Proceedings of the Third International Conference on Ciguatera Fish Poisoning

Puerto Rico 1990

Edited by
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Polyscience Publications, Quebec

Production Credits
Proofreading: Carmen Tosteson, Val Hensley
Design and Layout: Alexis Tosteson García, Angel Aníbal Escobar
Cover Art: Bonnie Dennis Bower
Cover Design: Angel Aníbal Escobar

ISBN 0-921317-35-2

Polyscience Publications, Inc.
Morin Heights, Quebec, Canada
Printed in Canada.

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Preface

Ciguatera fish poisoning is a human health problem that affects all persons living in tropical seas for whom marine fish represent a significant source of food. Ciguatera traditionally was limited to tropical regions, however, modern improvements in refrigeration and transport have augmented commercialization of tropical reef fishes and increased the frequency of this type of fish poisoning in temperate portions of the earth. Since the early reports of this poisoning in the tropical Pacific in the 17th century, ciguatera has come to have an impact of global proportions.

A broad and detailed treatment of the present status of research on ciguatera fish poisoning is given in this volume in 18 papers presented at the Third International Conference on Ciguatera Fish Poisoning held in La Parguera, Puerto Rico, April 30 through May 5, 1990. Laboratories, agencies and universities represented at this Conference stretched from Australia, Japan and the islands of the south Pacific to Hawaii, the United States, Canada and through the islands of the Caribbean to France and the Mediterranean Sea. The Proceedings are divided into four sections covering; (1) the chemistry of fish and dinoflagellate toxins, (2) the pharmacology and toxicology of ciguatoxic fish and dinoflagellate toxins, including the action of these toxins on membrane channels, their pathomorphological effects on the organs and tissues of the mouse, and a correlation of toxin potency with computer generated conformational models of these toxins, (3) ecological and fisheries aspects of ciguatera and the toxic dinoflagellate vectors of this poisoning, and (4) the societal impact of ciguatera in French Polynesia, Canada, the Dominican Republic, Puerto Rico and St. Vincents and Grenadines. These presentations address global issues dealing with epidemiology, public health, socioeconomic and legal aspects of ciguatera fish poisoning, a review of past and current trends, with a description of local and international programs presently being implemented to deal with ciguatera in these areas. We hope that this volume will be of use to research scientists working in this field as well as professionals and students in fisheries and public health interested in the origin and impact of ciguatera fish poisoning.

It would be difficult to name all of the persons who contributed to the success of the Third International Conference on Ciguatera Fish Poisoning and the production of these Proceedings. Thanks are due to all of the contributors to the Proceedings for their help in manuscript review, encouraging comments and patience with this process. Special gratitude goes to Mr. Alexis E. Tosteson, Administrative Director of the Conference, for his efforts and diligence in the preparation of these Proceedings.

La Parguera
Puerto Rico

T.R. Tosteson

Acknowledgements

Various organizations contributed to the Third International Conference on Ciguatera Fish Poisoning and to the preparation of these Proceedings. We would like to acknowledge the following:

Sponsors

U.S. Army Research Office

U. S. Army Chemical Research, Development and Engineering Center

Sea Grant College Program of the University of Puerto Rico

National Sea Grant College Program

Contributors

Fundación Educativa Ana G. Méndez

Caribbean Fishery Management Council

Intergovernmental Oceanographic Commission of Unesco

Experimental Program to Stimulate Competitive Research in Puerto Rico

Faculty of Arts & Sciences, Mayagüez Campus, University of Puerto Rico

Agricultural Development Administration, Department of Agriculture, Commonwealth of Puerto Rico

Center for the Enhancement of Teaching, University of Puerto Rico

Banco Popular de Puerto Rico

Servicios de Seguros de Salud de Puerto Rico

Corporation for the Technological Development of Tropical Resources, Commonwealth of Puerto Rico

Chiral Corporation

Upjohn Pharmaceutical

Regional Co-ordinating Unit of the Caribbean Environment Program of the United Nations Environment Program, Kingston, Jamaica

This publication is a result, in part, of funds sponsored by the U. S. Army Research Office, Grant Num. DAAL03-89-G-0112, and NOAA, National Sea Grant College Program Office (NSGCPO), Department of Commerce, under Grant No. UPR-SG-04-F-158-44030 (Project No. B/C-74-10).

