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A MULTIDISCIPLINARY STUDY OF CIGUATOXIN AND RELATED LOW MOLECULAR WEIGHT TOXINS FROM MARINE SOURCES

ANNUAL REPORT

LESLIE E. BAILEY
YOSHITSUGI HOKAMA
JAMES T. MIYAHARA
PAUL J. SCHEUER

AUGUST 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5236

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The work in this report deals with the isolation and purification of ciguatoxin from ciguateric fish, the pharmacology and toxicology of ciguatoxin and other toxins extracted from various assays and progress made on a system for detection of ciguateric fish flesh. Ciguatoxin has been isolated from livers and viscera of eels obtained from Pacific Islands where ciguatera is endemic. Evaluation of the pharmacology and toxicology has been both in vitro and in vivo systems but has been hampered by a shortage of purified toxins. The immunological "stick method" of assaying for ciguatera in fish has yielded interesting and promising results. Work on all of these aspects of ciguatera toxicity and toxicity to other marine toxins is proceeding at an accelerated pace.

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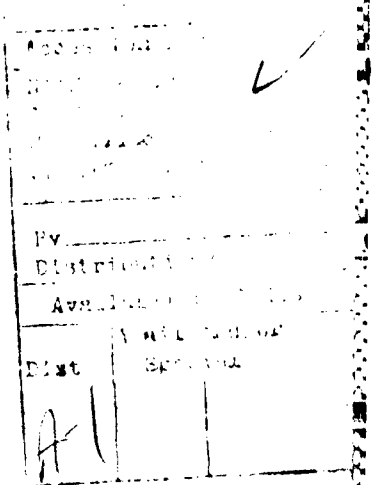

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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The Effects of Crude Tissue Extracts from Various Species on Several Bioassay Preparations

by

Leslie E. Bailey, Ph.D.

Ciguatoxin (CTX), a polyether substance similar chemically to the Na+ ionophore monensin, is believed to act by a similar mechanism to increase the release of acetylcholine, norepinephrine and other neurotransmitters to produce many of the cardiovascular and neurological signs and symptoms characteristic of this illness. However, not all effects of CTX either in vivo or in vitro are attributable solely to this mechanism, suggesting that ciguatera may be caused by a number of toxins such as ciguaterin, maitotoxin, scaritoxin, and okadaic acid as well as many not yet identified. CTX is difficult to wash out of tissues and many of its effects often persist for many hours, and at the same developing tachyphylaxis to repeated administrations of the toxin (1,2). On the other hand, supersensitivity similar to that known to occur in man to ciguatera toxins (3) has never been demonstrated in any preparation challenged with CTX. It is not known if this is true pharmacological supersensitivity or simply retention of "subclinical" amounts of CTX in the adipose tissue of those exposed to the toxin. The latter is more probable since fish exposed to toxin retain maximum toxicity for as long as 18 months.

The clinical management of ciguatera is supportive and symptomatic since no treatment is known to reverse or prevent all symptoms. Paradoxically, another marine toxin, tetrodotoxin, has been shown to prevent the increased permeability of voltage dependent sodium channels by CTX (1). However, this is not a practical therapeutic measure because of the toxicity of tetrodotoxin. Relief from some of the cardiovascular symptoms of ciguatera has been achieved by the use of atropine and the cholinesterase activating drug 2-PAM (4; Bailey and Miyahara, unpublished observations) indicating that at least one of the toxins has anticholinesterase activity. This toxin has not been identified but is found in a methanolic extract of tissues from affected fish (5).

METHODS

1) Anesthetized mongoose

Mongoose, 700 to 1000g, were anesthetized with 35 mg/kg sodium pentobarbital given intraperitoneally. The trachea was cannulated, catheters were inserted into the carotid artery and the femoral vein, EKG electrodes were attached and a thermistor inserted rectally. A Statham P23Db transducer was used for measurement of arterial pressure and a Grass PT5A transducer was used to measure respiration. In a few experiments contractions of the nictitating membrane were measured with a Grass FT03C force displacement transducer. Arterial pressure, rectal temperature, respiration and the EKG were monitored and analyzed on a DASA 9000 data acquisition system. Drugs and toxins were given intravenously and intraarterially.

2) Parallel bioassay

The preparation in this phase of the study is a modification of the blood superfused parallel pharmacological assay technique first developed by Gaddum (6) and refined by Vane (7). Figure 1 is a schematic diagram of the apparatus showing tissues superfused with Krebs-Henseleit solution. This technique has the added advantage that, unlike tissue baths, only small amounts of toxin are required and are added directly to the superfusate. This is of some importance when highly purified and scarce toxins such as CTX are used.

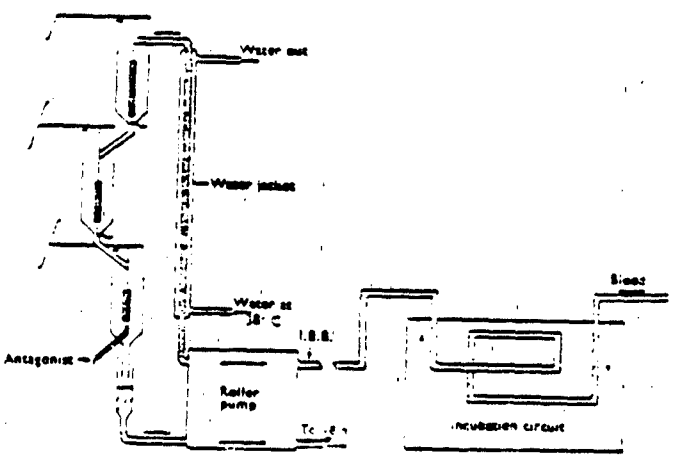

Figure 1. Schematic Diagram of Parallel Bioassay Apparatus (from ref. 7)

3) Isolated cardiac mitochondria and ventricular myocytes

Mitochondria from guinea pig hearts were prepared as described by Sordahl (8). Cardiac myocytes were prepared as described by Bailey and Fawzi (9). Oxygen consumption was measured on 3 ml suspensions of mitochondria or myocytes containing 10-20 mg protein using a Clark electrode and a YSI Model 53 Oxygen Analyzer.

4) Mouse bioassay

Mice, 20-25g, were given crude extract intraperitoneally as described by Kimura et al. (10). The dose of extract given was based on the recovery of crude extract, 1 to 2g/kg (10). Crude extract was dissolved in either 0.2% or 2% Tween 60. Mice were observed for 48 hours for signs and symptoms of toxicity. Time of death was used to estimate the LD_{50} by the method of Molenengo (11).

5) Guinea pig atria

The effect of crude extracts on contractile activity and intrinsic rhythm was assessed in guinea pig left and right atria. Guinea pigs were given 100 U/kg heparin intramuscularly 30 minutes before being anesthetized with enflurane. The animals were killed by a blow to the head, the heart removed and immediately immersed in cold (4 C) Krebs-
Henseleit solution. After contractile activity ceased the atria were dissected away from the base of the heart, and attached by thread to a plastic holder at one end and at the other to a Grass FT03D force displacement transducer. After immersion in the 20ml bath, the right atrium was allowed to beat spontaneously while the left was stimulated at twice threshold voltage at 2 Hz by punctate electrodes in the holder. Contractile activity was assessed at the resting tension which produced 50% of maximal contractile force. The temperature of the tissue was maintained at 30+-0.5 C throughout the experiment.

6) Preparation of crude extract

The procedure of Kimura et al. (10) was used to extract tissues from various species. In some extractions, phospholipids were precipitated by cooling the extract to -20 C. Extracts were prepared from flesh and viscera of kole (Ctenochaetus strigosus), a salt water fish obtained off the Waianae coast of Oahu, and tilapia (Tilapia sp.) a fish obtained from a land locked freshwater pond on the University of Hawaii campus. Extracts were also made of the viscera (liver and intestines) of guinea pigs, and the fruit of the avocado.

Authentic CTX was not available at the time the experiments outlined below were done and consequently the standard against which comparisons were made was a crude extract of kole suspected by the State Department of Health to have caused ciguatera. This material was kindly provided by Dr. Y. Hokama who authenticated its toxicity using tests in his laboratory. Throughout the text this extract is referred to as "authentic kole" while the extract obtained from kole speared at random along the same coast is called "kole".

Results.

1) Parallel Bioassay

A typical response of the three tissues to authentic 1mg/ml kole extract is shown in Figure 2. This extract consistently caused a delayed contraction of guinea pig ileum and tracheal rings and was without effect on guinea pig aortic chains. These responses were characterized by a 10-15 min delay before onset which became irreversible once established. No blocking agents tested, including phentolamine, methysergide, propranolol, indomethacin, or atropine prevented or reversed this response. In the presence of indomethacin, 4ug/ml, CTX frequently produced spontaneous rhythmic contractions of the ileum (Figure 3).

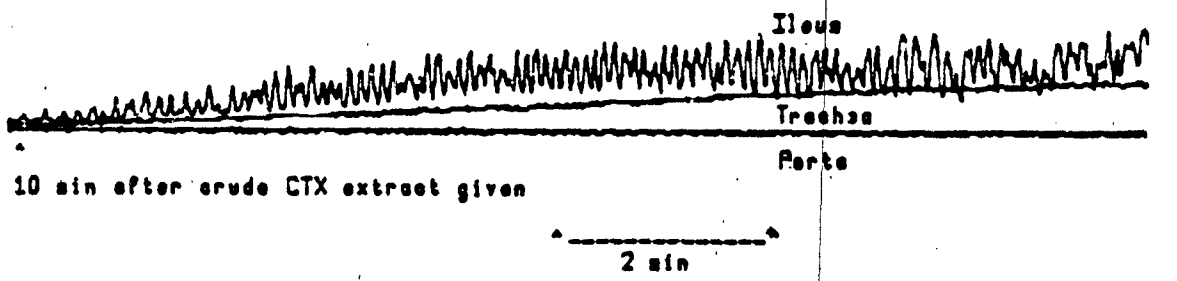

Figure 2. Late onset of contraction to 100 ng/ml authentic kole extract.
Tissues were exposed to extract for two minutes and washed out.

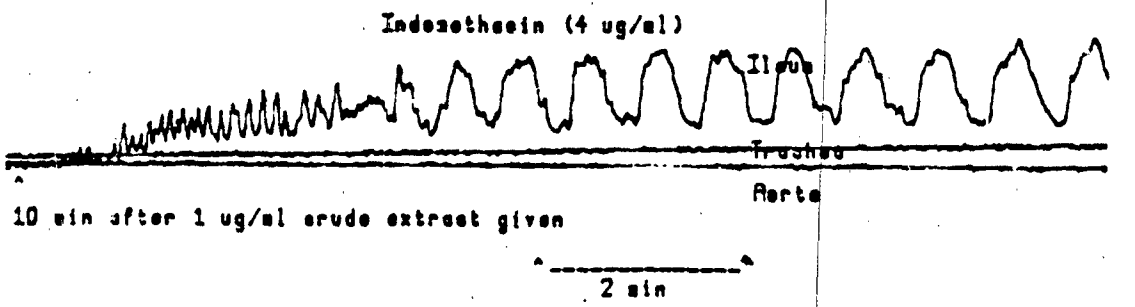

Figure 3. Rhythmic contractions in guinea pig ileum induced by 4mg/ml indomethacin given before 100 ng/ml authentic kole extract. Note that contraction did not occur until 10 minutes after exposure to the extract.

