the rate of excretion of CTX before and after mannitol.

In-vitro guinea-pig atria studies

The isolated guinea-pig left atria has been used recently by us to assess the positive inotropic effects of CTX [11]. CTX induced a direct, dose-dependent (10^{-10} - 10^{-9} M) positive inotropic response not reversed by extended washing with toxin-free Ringer. The effect was shown to be mediated via the opening of voltage-dependent myocardial Na channels [11]. The positive inotropic response of the guinea-pig atria therefore serves as an indirect (though physiologically relevant) measure of the quantity of CTX bound to the sodium channel that is mediating the physiological response.

As such this preparation is ideal to assess in vitro the interaction between CTX and mannitol.

We will quantify the dose-dependent effects of mannitol applied before or after the addition of CTX. Wash-out of the mannitol-CTX treatment will determine if the effect of mannitol remains upon wash-out. Rechallenging atria with CTX will confirm the response-status of the atria to CTX after the mannitol treatment.

We aim to monitor the [CTX] free in the bath throughout these experiments using a monoclonal antibody which interacts with CTX. The monoclonal antibody produced by an existing FIRTA grant will be used. HPLC procedures using our highly sensitive UV detector set at

215 nm will also be used to monitor the [CTX] in the Ringer. When [^{3}H] CTX is available this will be used to monitor the free and bound CTX with the advantage of increased sensitivity.

From our previous studies [11] it was shown that the [CTX] in the organ bath is considerably reduced during incubation of an atria. The possibility exists of detecting if any CTX is displaced from the atria by mannitol as described above.

We will determine if closely related sugars, e.g., mannose and sorbitol, effect the CTX-induced response. We aim to determine the class of compound which may be interacting with CTX binding or its effect. Knowledge of the class of compounds antagonising the binding of CTX may lead to further improvements in the treatment of ciguatera.

A toxin closely related to CTX is brevetoxin. The possibility exists that these toxins share an overlapping binding site [1]. We will determine the efficacy of mannitol to reverse the quasi-irreversible positive inotropic effects of BTX [26]. Tritiated brevetoxin is available (see following section) and will be used to quantify the bound versus free [brevetoxin] if mannitol is shown to reverse the physiological effect of brevetoxin.

Procaine inhibits the binding of brevetoxin to nerves if it is applied before but not after the addition of brevetoxin [27]. We will determine if prior application of procaine (10^{-4} g/ml) inhibits the binding of CTX and brevetoxin. This will be accomplished by adding procaine 30 min prior to a 20 min incubation with CTX or brevetoxin. Procaine and CTX or brevetoxin will be removed by extensive washing with toxin-free Ringer. The inotropic response remaining will indicate the extent of binding of these toxins under the influence of procaine.

Lidocaine appears as a selective blocker of CTX-modified Na channels [18] and has been proposed as potentially useful in the treatment of ciguatera [1,18]. The effect of lidocaine and related drugs including dibucaine, quinidine, propranolol and amitriptyline on the binding of CTX and brevetoxin will be assessed using the protocol described for procaine. The potency of these drugs to reverse CTX-induced positive inotropy will be determined. The drugs most effective at antagonising the effects of CTX may lead to additional classes of drugs being found which are therapeutically useful in the treatment of ciguatera.

In vitro guinea-pig ilea studies

The effects of CTX on the guinea-pig ilea have been described in detail by Lewis and Endean [15]. Using this preparation as a model will allow the interactions between CTX and mannitol on ganglia and cholinergic nerves of mammals to be established. Details of the protocol will parallel studies on the guinea-pig atria.

Rat brain synaptosomes

Synaptosomes will be prepared fresh daily from rat brain using the procedure described by Dodd et al [28] and integrity assessed by ^{22}Na influx determinations [29] in the presence of tetrodotoxin.