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by sponsoring and contributing agencies and organizations. Recommendations are those of the authors and are not necessarily endorsed by sponsoring and contributing agencies and organizations.

Chemistry of Fish and Dinoflagellate Toxins

Structure Determination of Ciguatoxin of Moray Eels and *Gambierdiscus toxicus*

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Abstract

Ciguatoxin isolated from viscera of the moray eel *Gymnothorax javanicus* and its congener (tentative codename CTX-4B) isolated from the causative dinoflagellate *Gambierdiscus toxicus* were subjected to structure elucidation. The molecular formulae of $C_{60}H_{86}O_{19}$ and $C_{60}H_{84}O_{16}$ were determined for ciguatoxin and CTX-4B respectively, by HR-FABMS and ^1H NMR measurements. ^1H NMR signals of protons around a nine-membered ring were broadened or disappeared due to a slow conformational change of the ring, but spectra quality was improved by measuring at low temperature. By extensive use of ^1H NMR 2D-correlation and NOE experiments carried out on 0.35 mg of ciguatoxin and 0.74 mg of CTX-4B, the two toxins were found to have brevetoxin-like polyether structures comprised of thirteen rings (7/6/6/7/7/9/7/6/8/6/7/6-spiro-5). CTX-4B was in a form less oxidized than ciguatoxin at two terminals of the molecule. Their relative stereochemistries, except for C2 of ciguatoxin, were clarified by detailed analyses of ^1H NMR NOE experiments. The primary alcohol at the terminal of the ciguatoxin molecule suggested its usefulness for preparing a flourescent derivative or a toxin-protein conjugate to be used for immunization.

Introduction

Ciguatoxin (CTX) is a term first proposed by Scheuer for the principal toxin of the red snapper *Lutjanus bohar* and then for the toxin in the liver of the moray eel *Gymnothorax* (=Lychodontis) *javanicus* [1,2]. The purified toxin was obtained as fine crystals and suggested by mass spectral measurements to have a molecular formula of either $C_{53}H_{77}NO_{24}$ (1112.2) or $C_{54}H_{78}O_{24}$ (1111.2). The polyether nature of the molecule was indicated by ^1H NMR measurements. However, the chemical structure of the toxin has remained undetermined due to the extreme difficulty in obtaining the toxin as well as the complex structure of the molecule. The toxin was presumed to be acquired by fish through the food chain. As to the origin of the toxin, Yasumoto discov-

ered in collaboration with the Tahiti group, the epiphytic dinoflagellate *Gambierdiscus toxicus* as the probable primary source. The toxin detected in the dinoflagellate resembled CTX in some chromatographic properties but remained ambiguous with regard to structural relationship with CTX.

In the present study structure elucidation was carried out on CTX obtained from moray eel viscera and one of the toxins, code named CTX-4B (previously GT-4b), isolated from *G. toxicus*. By extensive use of ^{1}H NMR 2D-correlation and NOE measurements, we determined the structures of the two toxins. They shared a brevetoxin-like skeleton comprised of thirteen ether rings. Their relative stereochemistries were determined, except for C2 of CTX, by detailed ^{1}H NMR NOE experiments. The primary alcohol present at one terminal of CTX molecule seemed to be useful for fluorescence labelling and for preparation of CTX-protein conjugate for immunization.