Treatment of the tissues with extracts from tilapia, kole, guinea pig viscera and avocado produced responses similar to those observed with authentic kole extract.

Pretreatment with authentic kole crude extract caused sensitization of the tissues to agonists such as carbachol (Figure 4), norepinephrine (Figure 5), 5-hydroxytryptamine, histamine and isoproterenol.

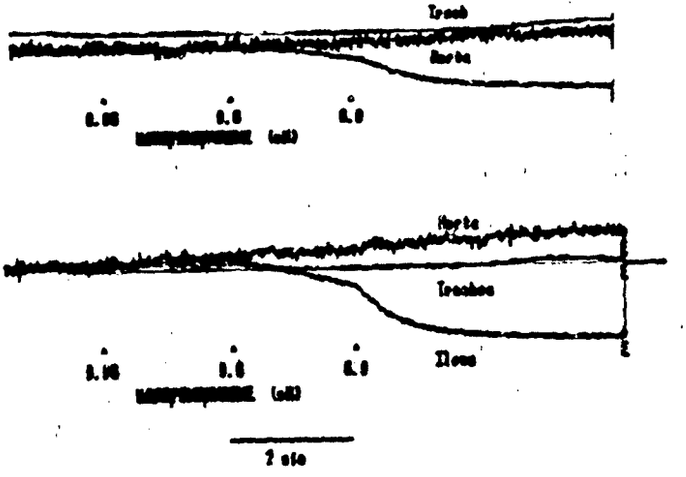

Figure 4. Increased responsiveness of tissues to 0.06, 0.6 and 6 uM norepinephrine after two minutes pretreatment with authentic kole.

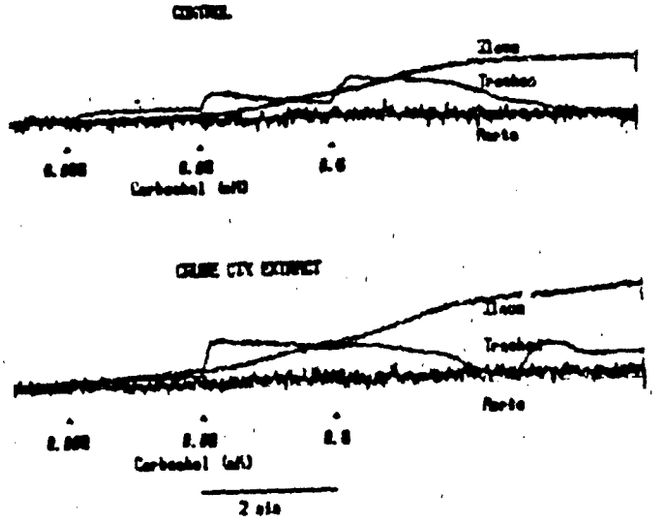

Figure 5. Increased responsiveness of tissues to 0.006, 0.06 and 0.6 uM carbachol after two minutes pretreatment with authentic kole.

2) Anesthetized mongoose.

Authentic kole extract, 5 to 20 ug/kg crude extract, caused characteristic cardiorespiratory responses when injected intravenously or intraarterially. A typical response of arterial pressure to a sublethal concentration (5 ug/kg) of authentic kole crude extract is shown in Figure 6. The immediate effect after administration of 20 ug/kg of extract was a brief period of hypotension followed by respiratory arrest at the maximum elevation of arterial pressure. Support of respiration was essential since this effect appeared to be irreversible. In animals artificially respired, the hypertensive response and tachycardia continued for several minutes. This effect was followed by a gradual decline in blood pressure during the next one to two hours, and ultimately death despite continued artificial respiration. Propranolol, 10 umole/kg, blocked the hypertensive, positive chronotropic and inotropic responses and atropine, 20 umole/kg prolonged the gradual decline of arterial pressure by approximately twofold. All extracts caused some degree of A-V dissociation and occasional ventricular ectopics. Changes in core temperature were inconsistent but tended to decrease. Pretreatment with antagonists including diphenhydramine, phentolamine, indomethacin and others had no effect on these responses.

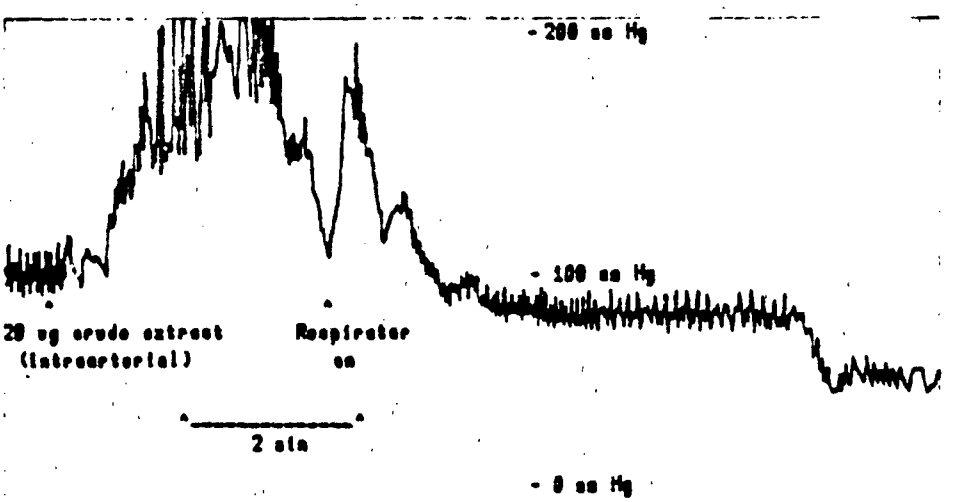

Figure 6. The effect of intraarterial injection of 20 ug/kg of authentic kole crude extract on arterial pressure. This sublethal concentration produced the characteristic cardiorespiratory response of hypotension followed by hypertension with superimposed respiratory depression. Mean bloood pressure before injection was 100 mm Hg and decreased to 80 mm Hg followed by an increase to 180 mm Hg.

Similar to observations made on the parallel bioassay system, intravenous injection of the same quantities (10-20 ug/kg) of crude extract prepared from guinea pig viscera, tilapia, and kole caused cardiorespiratory effects indistinguishable from those of the authentic kole extract and all ultimately caused death. Avocado extract differed only in the amount necessary to produce these effects, 80 - 100 ug/kg in two animals.

Injection of extracts into the carotid artery caused immediate respiratory arrest. The hypotensive response was either absent or less pronounced than that observed after intravenous injection. The onset of sympathetic discharge occurred earlier. When the extracts were given by gastric tube, the amount of extract required to produce similar cardiorespiratory effects was approximately ten times the parenteral dose, regardless of the source of extract.

3) Mouse bioassay

Intraperitoneal injection of authentic kole extract into mice caused flexion, hyperexcitability, diarrhea, salivation, piloerection, ataxia and eventually death. Extracts from other kole, guinea pig viscera, tilapia and avocado produced a similar pattern of effects, however, the dose required was two to four times greater. Intraperitoneal injection of the vehicles used had no effect on the mice. The time of death vs. dose relationship for authentic kole, kole, tilapia and guinea pig viscera extracts is shown in Figure 6. Avocado extract, 1000 to 2000 mg/kg, caused similar effects and had the same endpoint, i.e., death. However, the mean survival time of the animals was 19.8+-3.8 hours or ten to twenty times that measured with the other extracts.

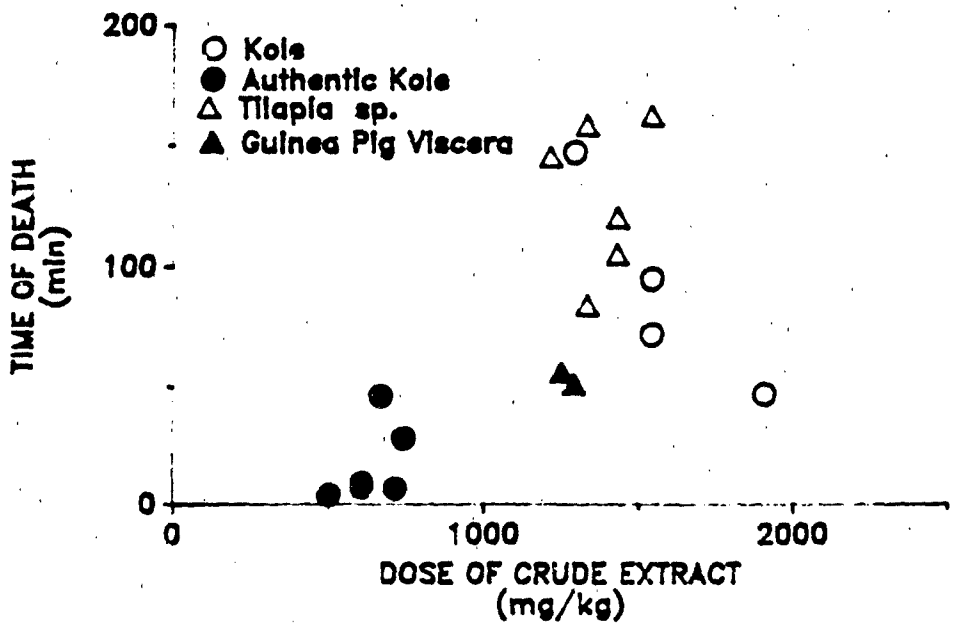

Figure 6. Dose:Time of Death relationship for crude extracts from a number of species

The same data are plotted as dose vs. time of death/dose in Figure 7. From this plot, the estimated LD_{50} for authentic kole was approximately 600 mg/kg crude extract. The LD_{50}s for the kole, guinea pig viscera, and tilapia extracts was approximately 1200 mg/kg or about twice that of the authentic extract. The LD_{50} for the avocado extract was approximately 2200 mg/kg crude extract. Clearly, when time of death or death was used as the endpoint in the mouse bioassay, the crude extracts from all sources differed only in potency.

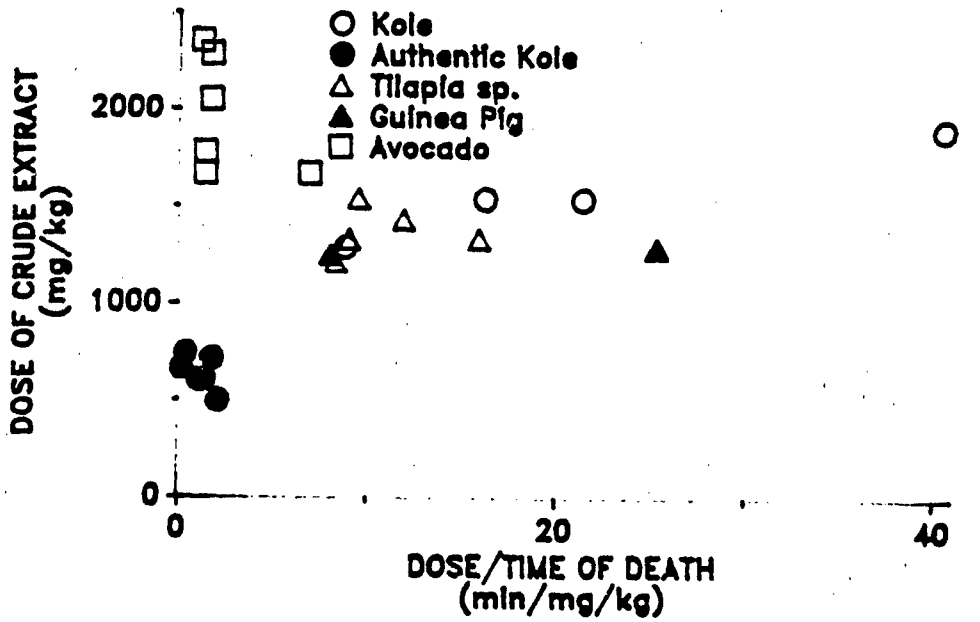

Figure 7. Dose/Time:Time of Death relationship for crude extracts from a number of species

4) Guinea pig atria

Authentic kole extract, 0.1 mg/ml, caused a significant positive inotropic and chronotropic response in the atria. These effects were not observed after treatment with as much 1mg/ml of the other extracts.