[^{3}H] Brevetoxin (PbTx-3) will be prepared synthetically from PbTx-2 by chemical reduction with tritiated NaB^3H_4 [29,30]. PbTx-2 has been provided by Professor Nakanishi, New York, and additional supplies are available from him as well as from Drs M. Poli and D. Baden in the United States. We have also started to develop collaborative arrangements with these latter researchers. The synthesis of labelled and unlabelled PbTx-3 will be performed under the supervision of Dr Ron Quinn at Griffith University who is involved in supervising similar work as part of our antibody study. Purity will be assessed by HPLC. Binding studies will be performed with the assistance of Dr M. Waters, University of Queensland, using his facilities.

Binding studies will involve co-addition of varying concentrations of CTX and [^{3}H] PbTx-3 (5 nM) to synaptosomes in 0.9 ml of binding medium consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, 1 mg/ml BSA, and 0.01% non-ionic detergent as an emulsifier. Incubation will be for 1 hr at 4^{o}C followed by rapid centrifugation (15,000 x g, 2 min), supernatant aspiration and pellet washing (5 mM HEPES (7.4), 163 mM choline chloride, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1 mg/ml BSA) [29]. The radioactivity will be measured by liquid scintillation in the presence of Dimilume. Non-specific binding will be determined using a saturating concentration of unlabelled PbTx-3 (10 um) and will be subtracted from total binding to yield specific binding. Free [^{3}H] PbTx-3 will be determined by directly assaying an aliquot of the supernatant prior to aspiration. The influence of mannitol and procaine on [^{3}H] PbTx-3 binding will be assessed in a similar manner. This approach has been used successfully to define the brevetoxin binding site [29].

Production of tritiated CTX could be possible by taking advantage of a reactive hydroxyl group on CTX [31]. If this proves possible the reverse of the above experiments looking at displacement of CTX by varying concentrations of unlabelled PbTx-3 and procaine will be performed.

11. PROPOSED COMMENCEMENT DATE AND ANTICIPATED COMPLETION DATE

1 July 1988 to 30 June 1991.

12. FUNDS REQUESTED

	1987-88	1988-89	1989-90
	$	$	$
(a) Total salaries and wages	32 802	32 802	32 802
(b) Total operating expenses	5 500	5 500	5 500
(c) Travel expenses	2 000	2 000	2 000
(d) Total capital items	4 600	-	-
GROSS TOTAL COST	44 902	40 302	40 302
Appendix B (additional optional cost)	7 420	-	-
GROSS TOTAL COST WITH OPTION	52 322	40 302	40 302

13. FUNDS TO BE PROVIDED BY APPLICANT

	1987-88	1988-89	1989-90
	$	$	$
. Salaries and wages (R. Lewis, 20% of time J. Pearn, 10% of time N. Gillespie, 5% of time)	15 000	15 000	15 000
. Operating and administrative (SFRC, Deception Bay)	15 000	15 000	15 000
. Capital (Pharmacology set-up, chart recorders, freezers, etc.)	20 000	-	-

14. CO-OPERATING AGENCIES AND THEIR FUNCTIONS

Department of Physiology, University of Queensland - facility to perform binding studies.

School of Sciences, Griffith University - facility to perform the chemical synthesis of radiolabelled toxins.

Medical Practitioners in Queensland, Northern Territory and New South Wales - perform clinical trials of mannitol.

15. SIMILAR WORK BEING UNDERTAKEN IN AUSTRALIA

This is the first study to be carried out to experimentally assess how mannitol treats ciguatera and represents the first controlled clinical trial of a treatment for ciguatera. Dr M. Capra of the Queensland Institute of Technology is presently supported by FIRTA (# 87/58) to find the mechanism by which fish gain protection from ciguatera, with the potential, in the longer term, of finding a

treatment for ciguatera. There is no overlap between his proposal and this proposal looking at mannitol. Findings from these separate lines of research are likely to provide complementary results towards an enhanced understanding of how to treat ciguatera.

16. PLANS FOR REPORTING OR PUBLISHING RESULTS

Publication as papers in scientific journals is planned. Summaries of results will be reported in the "Medical Journal of Australia", "Australian Fisheries" and at relevant conferences.