METHODS

Chromatographically pure CTX (350 μg) was obtained from viscera (145 kg) of the moray eel *Gymnothorax javanicus* collected in French Polynesia as described previously[3]. Wild specimens of *G. toxicus* were collected in the Gambier Islands, French Polynesia, during 1977-1979 by scrubbing the surface of dead corals followed by sedimentation and sieving[4,5]. Isolation of toxins from *G. toxicus* was carried out following the method used for CTX with a slight modification. A CTX congener (740 μg) purified from *G. toxicus* was used for structure elucidation[6,7]. This congener corresponds to CTX-4B according to our new code names proposed for CTX congeners[8]. NMR measurements were carried out with a JEOL GSX 400 (400 MHz). Spectra were taken in pyridine-d_5, CD_3CN solutions to achieve better separation of heavily overlapping proton signals. Measurements of proton signals were done at both 25°C and at -25°C to observe signals of protons on and around a nine-membered ring, which due to a conformational change causes broadening or even disappearance of those signals. FAB-mass spectra were taken on a JEOL JMS DX-303HF mass spectrometer.

RESULTS AND DISCUSSION

Toxicity and physicochemical properties

The lethal potency of CTX in mice (i.p.) was estimated to be 0.35 μg/kg, and that of CTX-4B 4 μg/kg [3]. CTX was obtained as an amorphous white solid; negative reaction to ninhydrin or Dragendorff's tests; no UV maximum above 210 nm; IR (film) 3400, 1111 and 1042 cm^{-1} (no bands between 1600-1800); high resolution FAB-MS m/z 1111.584 MH$^+$ (calculated for $C_{60}H_{87}O_{19}$, 1111.584). CTX-4B was suggested to have a trans-butadiene [UV_{max} 223 nm

(CH_3CN, ε 22000)]; high resolution FAB-MS m/z 1061.587 MH^+ (calculated for $C_{60}H_{85}O_{16}$, 1061.584).

Molecular formula

No evidence was obtained by the color reagents and IR spectra measurements to support the presence of nitrogen atoms in CTX. The molecular formula of CTX suggested by high resolution FAB-MS (HRFABMS) was supported in a subsequent NMR study. Partial structures deduced from ^1H and ^{13}C NMR spectra contained at least 59 carbons and thus left only 28 daltons unaccounted for. Hence the presence of nitrogen or sulfur atoms in CTX was ruled out.

Functional groups

Comparison of ^1H NMR spectra (in pyridine-d_5 and CD_3OD) with those reported by Tachibana confirmed that our CTX was identical with that of the Hawaii group[9]. The presence of five methyl (1 singlet and 4 doublets), five hydroxyls, and four double bonds (1 trans and 4 cis) was shown in the ^1H NMR spectra. To confirm the number of double bonds, 1 μg of CTX was subjected to catalytic hydrogenation. The reduction product gave a cationated ion $(M+Na)^+$ at m/z 1143 in FAB-mass spectra, suggesting the presence of five double bonds in the CTX molecule.