5) Isolated mitochondria and ventricular myocytes.

Authentic kole extract, 0.1 mg/ml, had no effect on respiration in cardiomyocytes, but reduced state 3 mitochondrial oxygen consumption from 47.6+-5.9 to 5.9+-1.8 uatoms/min/mg protein. Extracts from other species were not tested.

DISCUSSION

Although the results are incomplete, three tentative conclusions can be drawn from these experiments:

1) The mouse bioassay using death as an endpoint, the intact mongoose and the parallel bioassay are not specific for CTX content of tissues.

2) The extraction procedure either extracted or, less likely, synthesized a highly toxic lipophilic substance from fish, mammalian and vegetable tissues.

3) Further investigation of the pharmacology and toxicology of CTX and other chemically similar toxins requires the use of purified compounds.

The guinea pig atria assay was the only one of the several bioassays tested which appears to be specific for detection of CTX in crude extracts (12). The equivocal results obtained with various extracts on the parallel bioassay, the mongoose assay and the mouse bioassay were somewhat surprising. The comparisons were necessitated because of the unavailability of purified CTX or other purified toxins (see appendix 1) were not available during these studies . That is, a less satisfactory verification of specificity of a bioassay can be obtained by testing a crude extract from species not known to cause ciguatera. Consequently, viscera from a fresh water fish, tilapia, viscera from a mammal, guinea pig, viscera from kole not known to be toxic and vegetable matter with high lipid content, avocado, were extracted by a standard procedure (10). The kole speared at random may have contained CTX, but this was not verified. The effects of the extracts on the three bioassay systems tested, parallel bioassay, mongoose assay and mouse bioassay did not differ from the results obtained from authentic kole extract known to contain CTX by tests done in Dr. Hokama's laboratory. Thus, one is forced to conclude that these three bioassays appear to be inappropriate for assessing the presence or absence of CTX extracted by this procedure from flesh of suspect fish.

The active but unknown principle revealed by the extraction procedure is of some interest. In intact systems, the mouse and mongoose, it caused rapid and irreversible respiratory arrest in low concentrations. This effect may be mediated by an action on the central nervous system since the onset of respiratory arrest occurs more rapidly if the extract is given intraarterially rather than intravenously. Furthermore, a direct peripheral effect on respiration seems unlikely since much higher concentrations are required to depress neuromuscular transmission or muscle contraction of the rat phrenic nerve-hemidiaphragm preparation in vitro. The sympathetic discharge may also be mediated by a direct action of the active ingredient in the extracts on the central nervous system, however, the direct stimulation of norepinephrine release from peripheral nerve endings cannot be ruled out. Clearly any conclusion about the site of action of an unknown active ingredient in a lipophilic extract of various tissues is only speculation based on these incomplete results. The other effect caused by the extract, which may or may not be caused by the same principle, is the sensitization of smooth muscle to other agonists and the late onset response in bronchial, aortic and gastrointestinal smooth muscle. The latter response does not appear to be mediated by an interaction between classical receptors since the effect is not blocked by any antagonist tested.

The observation that authentic kole extract inhibited oxidative metabolism in cardiac muscle mitochondira but not in cardiomyocytes indicates the toxin cannot enter cells but will uncouple oxidative phosphorylation if it does gain access. The active principle in these experiments could not be identified nor were experiments done with the so-called "inactive" extracts.

Future Plans

The apparent lack of specificity of the various bioassays for CTX, especially the mouse bioassay because of its widespread use, must be thoroughly documented and published. Dr Paul Scheuer very generously gave me on August 28, 1986 2 ug of purified CTX which I shall use to test the authenticity of these puzzling observations. In addition, some effort will be directed toward better understanding of the very potent respiratory depressant in all crude extracts tested. These studies will be completed during the next year and certainly by July 1, 1987.

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ASSESSMENT OF IMMUNOLOGICAL ASSAYS FOR THE DETECTION OF CIGUATOXIN AND RELATED POLYETHER TOXINS IN FISH TISSUES

Yoshitsugi Hokama, Ph.D.
Department of Pathology
John A. Burns School of Medicine
University of Hawaii at Manoa

Abstract

This report covers the period from August, 1985 through July, 1986. It consists of all phases of the proposal: 1) Data from the analysis of fishes from the State Department of Health, the State Department of Land and Natural Resources, Division of Aquatic Resources, and from private fishermen, by the stick enzyme immunoassay. 2) Preliminary data from the conversion of cultured G. toxicus extracts suggesting changes from non-CTX-like to CTX-like activity. These include changes in solubility (from methanol soluble to chloroform soluble) and in the stick enzyme immunoassay reactions (from essentially negative to positive). 3) immunofluorescence study of guinea pig tissues (ileum, colon and atrium), treated with CTX (Miyahara) and examined with fluorescein labelled sheep anti-CTX. These tissues showed increased staining of the membrane in certain areas after treatment with CTX.

The results of phases 2 and 3 are preliminary and will require further extensive studies. The results of phase 1, the stick enzyme immunoassay studies, supports our previous studies and we are currently in the process of evaluating the stick enzyme immunoassay in a national and international collaborative study.

Introduction

The application of immunological techniques to the detection of low molecular weight compounds including carbohydrates, peptides, fats and drugs has increased markedly in the past decade. The ability to conjugate these small molecules covalently to appropriate antigenic carriers via their functional groups has led to the production of specific antibodies to the haptenic molecules.

The availability of a specific antibody to these low molecular weight compounds can be utilized for the development of sensitive and specific immunological procedures such as radioimmunoassay (RIA) and enzyme immunoassay (EIA). In addition, the antibodies are useful in examining other areas of study, including tissue distribution, synthesis, and in some instances function of the molecule through agonistic or competitive studies.

The application of sensitive immunological methods to the detection of marine toxins such as ciguatoxin (CTX), saxitoxin and tetrodotoxin merits strong consideration in light of the minute amounts of toxins in fish or shellfish tissues that have constituted serious hazards to the consumer and created anxiety within the fishing and shellfish industries.

Background

Utilizing immunological approaches, a RIA procedure was developed in 1977 (1,2). This procedure was evaluated from 1979 to 1981 by utilizing it for the screening of Seriola dumerili (amberjack) prior to marketing the fish (3,4). The test proved to be successful in removing toxic amberjacks from the market and providing protection to the consumer against CTX poisoning for the period of the study. Over 5000 amberjacks weighing from 1-100+ lbs. were examined. Of these, 85% were sold to the public without any reported incidence of poisoning (3). Economic factors and the complication of the RIA procedure precluded further use of the RIA.

Shortly thereafter, an EIA procedure was developed (5) using the same sheep anti-CTX antibody that was produced for the RIA. Numerous studies have been carried out with this procedure (6,7). The EIA system has demonstrated the ability to react with CTX and related polyethers, including brevetoxin, okadaic acid (8,9,10) and even maitotoxin (9). The EIA system has been shown to detect approximately 5 pg of purified CTX by competitive inhibition assay (7).

These immunological procedures have proven to be superior to other animal and physiological assays (11,12,13,14) with regard to specificity, sensitivity and simplicity.

Recently, a stick test EIA has been devised which is simple, rapid and has potential as a field assay system (15).

Objectives

A. Further develop and evaluate the stick test EIA procedure for screening of toxic fishes. This will aid in the accumulation of toxic samples for fractionation and isolation of the purified toxin.

B. Investigate the possibility of CTX-like moieties being epitopes of maitotoxin, via degradation of maitotoxin with liver tissues from fishes.

C. Use of labelled anti-CTX antibodies for the examination of tissue distribution and localization of CTX and related polyethers in fishes, experimental animals and in vitro pharmacological treated tissues.

Results and Discussion

A. Stick Enzyme Immunoassay

We have continued to assess and refine the stick test procedure during the past fiscal year. This annual report consists of the following:

1. Evaluation of reef fishes from a specific targeted area, Barbers Point on the leeward side of Oahu, as part of an ongoing survey
2. Evaluation of fishes implicated in ciguatera fish poisonings and fishes from associated corresponding catches, submitted by the Department of Health (DOH), State of Hawaii
3. Evaluation of fishes submitted by private individuals
4. Comparison of hybridoma 5C8 anti-CTX antibody and Sheep anti-CTX antibody
5. Comparison of Pentel Correction Fluid and Liquid Paper as a stick coating agent

1. Evaluation of reef fishes from Barbers Point survey.

Table 1 summarizes the results of the stick enzyme immunoassay for the Barbers Point study from Sept. 1, 1985 to July 31, 1986. These fishes were collected by the Department of Land and Natural Resources (DLNR), State of Hawaii, from the Barbers Point study site and three other "control" sites - Brown Camp, Ewa and Mokumanu. A total of 435 fishes were examined using hybridoma 5C8 anti-CTX antibody. For the purposes of scoring fish toxicity by the stick test, stick test results in the borderline positive to high positive range are considered toxic, and are rejected for consumption. The Barbers Point study site had the highest overall rejection rate, 56 per cent for all species combined. Ctenochaetus strigosus (surgeonfish) from Barbers Point also had the highest rejection rate, 62 per cent, of surgeonfish from all sites.

2. Evaluation of fishes from DOH.

Table 2 summarizes the results of stick tests done on fishes submitted by the DOH implicated in cases of ciguatera fish poisoning. Of a total of 16 cases where samples from consumed, clinically toxic fishes were submitted for analysis, all 16 were in the borderline positive to high positive range for ciguatoxin and related polyether toxins. Of a total of 4 cases where only samples from corresponding catch fishes that were not consumed were submitted, 3 were in the borderline to high positive range and one was in the negative range for ciguatoxin and related polyether toxins. The combined total of 20 cases involved approximately 98 individuals. The most extensive case involved 20 individuals affected out of a total of 28 individuals exposed, from a single Caranx sp. (jack).

3. Evaluation of fishes submitted by private individuals.

Table 3 summarizes the results of stick tests done on fishes submitted by private individuals prior to consumption. The 97 Elagatis bipinnulatus (rainbow runner) were all caught off Palmyra Island in late March 1985, and were submitted as a group. Of the Lutjanus bohar (snapper), the three negative fish were also from Palmyra Island and were submitted with the rainbow runners. The seven positive snappers were from Palau. The Seriola dumerili (amberjack) and Caranx sp. (jack) were submitted by various individuals, from Hawaiian waters.

4. Comparison of hybridoma 5C8 anti-CTX antibody and Sheep anti-CTX antibody.

Table 4 summarizes the comparison of hybridoma 5C8 versus Sheep antibody. For several different types of fishes, there appears to be an overall greater sensitivity with the hybridoma 5C8 antibody as compared to the Sheep antibody. The hybridoma 5C8 antibody was produced from mouse ascitic fluid. Currently, production of antibody from in vitro tissue culture supernatant medium is being investigated.