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APPENDIX A

18. DETAILED STATEMENT OF FUNDS REQUESTED

(Project: Experimental and clinical studies of mannitol in the treatment of ciguatera)

	1988-89	1989-90	1990-91
	$	$	$
(a) Salaries and wages			
Temporary Fisheries Biologist Div. II (S5-5)	26 668	26 668	26 668
On-costs (23%)	6 134	6 134	6 134
Subtotal	32 802	32 802	32 802
(b) Operating			
Chemicals and drugs	2 500	2 500	2 500
Glassware	200	200	200
Guinea-pigs	500	500	500
Mice	300	300	300
Fish collection and transport	2 000	2 000	2 000
Subtotal	5 500	5 500	5 500
(c) Travel expenses			
Five return trips by air @ $400 average	2 000	2 000	2 000
Subtotal	2 000	2 000	2 000
(d) Capital items			
3 x F-60 isometric force transducer Narco	2 700	-	-
1 x SIU5 Stimulus isolation units	600	-	-
1 x S44 Stimulator (Grass)	1 300	-	-
Subtotal	4 600	-	-
GRAND TOTAL	44 902	40 302	40 302
GRAND TOTAL WITH OPTION IN APPENDIX B	52 322	40 302	40 302

JUSTIFICATION OF BUDGET

Research officer

The project requires a recent PhD graduate with in vivo and in vitro pharmacology/physiology experience. Experience with purification procedures would be an advantage. They will be responsible for performing the experimental studies. Mr A. Wong Hoy is an ideal candidate for this position and he has indicated his availability for this position for three years.

Capital

We require three force transducers and a stimulator to upgrade our existing system. Running experiments four at a time (two atria and two ilea) will greatly increase the experimental efficiency of the laboratory. We have the necessary infrastructure, including chart recorders, to support this additional equipment.

Operating

Support is required for the supply of consumables used during this project. Moray eels need to be purchased to augment supplies of CTX obtained during the FIRTA funded antibody project.

Travel

Travel by air is required to visit centres along the Queensland coast to discuss in person the results of the clinical trials with co-operating physicians.

This budget does not allow for inflation.

APPENDIX B

OPTIONAL COMPONENT TO THIS PROJECT

It is proposed to gain first-hand information concerning the use and success of mannitol in the treatment of ciguatera in the Marshall Islands. To our knowledge this is the only place using mannitol to treat ciguatera (apart from our own study in Australia). In our most recent telephone conversation to Dr L. Jain of the Armer Ishoda Memorial Hospital, he reported over 100 cases of ciguatera had been successfully treated with mannitol in the Marshall Islands. To date they have not published their work.

Specific Objectives

(a) Collaborate with Doctors Luis Jain and Neal Palafox during the treatment of cases of ciguatera in the Marshall Islands with mannitol.

(b) Interview patients treated with mannitol and obtain details of the change in symptomology before and after mannitol treatment using our standard reporting forms.

(c) Interview cases of ciguatera not treated with mannitol as the control group.

(d) This will allow the results of the mannitol treatment in Australia and the Marshall Islands to be compared directly. In addition, the pattern of symptomology of ciguatera in these two countries will be directly compared to determine if we are looking at the same syndrome in each place. These results will help speed progress towards the implementation of a treatment for ciguatera in Australia.

(e) For an additional $300 in airfares we can visit the country of Kiribati. Mr T. Tabano of the Atoll Research and Development Unit in Kiribati is collecting toxic moray eels for us. These eels have provided the bulk of our ciguatoxin to date and we are keen to reinforce this link, which is essential for the successful completion of this project and the FIRTA funded antibody project. The cost to the antibody project of obtaining eels from this source will be in excess of $5 000.

(f) Results of this trip will be published as a Study Tour Report which will be included with the progress report for this proposal.

(g) DETAILS OF FUNDS REQUESTED 1988-89

Airfare (two persons: R.J. Lewis and/or J. Pearn and/or N.C. Gillespie)	4 640
Accommodation (14 nights @ $50/night/person)	1 800
Living expenses (@ $35/day/person)	980
TOTAL	$ 7 420

Appendix XV

Proceedings of the Ciguatera Management Workshop