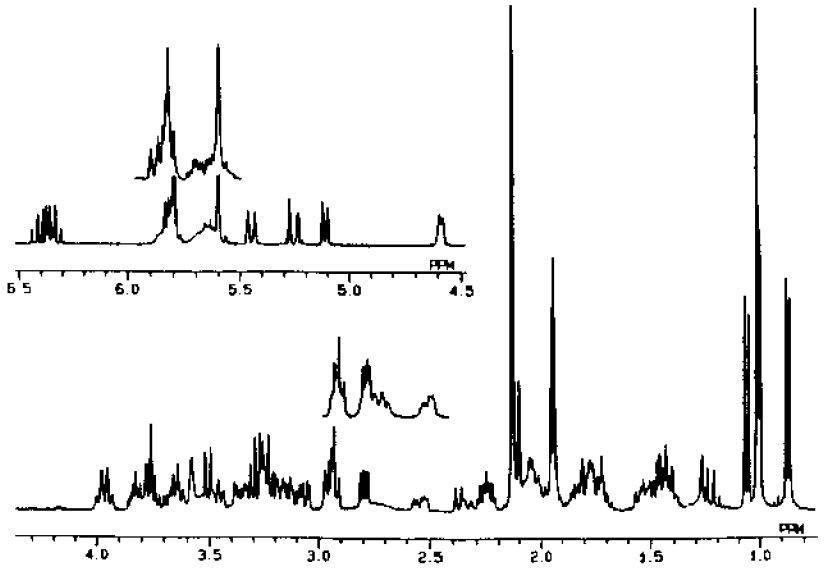

Fig. 1. NMR Spectra of CTX-4B.
^1H NMR spectra of CTX-4B (400 MHz) in CD_3CN at 20°C. Two inset partial spectra were measured at -25°C.

The olefinic unobserved by measurements at 25°C, appeared in spectra measured at -25°C (Figure 1). As there was no sign of carbonyl in the IR spectrum, we inferred that unsaturation in the CTX molecule was ascribable mainly to C=C bonds and ether rings. Hence CTX probably has thirteen ether rings fused in a laddershape, mimicking brevetoxin-B and yessotoxin[10,6].

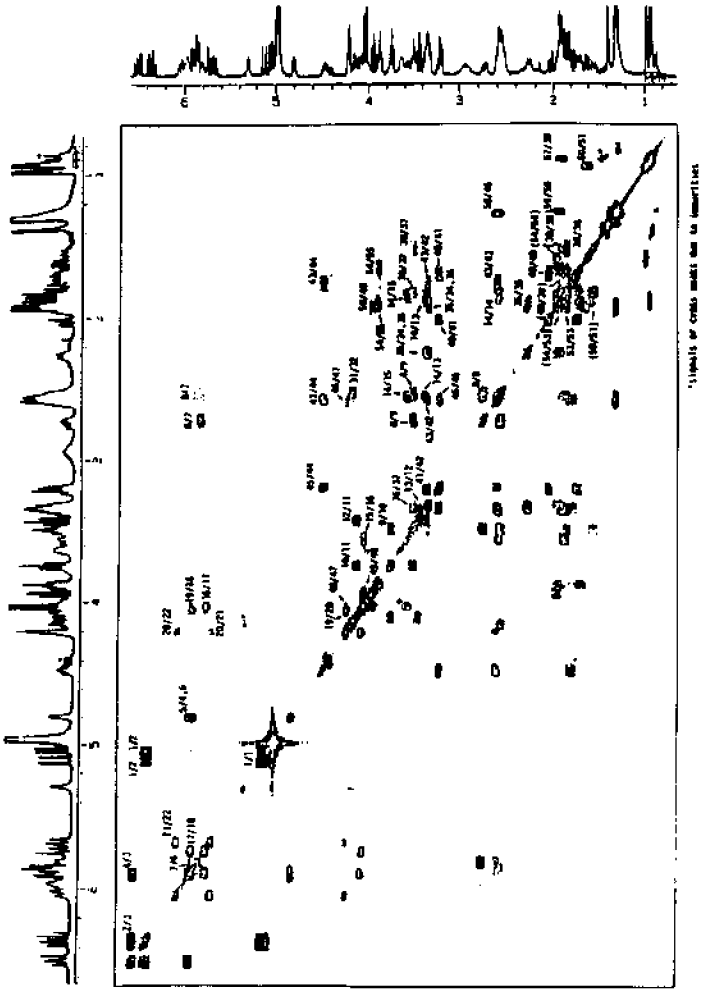

Fig. 2. NMR-COSY Map of CTX-4B.
^1H-^1H COSY map of CTX-4B (400MHz) in pyridine-d_5 at 25°C and the assignment of cross peaks. Figures denote the carbon numbers of coupled protons giving a cross peak. Asterisks express signals and cross peaks due to impurities. Figures in parentheses were assigned with additional use of 2D-HOHAHA and relayed COSY.