5. Comparison of liquid correction fluid versus oil base opaquing fluid as a stick coating agent.

A problem with the stick enzyme immunoassay procedure has been the instability of the coating agent used, a liquid correction fluid. Correction fluid coats have a tendency to become brittle, with cracking and flaking which can cause false positive readings. Due to the solvent base used in the correction fluid, the coat is also slightly soluble in the methanol used as a fixative in the test procedure. The solvent base also causes inconsistencies in the preparation of stick coats.

To minimize these coat-related problems, a more stable coating agent was investigated. We are investigating an oil based opaquing fluid which maintains its homogeneity without the addition of solvent. This allows the preparation of a coat which is more homogeneous and reproducible, and which essentially eliminates the non-specific background binding of the conjugated antibody. The opaquing fluid coat is also more resistant to cracking and flaking, and is not soluble in the methanol fixative.

Table 5 summarizes the comparison of solvent base and oil base fluids as a coating agent. Both coating agents were tested against a known positive S. dumerili (amberjack) flesh sample, using hybridoma 5C8 antibody. There appears to be no significant difference in terms of test results between the two coating agents,

Table 6 summarizes the examination of fishes that were consumed, with clinically toxic or non-toxic results, using oil base coated sticks and

hybridoma 5C8 antibody. The results with oil base coats fall within the ranges of toxicity scoring established with solvent base coats and hybridoma 5C8 antibody, as to the end-point readings of each toxicity category.

The oil base coat appears to be more consistent than the solvent base, with retention of specificity and sensitivity for detection of ciguatoxin and related polyether toxins directly from fish tissues.

6. Collaborative laboratory study for assessment of the stick test.

Preliminary preparations to set up collaborative studies for assessment of the stick test have been initiated. Stick test "kits" containing necessary reagents and supplies have been provided to participating laboratories as follows: 1) one in Hawaii; 2) one in Australia; 3) two in the East Coast of the U.S.; and 4) one in Palau. All stick test kits were sent via U.S. air mail, and all arrived at their various destinations in good condition. We were interested in the experiences of the collaborative laboratories in setting up the stick test in their own facilities. The major problem area encountered by the laboratories was understanding the written test procedure. With feedback received from the collaborative laboratories, we have modified the written instructions to clarify ambiguous areas and have included more figures and drawings. The current laboratory procedure is included in Appendix A.

Another problem area reported by a few of the collaborative laboratories was scoring test reactions according to the standard color chart included in the kits. The standard color chart we have prepared from commercially available color chips does not precisely match the hue of the test reactions. This appears to cause some difficulty in scoring the test reactions, although we have indicated to the collaborative laboratories that the scoring is based on intensity of color, rather than hue. Possible solutions to this problem include obtaining color chips of the precise hue, preparing liquid standards in test tubes to match the test reactions, and converting the color standards to spectrophotometer readings.

B. Biosynthetic degradation of maitotoxin (MTX).

As reported in the first-second quarterly report, initial studies on in vitro metabolic degradation of maitotoxin were carried out. MTX was obtained in crude extracts of axenic cultures of T-39 Gambierdiscus toxicus, 1 x 10^7 cells in methanol. The crude extract was incubated with homogenized liver tissue from C. strigosus, a herbivore, then extracted again. Controls of crude extract with heat-treated liver tissue homogenate and crude extract with saline were also processed in the same manner.

The end products of liver degradation were tested by: 1) stick test EIA; 2) mouse bioassay; and 3) hemolysis of human peripheral red blood cells. The end products were also tested by Dr. Miyahara with guinea pig atria bioassay (see Dr. Miyahara's report). Table 7 summarizes the results of these preliminary studies. There appears to be some transformation of MTX-like activity to CTX-like activity in crude extracts treated with liver tissue, while heated liver and saline treated end products show little change from the original crude extracts.

C. Immunofluorescent labelling.

As reported in the first-second quarterly report, initial studies on distribution and localization of CTX in tissues were carried out using fluorescein labelled sheep anti-CTX antibody. Frozen sections of guinea pig ileal and colonic tissues were exposed to crude extracts of stick test-positive C. strigosus and S. dumerili samples. These exposed sections and unexposed control sections were then treated with sheep anti-CTX-FITC conjugated antibody, and examined under fluorescent microscopy. Preliminary observations indicate that there is an increase in fluorescent staining of the membrane surfaces in the serous and other exposed membranous areas.

Table 1. Stick test analysis of fishes from Barbers Point study, Sept. 1, 1985 to July 31, 1986.

Source	Species	Tot. #	Stick Test		% Reject
			-	+	
Ewa	C. strigosus	110	67	43	39
	L. kasmira	64	35	29	45
	M. kuntee	6	4	2	33
	A. dussumieri	3	1	2	66
	P. porphyreus	3	2	1	33
	TOTAL	186	109	77	41
Barbers Point study site	C. strigosus	69	26	43	62
	L. kasmira	41	23	18	44
	M. murdjan	10	6	4	40
	A. olivaceus	3	0	3	100
	A. nigrosis	3	0	3	100
	M. kuntee	2	2	0	0
	TOTAL	128	57	71	56
Brown Camp	C. strigosus	79	32	47	60
	A. nigrosis	11	8	3	27
	P. bifasciatus	2	1	1	50
	Chromis verator	1	1	0	0
	P. multifasciatus	1	1	0	0
	L. kasmira	1	0	1	100
	TOTAL	95	43	52	55
Mokumanu	C. strigosus	20	11	9	45
	M. kuntee	3	3	0	0
	A. nigrosis	2	2	0	0
	A. dussumieri	1	0	1	100
	TOTAL	26	16	10	38
GRAND TOTAL		435	225	210	48

Table 2. Stick test analysis of fishes from DOH
Sept. 1, 1985 to July 31, 1986

Date	Species	Source	#III/ # Exposed	Stick Test
(1985)				
9-6	C. strigosus	Kauai	7/7	No sample
	Corresp.			
	C. strigosus - 24			22 +
				2 -
9-18	C. strigosus - 1	Maui	1/3	+
9-20	Caranx sp. - 1	Maui	?	+
11-7	A. virescens - 1	Miloili	2/9	+
11-7	Caranx sp. - 1	Puako	2/2	+
11-20	Mullidae	?	1/1	No sample
	Corresp.			
	Mullidae - 1			+
(1986)				
2-5	Sphyraena sp.	Hookena	2/2	No sample
	Corresp.			
	Sphyraena sp. - 1			+
2-5	Caranx sp.	Kailua, Big Is.	2/5	+
	Corresp.			
	L. fulvus - 1			+
2-5	Caranx sp. - 1	Napoopoo	7/7	+
2-5	C. argus - 3	KTA Mkt	7/10	3+
2-6	S. dumerili - 1	Midway	4/4	+
2-14	Myripristis sp. - 1	Bob's Mkt	1/4	+

3-13	A. furcatus - 8	Kahoolawe	3/4	8+
	Corresp.			
	L. kasmira - 9			8+
				1-
	Caranx sp. and	Ule Point	?	5+
	A. virescens			5-
	(mixed fillets - 10)			
3-18	Mullidae - 1	BW Mkt	2/2	+
3-18	Caranx sp. - 1	Kona	20/28	+
5-5	A. virescens - 1	Oahu	3/4	+
5-22	Caranx sp. - 1	Kawaihae	2/2	+
5-22	Caranx sp. - 1	Kawaihae	2/2	+
6-5	L. kasmira	Kapoho	1/2	No sample
	K. sandvicensis			
	Corresp.			
	L. kasmira - 2			2-
	K. sandvicensis - 2			2-
	Myripristis sp. - 2			2-

Total number of cases investigated, Sept. 1, 1985 to July 31, 1986

	Tot.#	#(-)	#(+)	% Reject
Consumed samples	15	0	15	100
Corresp. only	4	1	3	75

Table 3. Stick test analysis of fishes submitted by private individuals

Species	Tot. #	#(-)	#(+)	% Reject
S. dumerili (amberjack)	21	5	16	76
Caranx sp. (jack)	33	15	18	54
E. bipinnulatis (rainbow runner)	97	68	29	30
L. bohar (snapper)	10	3	7	70
TOTAL	161	91	70	43

Table 4. Comparison of hybridoma 5C8 and Sheep anti-CTX antibodies

Species	Tot.	Sheep		5C8	
		-	+	-	+
C. strigosus (surgeonfish)	120	72	48	47	73
E. bipinnulatis (rainbow runner)	95	88	7	68	26
L. kasmira (snapper)	41	26	15	14	27
M. cephalus (mullet)	28	28	0	18	10
S. dumerili (amberjack)	21	15	6	7	14
Caranx spp. (jack)	12	5	7	5	7
L. bohar (snapper)	7	1	6	0	7

Table 5. Comparison of oil base opaquing fluid and solvent base correction fluid

Coating Agent	Mean Stick Value ± S.D.
Solvent base	4.2 ± 1.0
Oil base	4.4 ± 0.8

Table 6. Examination of toxic and non-toxic fishes with oil base opaquing fluid coated sticks
(Toxicity ranges: toxic ≥ 1.3; non-toxic ≤ 1.2)

Species	Tot.*	Mean	S.D.
TOXIC			
Sphyraena sp.	1	1.6	0.4
Caranx sp.	5	2.4	0.9
S. dumerili	1	2.8	0.9
C. argus	1	1.2	0.4
L. kasmira	2	1.8	0.1
Mulloidichthys sp.	1	2.3	0.6
TOTAL	11	2.0	0.6
NON-TOXIC			
E. bipinnulatis	6	0.5	0.0
Caranx spp.	12	0.7	0.3
L. kasmira	5	0.9	0.3
Mulloidichthys spp.	2	0.0	0.0
Sphyraena sp.	1	0.5	0.0
S. dumerili	9	1.0	0.2
TOTAL	35	0.6	0.2

Table 7. Biosynthetic degradation of G. toxicus crude extracts with homogenized liver tissue

	Stick Test	Mouse Bioassay	H RBC Hemolysis
chloroform fx	-	+	+
methanol fx	+	+	-

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The Effect of Ciguatoxin-like Substances Extracted from Ctenochaetus strigosus and Seriola dumerili

Miyahara, J.T., Kurihara, J.E. and Shang, E.S.

Ciguatoxin (CTX), the potent lipid soluble toxin (LD50 of approximately 0.5 µg/kg in mice), is thought to be the causative agent of the common food poisoning called ciguatera. Although CTX is presumed the major culprit, evidence would suggest other toxins are also involved in this poisoning. For example, the signs and symptoms of ciguatera can vary widely depending on the fish involved, herbivorous or carnivorous fish. While CTX can be extracted from the wild strains of Gambierdiscus toxicus, other toxins such as maitotoxin and scaritoxin are also present and in culture conditions this dinoflagellate produces mainly maitotoxin with little or no CTX (Yasumoto et al., 1979). Therefore this investigation was undertaken to compare the pharmacology of different toxic fish extracts on a variety of isolated tissues. This report is on the effects of extracts from a herbivore Ctenochaetus strigosus (kole) and a carnivore Seriola dumerili (kahala) on the isolated guinea-pig atria and vas deferens.

Methods

Guinea pigs of either sex weighing between 250 to 400 gms were used in this study. After the animal was killed by a blow on the head, the heart was quickly removed and the right and left atria were dissected, separated and mounted in 25 ml tissue chambers with Krebs-bicarbonate solution, constantly bubbled with 95% O_2-5% CO_2 and kept at 30°C, pH 7.4. Atrial contraction and tension change were recorded isometrically through a force displacement transducer and displayed on a Grass (Model 7) polygraph. The left atrium was electrically stimulated by a Grass stimulator through the platinum electrodes in the plexiglas tissue holder. The right atrium was prepared and suspended to record the rate as well as the force of spontaneous contractions.