Proton connectivity

^{1}H NMR chemical shifts and coupling constants were almost identical between CTX and CTX-4B except for the two terminal parts of the molecules. Therefore, the structure elucidation of the common part will be discussed using the data obtained with the latter. The skeletal structure was established mainly on the basis of ^{1}H-^{1}H 2D NMR data obtained from COSY (Figure 2), relayed COSY, and 2D HOHAHA experiments. Interpretation of ^{1}1H-^{1}1H COSY measured in pyridine-d_5 is shown in Figure 3A. First, four fragments, C1-C32, C34-C38, C40-51 and C53-55 were assigned. The positions of OH groups were clarified by the COSY measured in pyridine-d_5. 2D-HOHAHA experiments revealed multiple relayed couplings of H-37/Me-57

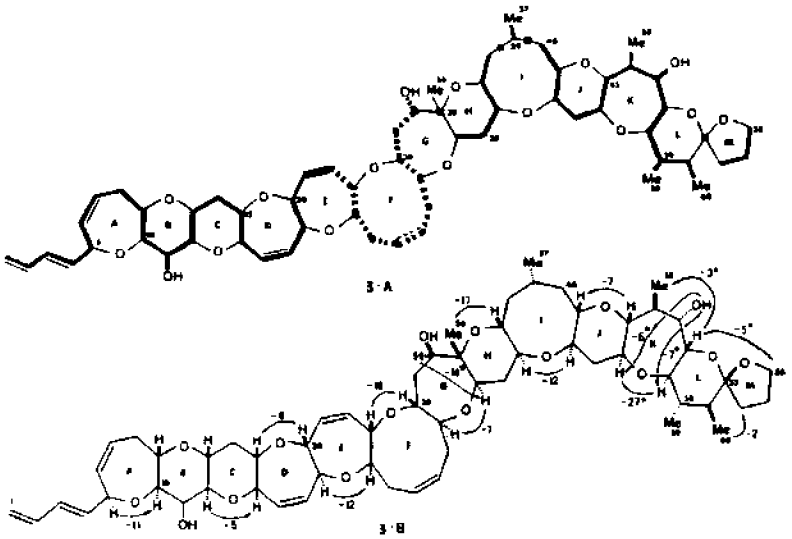

Fig. 3. CTX-4B: Results of 2D Correlation and NOE Experiments.
3-A, Heavy lines indicate the connectivities assigned on the basis of ^{1}H-^{1}H COSY, relayed COSY and 2D-HOHAHA at room temperature, while broken lines denote those as-25°C. 3-B, Arrows and figures denote irradiated protons (tail)/ observed protons (head) in NOE difference experiments and NOE's in percentage measured in CD_3CN at -25°C. The figures with an asterisk are those measured in pyridine-d_5 at -25°C.

and H-41/Me 57, allowing us to connect H-37 through H-41. Moreover, NOE (Figure 3B) observed between H-36 and H-42 suggested that C39 probably resided on an eight-membered ether ring. The eight-member ring I neighbored by two six membered rings (H and J) was inferred to take a stable crown conformation as in brevetoxins[11]. MM_2 calculations also supported this

conclusion. ^{1}H NMR spectral simulations done for H2-38/H-39(Me-57)/H-40, and H-39/H2-40/H-41 using parameters obtained from the MM2, calculations and COSY experiments enabled us to establish the structure of the ring I, including the equatorial orientation of Me-57. A long range coupling of Me-56/H-32, and a large NOE on Me-56/H37 allowed us to connect H-32 through H-37. Coupling constants of H-34 through H-37 were also typical for a 2,2,3,5,6-pentasubstituted tetrahydropyran. The presence of a signal at δ 109.2, typical for a spiro carbon of 5/6 membered rings, in a ^{13}C NMR spectrum and an NOE on Me-60/H-53 revealed the spiro-fused ring M.

Ether linkage and stereochemistry

Positions of ether rings were clarified by analyses of coupling constants between angular oxymethine protons (ca. 9 Hz) and by NOE experiments on angular protons or a methyl (Me-56). Geometries of the double bonds were assigned by their vicinal coupling constants. The coupling pattern of H-11 (triplet, 9Hz) indicated the 11-OH to be equatorial, and a coupling constant of H-51 (doublet, 11Hz) revealed diequatorial orientation of Me-59 and Me-60. A larger NOE was observed on Me-60 than on H-54 when Hß-53 was irradiated and this order in NOE intensities was reversed when Hα-54 was irradiated. The coupling pattern of Hß-53 (dd, 10, 2 Hz) and H-53 (dd, 10, 4 Hz) corresponded to trans-vicinal and cis-vicinal couplings, respectively, on tetrahydrofuran. Thus, the hydroxyl group at C-54 was shown to be a ß-substituent. A probable W-type coupling observed between H-32 and Me-56 suggested an equatorial orientation of OH-32. This was further supported by a large NOE on H-32/H-34. A prominent NOE on H-48 observed upon irradiation of Me-58 suggested a ß-orientation of Me-58. The α orientation of

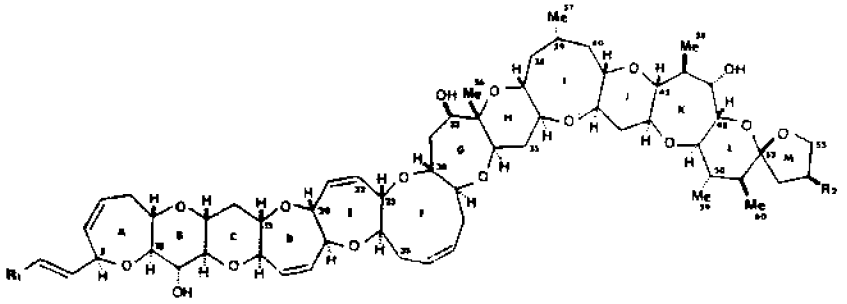

Fig. 4. Structures of CTX and CTX-4B.

Table 1. ^{1}H NMR Chemical Shifts[a] and Coupling Constants[a] of Ciguatoxin[1] and GT4b2