Crude gut (stomach and intestine) extracts of kole and kahala, implicated in outbreaks of ciguatera and found to be highly toxic by both the mouse and stick (Hokama, 1985) tests, were used in this study. The extraction scheme followed that of Scheuer et al. (1967) with minor modifications.

Minced tissue (Flesh or Gut)

↓ Acetone at room temp
2 x filtration

Acetone filtrate

↓ Evaporate 37°C

Aqueous mixture

↓ Extract c̄ $CHCl_3$
Backwash c̄ 20% brine
Discard brine
Conc. $CHCl_3$

$CHCl_3$ soluble fraction

↓ Extract c̄ hexane
Backwash with aqueous MeOH (80%)
Discard hexane
Conc. MeOH

Crude extract

The crude extract was dissolved in methanol and diluted to make a stock solution of 100 mg/ml extract in 10% methanol. The extract concentrations used in the experiments were calculated on µg/ml final bath concentration.

Results

Effects of extracts on atria

The two extracts caused a concentration-dependent positive inotropic effect on the left atria and both inotropic and chronotropic effects on the spontaneously-contracting right atria. In Fig. 1 are shown the time course of the responses elicited by kole and kahala extracts. With the kahala extracts the response was fast rising, often two-phased with a peak (contraction, 300 to 400% over control) at about 3 min after administration, followed by a trough and a lesser peak at 10 to 12 min. Thereafter the contractions remained at this elevated state for the duration of the experiment (2 to 3 hr). The effect of kole extract was similar, a long-lasting increase (contraction, 300 to 400% over control) in atrial contractions; however, the configuration of the response was quite different. In contrast to kahala extract the kole effect was slower to rise and with a single peak at 6 to 12 or more min.

Effects of tetrodotoxin (TTX)

Pretreatment of atrial strips with TTX up to 10^{-6}M concentration had little effect on the positive inotropy and on the configuration of the responses to kole and kahala extracts. TTX lowered the control response, but was still capable of producing a dose-dependent increase in force of contraction (Fig. 2). On the other hand post treatment of TTX was very effective. With increasing concentration (from 10^{-7} to 10^{-6}M) TTX caused a progressive decrease in atrial contractions of both kole and kahala extracts until the positive inotropic response was completely reversed (Fig. 3). Thereafter rinsing the tissue to remove TTX led to a rebound increase in force of contraction.

Effect of reserpine

In these experiments the guinea-pigs were pretreated with reserpine (i.p., 2 mg/kg) for two days to deplete their neuronal catecholamine stores. After such treatment the intensity of inotropic effect (contraction, 200% over control) evoked by the kole and kahala extracts was decreased; however, the pattern of response remained almost unaltered, a rapidly rising double peak for kahala and slow developing single peak for kole (Fig. 4). On the right atria these extracts still caused a slight increase in the force and rate (130% of control) of contractions, suggesting the reserpine pretreatment only decreased but did not completely deplete the mediator of the cardiotonic effects.

Effects of adrenoceptor blockade

Adrenoceptor blockade was accomplished by a combination of propranolol (10^{-5}M) and phentolamine (5×10^{-6}M). Pretreatment with these adrenoceptor blockers decreased the atrial contractions and the positive inotropy but had little effect on the pattern of response to the fish extracts. The kahala extract response after adrenoceptor blockade still had a rapid onset and time to peak while that of kole remained on a slower time course. As with TTX, the adrenoceptor blockers were more effective in post treatment after the toxin extract response was well-manifested than in pretreatment.

Effects of extracts on vas deferens

The isolated guinea-pig vas deferens was relatively unreactive to both kole and kahala extracts. The vas deferens responded maximally to norepinephrine and to KCl but even with high concentrations of extracts, the contraction was barely detectable. Although unresponsive to the extracts, after a single exposure the vas deferens became supersensitive to norepinephrine and generated TTX-resistant, spontaneous contractions.

Discussion

Fat-soluble toxic extracts were extracted from a carnivore (kahala) and a herbivore (kole) which had caused cases of ciguatera in Hawaii. On the isolated atria these extracts evoked a long-lasting positive inotropy (on left atria) and positive inotropy and chronotropy (on right atria) similar to the "classical" CTX isolated from the moray eel (Miyahara et al., 1985). Unlike the moray eel CTX, however, the responses to the kole and kahala extracts, although reversed by post treatment, were not readily blocked by pretreatment of TTX, reserpine or adrenoceptor blockers. Moreover while the configuration of kahala extract response (rapid rise and double peaks) resembled that of classical CTX, that of kole (slow onset and slow to peak) departed from the usual pattern.

It has been suggested that CTX has a dual effect, an indirect and direct effect, on the isolated atria (Lewis). The indirect effect is the depolarization of presynaptic terminals which leads to the release of norepinephrine and accounts for the rapid initial portion of the toxin response. The direct effect is the activation of the musculature which gives rise to the sustained increase in atrial contraction. According to this notion the indirect and direct effects of the toxin response should be readily separated. Low concentrations of TTX (10^{-7}M), reserpine pretreatment and adrenoceptor blockade would affect only the transmitter-mediated phase and leave the direct portion of the response unaltered. In this study, however, the toxin responses were not separable into two phases. After TTX, reserpine or adrenoceptor blockade, kole and kahala toxins maintained their original pattern of response.

Moray eel CTX causes the release of norepinephrine contraction of smooth muscle and supersensitivity of the vas deferens. In our experiments even with massive concentrations, neither the kole nor kahala extract had any effect on the contractility. These vas deferens strips contracted only to norepinephrine and KCl and showed supersensitivity to norepinephrine after exposure to the extracts but were not responsive to the toxins. All of these data in this study would suggest there are other toxins related to CTX that may contribute to the ciguatera syndrome. Therefore effort should be made to isolated and identify the toxins in these extracts.

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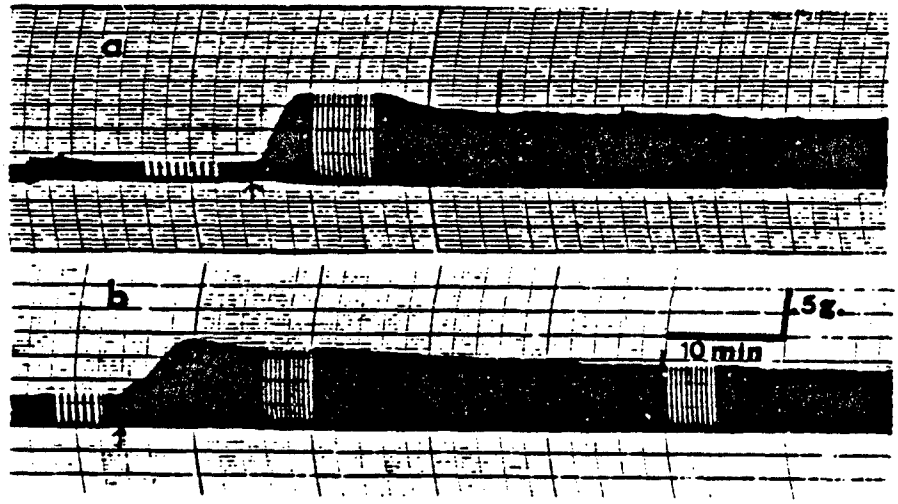

Fig. 1. Inotropic and chronotropic effects of isolated right atria to (a) kahala and (b) kole extracts.

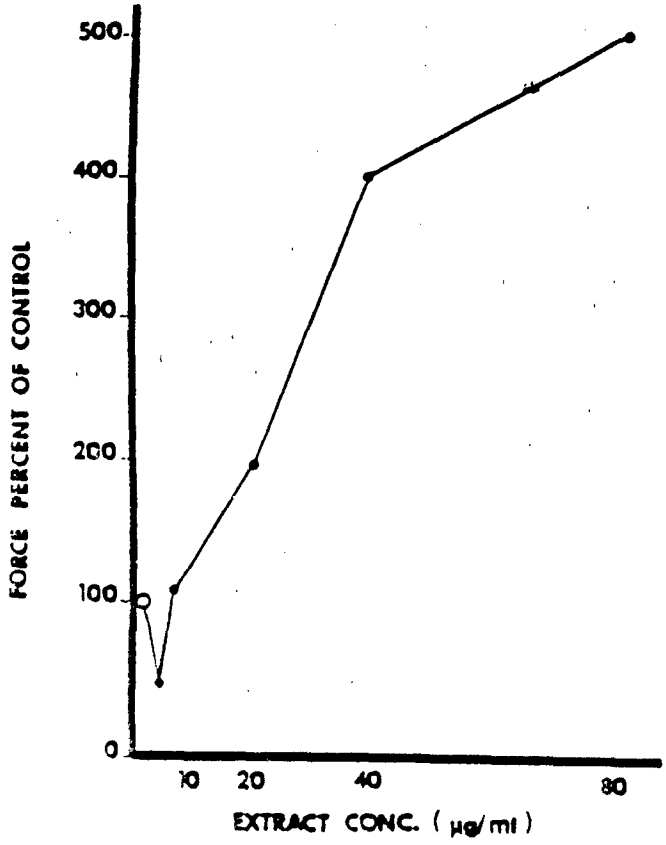

Fig. 2. Effect of TTX pretreatment on kol extract atrial contractions. TTX 5 x 10^{-7} M administered 20 min prior to initial application of extr

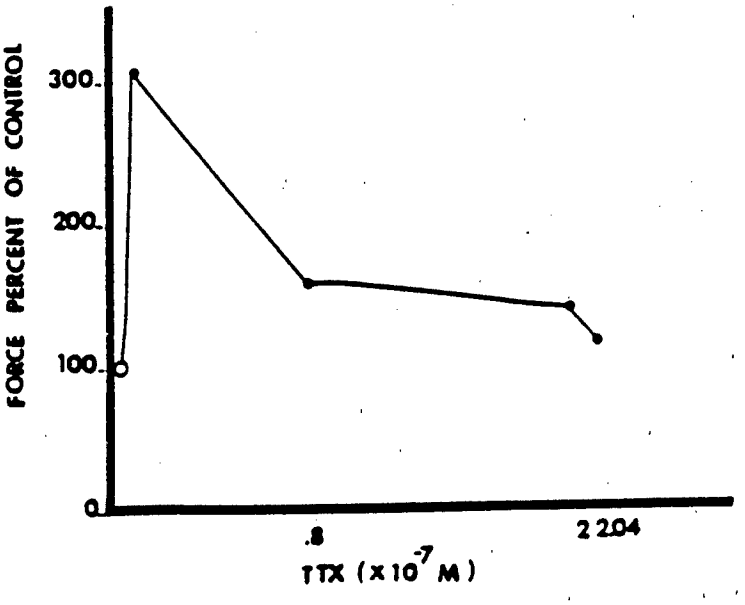

Fig. 3. Effect of TTX post treatment on kole extract (40 µg/ml) atrial contractions. TTX (x 10^{-7}M), cummulative bath concentration.