positn	2	pattern[b]	1	pattern	positn	2	pattern	1	pattern
1	5.04	(10, 2)	3.98	(-[c])	35	1.91	(q, 12)	1.92	(q, 12)
	5.11	(16, 2)	3.97	(-)		2.26	(12, 4, 4)	2.25	(12, 4, 4)
2	6.35	(16, 10, 10)	4.69	(m)	36	3.35	(-)	3.35	(-)
3	6.49	(15, 11)	6.37	(15, 5)	37	3.50	(10, 9, 4)	3.50	(-)
4	5.90	(15, 5)	6.38	(15, 5)	38	1.54	(13, 11, 8)[f]	1.53	(-)
5	4.80	(m)	4.86	(m)		1.84	(13, 10, 4)[f]	1.83	(-)
6	5.86	(-)	5.90	(11, 3, 2)	39	1.91	(q,t, 7, 8)[f]	1.90	(-)
7	5.81	(-)	5.77	(11, 8, 2, 1)	40	1.72	(13, 10, 8)[f]	1.72	(-)
8	2.55	(m)	2.54	(-)		2.03	(13, 10, 3)[f]	2.03	(-)
	2.73	(15, 7, 4)	2.72	(15, 7, 4)	41	3.20	(10, 10, 3)	3.21	(10, 10, 3)
9	3.50	(m)	3.49	(m)	42	3.34	(-)	3.35	(-)
10	3.74	(9, 9)	3.75	(9, 9)	43	1.77	(q, 12)	1.77	(q, 12)
11	4.11	(9, 9, 2)	4.10	(-)		2.59	(-)	2.59	(-)
12	3.43	(9, 9)	3.43	(9, 9)	44	4.47	(11, 9, 5)	4.45	(11, 9, 5)
13	3.36	(12, 9, 4)	3.35	(-)	45	3.19	(9, 5)	3.21	(9, 5)
14	1.85	(q, 12)	1.85	(q, 12)	46	2.59	(-)	2.59	(-)
	2.58	(12, 4, 4)	2.56	(-)	47	4.20	(3, 2)	4.21	(-)
15	3.55	(11, 9, 4)	3.55	(-)	48	4.03	(9, 1)	4.06	(10, 1)
16	4.03	(br.d, 9)	4.03	(br.d, 9)	49	3.94	(10, 10)	3.96	(10, 10)
17	5.74	(13, 2, 2)	5.73	(br.d, 13)	50	1.94	(q,t, 6, 11)[f]	2.01	(-)
18	5.89	(-)	5.89	(br.d, 13)	51	1.60	(q,d, 7, 11)[f]	1.67	(-)
19	4.08	(br.d, 9)	4.07	(br.d, 9)	53	ca.1.85	(-)	2.35	(13, 5)
20	4.22	(br.d, 9)	4.21	(br.d, 9)		ca.1.93	(-)	2.40	(13, 8)
21	5.67	(13, 2, 2)	5.67	(br.d, 13)	54	ca.1.68	(-)	4.86	(m)
22	6.10[d]	(br.d, 13)	6.10[d]	(br.d, 13)		ca.1.90	(-)		
23	4.03[d]	(br.d, ca. 8)[e]	4.03[d]	(-)	55	3.87	(-)	4.18	(10, 2)
24	3.68[d]	(-)	3.68[d]	(-)		3.88	(-)	4.19	(10, 5)
25	2.20[d]	(-)	2.20[d]	(-)	56	1.37	(s)	1.37	(s)
	3.01[d]	(-)	3.00[d]	(m)	57	0.92	(8)	0.92	(8)
26	6.05[d]	(11, 11, 5)[h]	6.05[d]	(-)	58	1.29	(8)	1.30	(8)
27	6.07[d]	(11, 11, 5)[h]	6.07[d]	(-)	59	1.28	(6)	1.32	(6)
28	2.36[d]	(-)	2.36[d]	(-)	60	0.97	(7)	1.24	(7)
	2.98[d]	(m)	2.98[d]	(m)	1-OH			6.40	(6, 4)
29	3.86[d]	(br.d, 9)[e]	3.86[d]	(-)	2-OH			6.67	(4)
30	3.68[d]	(9, 8, 7)[e]	3.67[d]	(-)	11-OH	7.34	(2)	7.32	(2)
31	2.65[d]	(13, 8, 6)[f]	2.66[d]	(-)	32-OH	5.29	(1)	5.29	(1)
	2.70[d]	(13, 8, 7)[f]	2.70[d]	(-)	47-OH	6.76	(3)	6.77	(3)
32	4.16	(8, 8, 1)[f]	4.16	(-)	54-OH			6.53	(4)
34	3.34	(12, 4)[g]	3.33	(-)					

[a]Proton NMR spectra were measured with a JEOL SGX 400 (400MHz) spectrometer in pyridine-d_5 at 25°C except for those with superscripts, d, e, g and h. [b]Multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet) and coupling constants in Hz. [c]Couplings were unassignable due to heavy overlappings of signals. [d]Measured in pyridine-d_5 at -20°C. [e]Obtained from NOE difference spectra in pyridine-d_5 at -20°C. [f]Obtained from decoupling difference spectra. [g]Coupling constants in CD_5CN at 25°C. [h]Coupling constants in CD_3CN at -25°C.

OH-47 was supported by NOE's detected both on H-44/OH-47 and H-49/OH-47. The orientation of C53 to ring L was assigned to be equatorial by NOE's on Me-60/H53 and on H48/H-55.