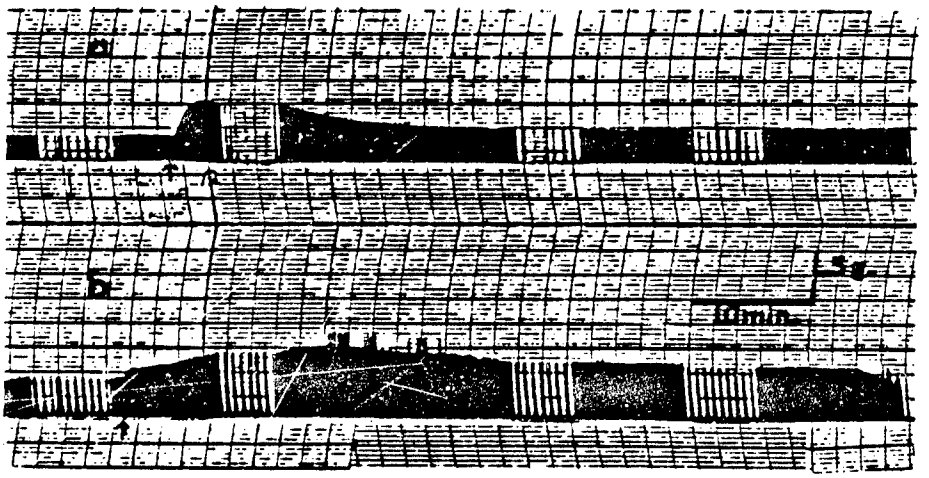

Fig. 4. Effects of reserpine pretreatment on the contractile response of atria to (a) kahala and (b) kole extracts. Guinea-pig was treated with reserpine (2.0 mg/kg/day) for two days and used on third day. Isometric contractions elicited by electrical stimulation (1.5 x threshold intensity, 4 msec duration and 1.5 Hz).

The Discovery of an Anti-ciguatoxin Effect From Black Tree Fungus (Auricularia polytricha) Factors

Shirai, L.K. and Miyahara, J.T.

The black tree fungus (BTF) Auricularia polytricha is an edible fungus of the class Basidiomycetes. It has a wide distribution from tropical to subtropical regions of the world and grows on a wide range of substrates, most of which are wood or wood-like substrata. BTF has been shown to have hypocholesteremic, antiplatelet aggregatory, cardiotonic and antiblastogenic activities. This study shows BTF also contains an anti-ciguatoxin effect.

Materials and Methods

To monitor the CTX antagonistic effects of BTF on the guinea pig ascending colon a preparation utilizing a modified method of Blattner et al. for the guinea pig ileum was employed. Colon segments were mounted vertically in 20 ml tissue baths and connected to a force displacement transducer leading to a Grass Model 7B polygraph for recording colonic activity. The Tyrode bathing solution was maintained at 30°C and bubbled with 95% O_2 - 5% CO_2 gas.

Left and right atrial strips from male guinea pigs weighing between 300 and 600 gm were used for cardiac function studies. The left atrium was cut into two equal strips then mounted vertically in 20 ml tissue chambers and connected to force displacement transducers. Electrical stimulation was provided through electrodes which delivered electrical pulses from a Grass stimulator. The right atrium was prepared and mounted in a chamber for the recording of spontaneous contractions. Changes in contractility and rate of contraction were recorded isometrically and displayed on a Grass Model 7B polygraph. Krebs bathing solution was maintained at 37°C and aerated with 95% O_2-5% CO_2.

Black tree fungus extracts were prepared as follows. Dried BTF was weighed, rehydrated in deionized water then homogenized to a viscous slurry. This suspension was centrifuged at 2000 rpm for 20 minutes, the supernatant decanted and placed in dialysis membranes (Spectra/Por 4) and dialyzed in deionized water for 48 hours at 4°C. The BTF dialysate obtained was then shell frozen and lyophilized until completely dried.

Chromatographic separation of the BTF dialysate was carried out on DEAE-Sephadex G-25 gel (Pharmacia Fine Chemicals, New Jersey) using a 150 ml bed volume. A weight of approximately 200 mg of dialyzed extract, resolubilized in 1.0 ml of deionized water, was used for each column run. Deionized water, pH 7.4, was used as the eluent. All column runs of the BTF dialysate extract were done at 4°C using a Pharmacia P-1 peristaltic pump. Fractions of 1.5 ml volume were collected and peaks were pooled after spectrophotometric examination at 280 and 260 nm using a DU-50 spectrophotometer.

Results

An active CTX antagonistic fraction was separated on DEAE-Sephadex A-25 anion exchange gel. A typical separation on this medium is shown in Fig. 1. The active material eluted from the column consistently from tubes 24 to 33. This corresponds to the second eluted peak, which will be referred to a Fraction II from hence on.

The crude BTF extract was applied to the atrial preparations during the "rise" of the CTX response and "after equilibration" to the CTX response. Data are represented as the percent increase in amplitude of the respective agent used (µg/ml).

Data obtained from the experiments on the distal colon were used to observe the antagonistic effects of BTF Fraction II on the CTX-induced increase in tone based on an acetylcholine control versus the negative log concentration of the BTF or CTX extract added to the physiologic bath.

Addition of the crude BTF extract "after equilibration" to the CTX response decreased the CTX induced increase in contractility of both the right and left atria (Fig. 2 and 3). Depression of inotropy ranged from 69% to 100% depending on the preparation used.

Administration of BTF extract during the CTX response "rise" did not cause an immediate rapid decrease as in the previous experiments. The initial dose of BTF had no effect on the CTX induced rise in contractility, but subsequent doses caused a gradual and dose dependent decrease in contractility of both the right and left atria (Fig. 4 and 5).

Addition of CTX (300 µg/ml) to isolated colon preparations caused a rapid and long lasting increase in tone, and in some cases, spontaneous activity of the colon strip. Administration of BTF Fraction II caused an immediate and persistent decrease in the tone of the colon preparation (Fig. 6 and 7). In those preparations in which CTX elicited increased myogenic activity, BTF similarly diminished the rise in spontaneous contractures.

The mechanisms of this antagonism of BTF on CTX elicited responses on the guinea pig atria and colon is not known and requires further study. Results obtained encourage further study in in vitro and in vivo preparations in experimental ciguatera. Areas of future investigation would include: demonstrating an active fraction of BTF which reverses CTX induced effects on the atria, further purification of the active fractions, determination of their mechanism of action, further trials on different in vitro and in vivo preparations, and possible identification of the active compounds.

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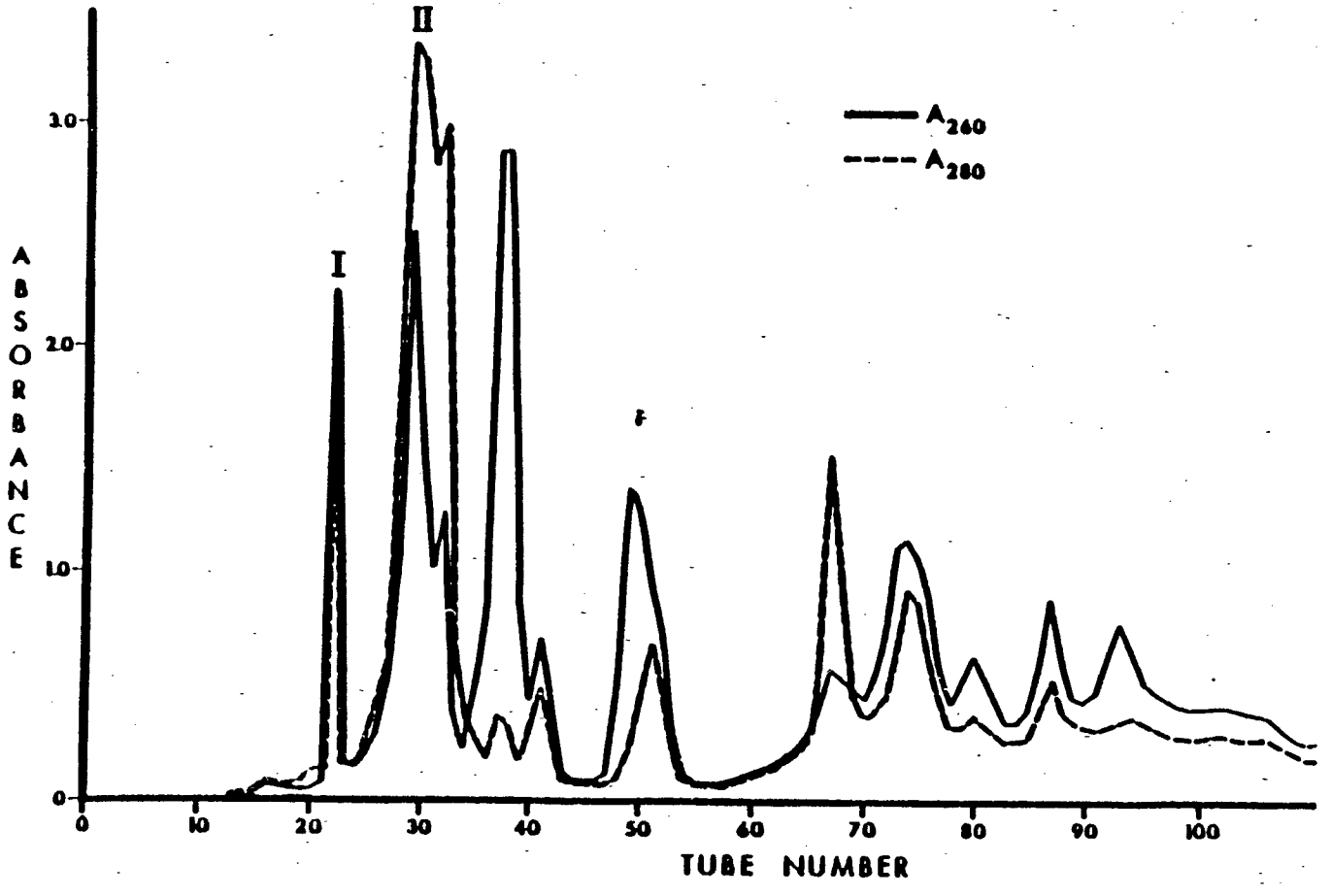

Figure 1. Separation of BTF dialysate on DEAE-Sephadex A-25.

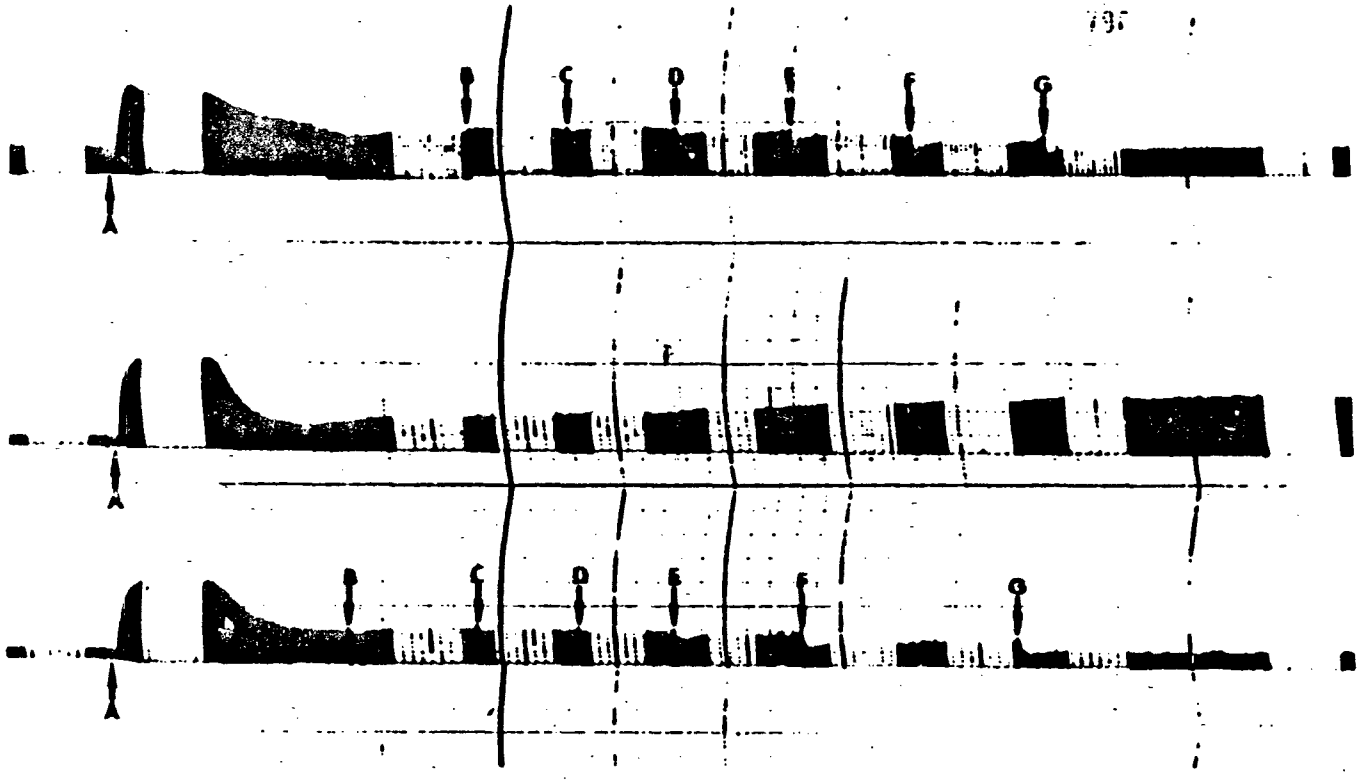

Figure 2. Physiograph recordings demonstrating the action of crude BTF extract following equilibration to the maximum CTX response on the right (bottom) and left (top and center) atria. A, CTX (200 ug/ml); B, BTF (99.5 ug/ml); C, BTF (196 ug/ml); D, BTF (385 ug/ml); E, BTF (741 ug/ml); F, BTF (1379 ug/ml); G, BTF (2424 ug/ml).

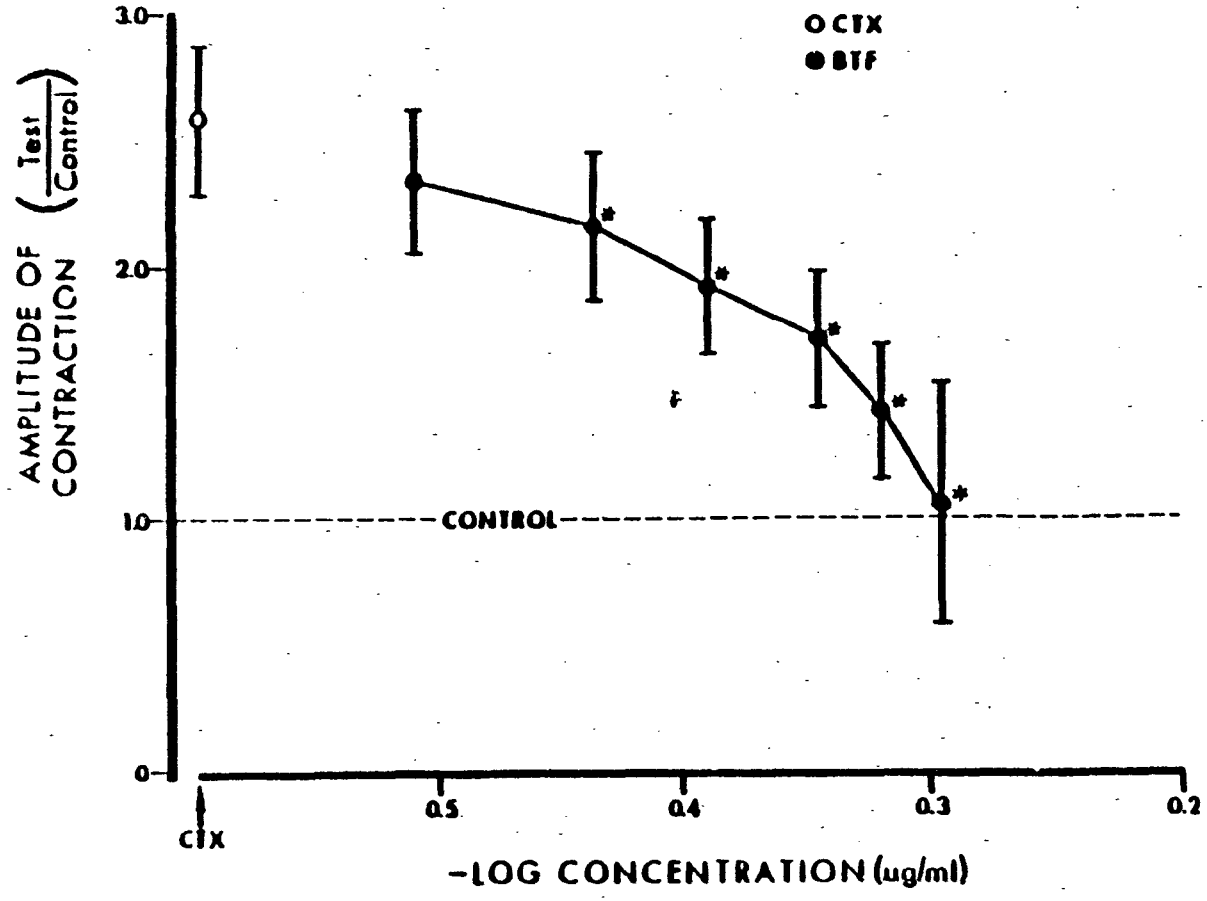

Figure 3. Response of guinea pig atria pre-treated with 200 ug/ml CTX to increasing concentrations of BTF extract (ug/ml) following equilibration to the CTX response. N = 5 for all points. *Statistically significant from pre-BTF levels.

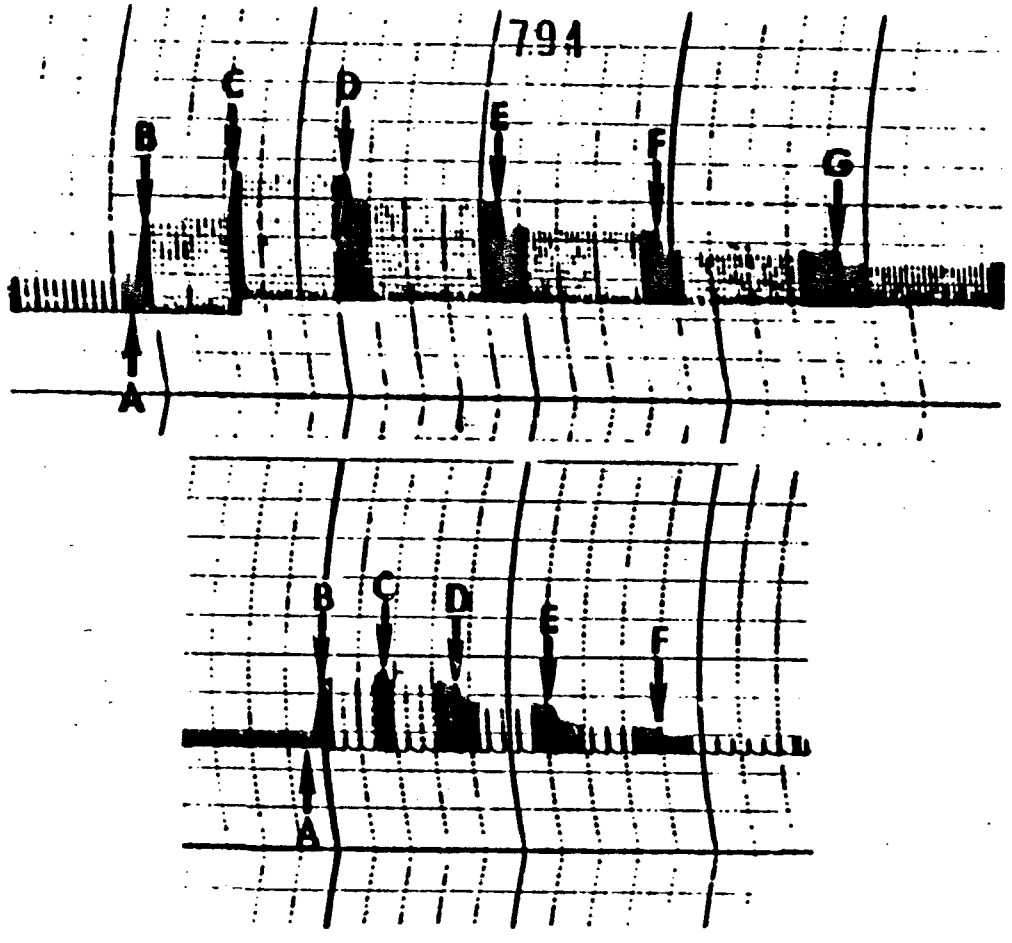

Figure 4. Physiograph recordings demonstrating the action of crude BTF extract applied during the rise of the CTX response on the right (top) and left (bottom). A, CTX (200 ug/ml); B, BTF (99.5 ug/ml); C, BTF (196 ug/ml); D, BTF (385 ug/ml); E, BTF (741 ug/ml); F, BTF (1379 ug/ml); G, BTF (2424 ug/ml).

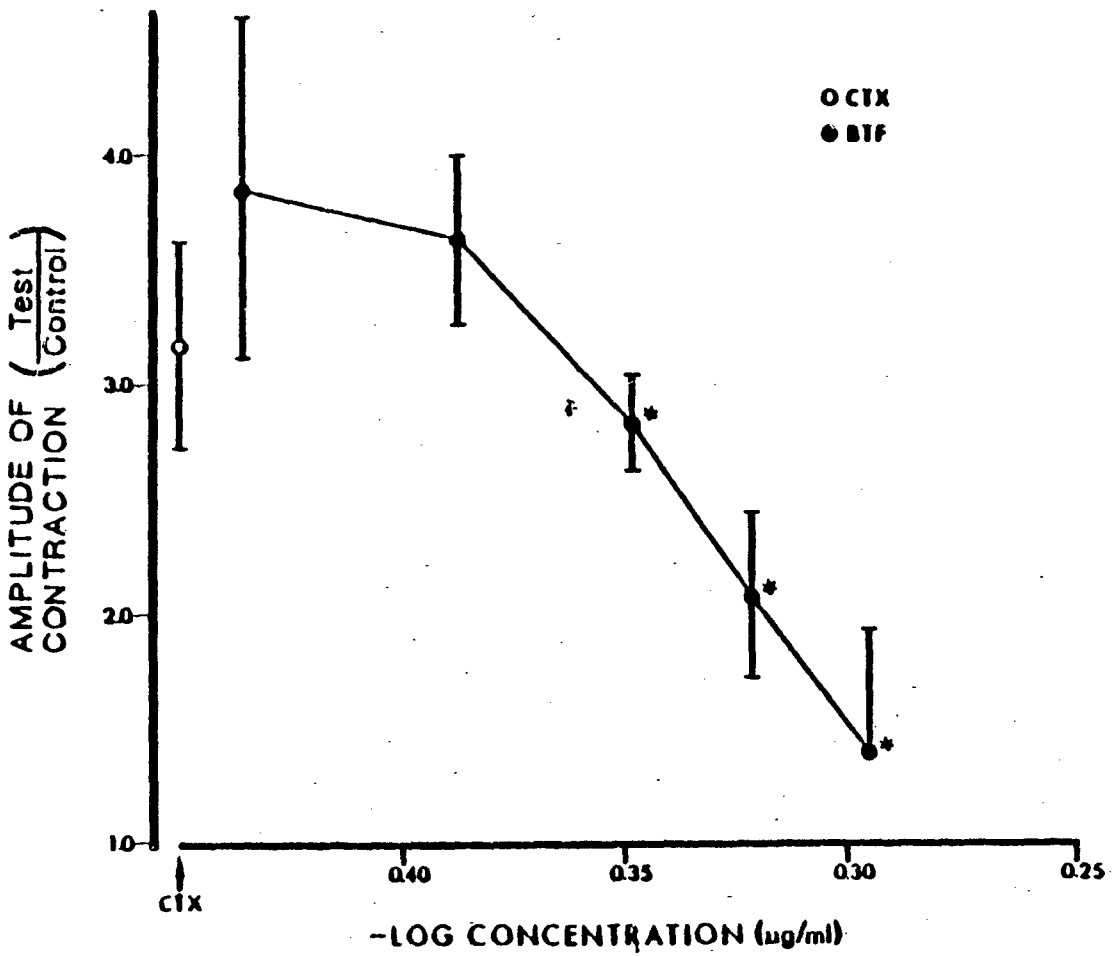

Figure 5. Response of atria pre-treated with 200 ug/ml CTX to increasing concentrations of crude BTF extract on the rise of the CTX response. N = 5 for all points. *Statistically significant from pre-BTF levels.

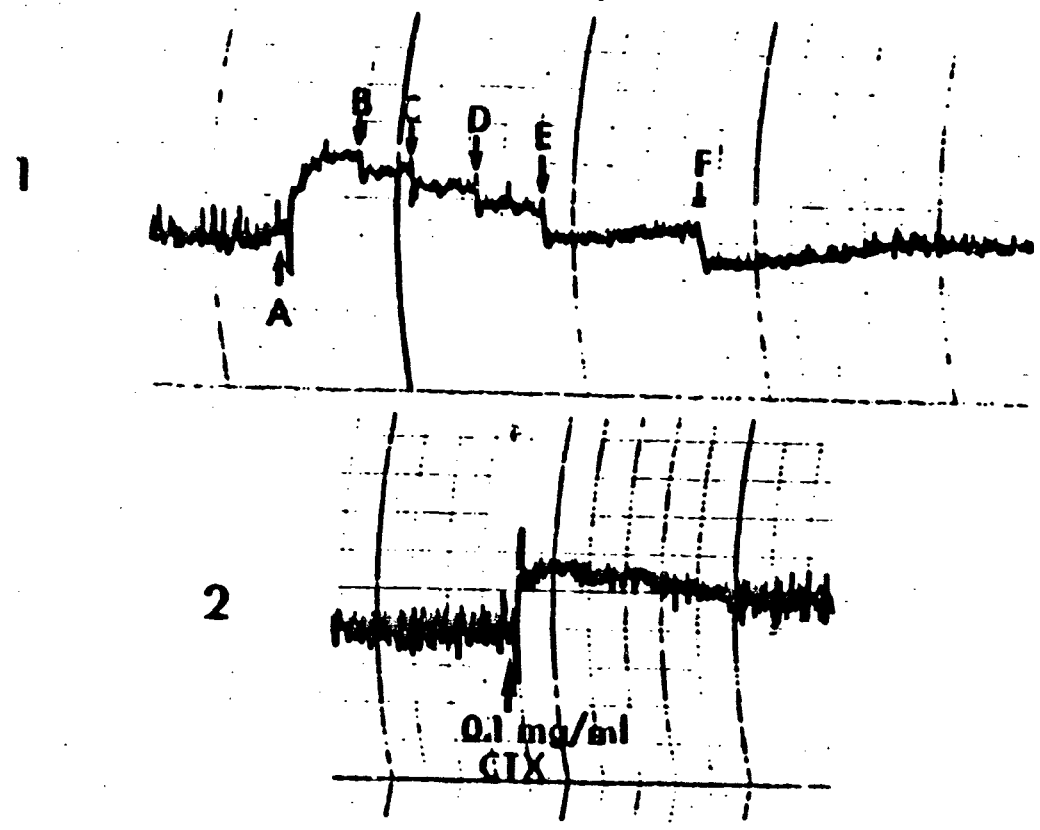

Figure 6. (1) Physiograph recordings demonstrating the action of BTF Fraction II on the ascending colon pre-treated with CTX. A, CTX (1000 ug/ml); B, BTF (100 ug/ml); C, BTF (200 ug/ml); D, BTF (400 ug/ml); E, BTF (794 ug/ml); F, BTF (1575 ug/ml).

(2) Control polygraph recording of guinea pig ascending colon treated with 1000 ug/ml ciguatoxin.

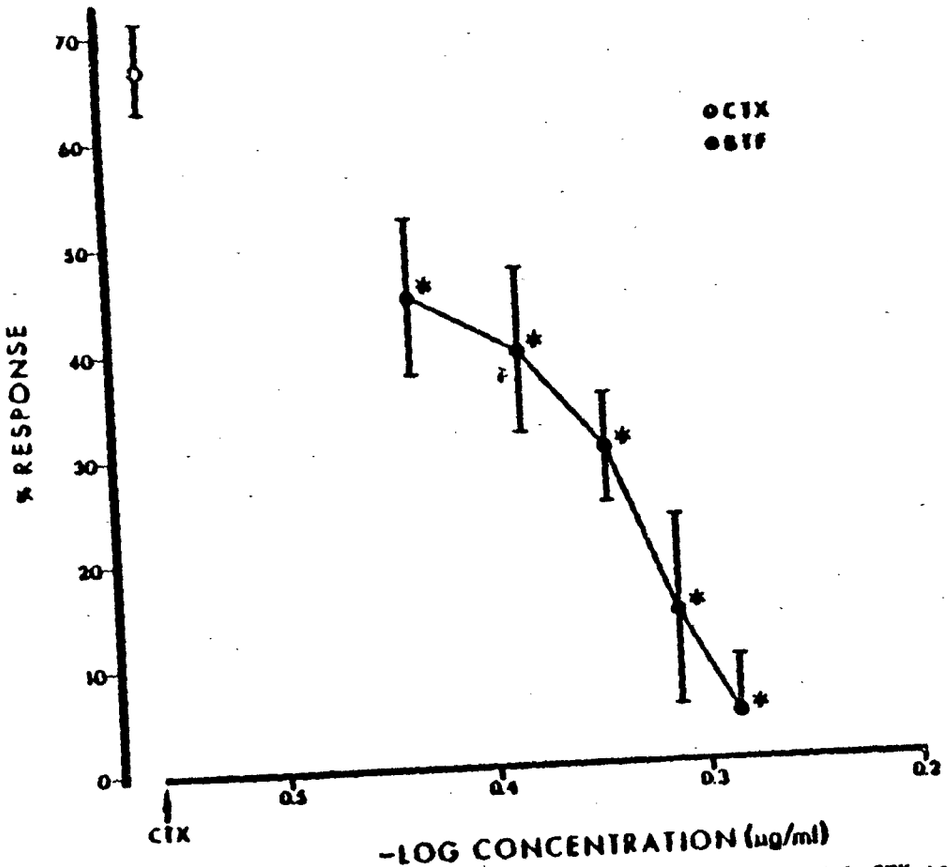

Figure 7. Response of the ascending colon pre-treated with CTX to increasing concentrations of BTF FractionII. N = 4 for all points. *Statistically significant from pre-BTF levels.

Abstract

From November, 1985 through July, 1986 we have received 30.9 kg of toxic eel viscera from Tarawa atoll, Republic of Kiribati. All of this material has been purified up to the final liquid chromatography step. The expected estimated yield is 600 µg of ciguatoxin (CTX).

The most likely molecular formula of CTX is $C_{59}H_{85}NO_{19}$. If this is confirmed by a duplicate experiment, CTX is the only known polyether toxin containing nitrogen.

Technical Report (Scheuer)

Statement of the Problem

Ciguatoxin (CTX) is a major marine toxin of unknown molecular structure. It is one of the most potent low molecular weight toxins (LD_{50} 0.45 µg/kg, ip mice; M Wt 1,111 daltons) and is responsible for human intoxication from ingestion of coral reef fishes in many parts of the world. Since a study of its mechanism of action, the design of a specific diagnostic test, and development of a rational therapy depend entirely on availability of pure toxin and partly on a knowledge of its molecular structure, isolation, purification, and molecular structure determination are the principal goals of this part of the multidisciplinary program.

Background

Outbreaks of ciguatera fish poisoning are unpredictable in time or place and persist for limited periods. Hence procurement of toxic fish for scientific research has been logistically difficult and unrewarding as the concentration of CTX in toxic fish ranges from 1 to 10 ppb (Tachibana, 1980). Yasumoto's discovery (Yasumoto et al., 1977) of a benthic dinoflagellate, Gambierdiscus toxicus, as the originating organism held initial promise that toxin procurement would be solved by laboratory culture. Yet despite vigorous efforts in Japan, France, and the United States G. toxicus cultures have not yielded significant amounts of the toxin (Scheuer and Bagnis, 1985). Consequently, toxic fish catches from known or suspected ciguateric areas have remained the only source of CTX.

Approach

(i) procurement

Because of our familiarity with the Pacific we have concentrated our procurement efforts on Pacific archipelagoes with a current ciguatera problem. Because of the distances involved and the difficulty with communication and transportation, we have attempted to set up a procurement operation by personal contact to be followed up by air shipment of toxic fish viscera. Viscera rather than flesh are known to be the best yielding source of CTX (Yasumoto and Scheuer, 1969).

(ii) purification

In order to minimize losses during our complex multi-step purification procedure we accumulate and combine toxin prior to column and prior to high pressure liquid chromatography and we carry out bioassay monitoring only when absolutely necessary.

(iii) molecular structure

Our molecular structure goals are being approached by two parallel avenues: high-field NMR experiments with pure toxin and crystallization attempts of a CTX p-bromobenzoate.

Results and Discussion

(i) procurement

Personal contacts by project personnel with knowledgeable residents in Kiribati, French Polynesia, and New Caledonia have resulted in a reasonably study supply of toxic eel viscera only from Tarawa atoll (173°E, 1°20'N), Republic of Kiribati. Since November, 1985 we have received 30.9 kg of frozen viscera with an expected estimated yield of 600 µg of CTX.

(ii) purification

All toxin has been purified up to the liquid chromatography step.

(iii) molecular structure

The molecular formula of CTX has been determined by high resolution experiments. It is $C_{59}H_{85}NO_{19}$ although this needs to be confirmed by a duplicate experiment.

Conclusions

The demonstration that a nitrogen atom is present in CTX is a significant achievement. A nitrogen function of as yet unknown nature sets this toxin apart from other known polyether toxins -- okadaic acid, the halichondrins and the brevetoxins.

Recommendation

We recommend that all possible steps be taken to increase our supply of toxic raw materials.

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