The structural difference between CTX and CTX-4B was readily elucidated by COSY analyses. CTX-4B of *G. toxicus* has a trans-butadiene at one terminus of the molecule and lacks a hydroxyl group at C54 at the other end of CTX. Thus, we successfully determined the structures of CTX and CTX-4B (Figure 4), except for the stereochemistry at C2 of CTX, with no more than 1 mg of material. ^{1}H NMR chemical shifts and coupling constants of CTX and the congener are given in Table 1. Further details of spectral data to support the structure assignments will be presented in a coming paper[7].

The difference in the extent of oxygenation between the two toxins led us to hypothesize an oxidative modification of toxins during the course of the food chain. It is interesting to note that lethal potency of CTX-4B increased about ten fold when it was oxidized to CTX. That may explain, at least partly, why the livers of moray eels are so toxic compared with those of herbivorous fish. Confirmation of the presence of a primary hydroxyl group at one terminus of CTX allowed us to design a fluorometric HPLC method for detection of nanograms of CTX[8] . Efforts are being continued to elucidate structures of other toxins.

Acknowledgements

We are most grateful to Professor J. Roux, Director of the Institute Louis Malarde, and to Dr. R. Bagnis for their continued support of the ciguatera project. Thanks are also due to Mr. J. Bennett for collecting the dinoflagellate and fish, and to Mr. P. Cruchet for his assistance in toxin purification. Discussions and suggestions provided by professor M. Hirama of Tohoku University are greatly acknowledged. This work was supported by the French Ministry of Research and Industry, Pasteur Institute of Paris, French Polynesia Government, and by a Grant-in-Aid for Overseas Research of the Ministry of Education, Science and Culture, Japan.

Literature Cited

1. Scheuer, P.J., W. Takahasi, J. Tsutsumi and T.Yoshida. 1967. Ciguatoxin: Isolation and chemical nature. Science 10:1267-1268.
2. Tachibana, K., M. Nukina, Y-G. Joh and P.J. Scheuer. 1987. Recent developments in the molecular structure of ciguatoxin. Biol. Bull. 172:122-127.
3. Legrand, A.M., M. Letandon, J. N. Genthon, R. Bagnis and T. Yasumoto. 1989. Isolation and some properties of ciguatoxin. J. Appl. Phycol. 1:183-188.

4. Yasumoto, T., I. Nakajima, R. Bagnis and R. Adachi. 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. Bull. Jpn. Soc. Sci. Fish. 43:1021-1026.
5. Yasumoto, T., I. Nakajima, Y. Oshima and R. Bagnis. 1979. A new toxic dinoflagellate found in association with ciguatera. In: Toxic Dinoflagellate Blooms, (Taylor and Seliger eds.). Elsevier, New York. pp. 65-70.
6. Murata, M., M. Kumagai, J.S. Lee and T. Yasumoto. 1987. Isolation and structure of yessotoxin, a novel polyether compound implicated in diarrhetic shellfish poisoning. Tetra. Let. 28:5869-5872.
7. Murata, M., A-M. Legrand, Y. Ishibashi, M. Fukui and T. Yasumoto. 1990. Structure and configurations of ciguatoxin from the moray eel *Gymnothorax javanicus*, and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. J. Am. Chem. Soc. In press.
8. Legrand, A-M., M. Fukui, P. Cruchet, Y. Ishibashi and Y. Yasumoto. 1991. Characterization of ciguatoxins from different fish species and wild *Gambierdiscus toxicus*. In: Proceedings of the Third International Conference on Ciguatera Fish Poisoning. (T.R. Tosteson ed.). Polyscience Publishers, Quebec, Canada. See pp. 27-36, these Proceedings
9. Tachibana, K. 1980. Structural Studies on Marine Toxins. Ph.D. Dissertation, University of Hawaii. 156 pp.
10. Lin, Y.Y., M. Risk, S.M. Ray, D.V. Engen and J. Clardy. 1981. Isolation and structure of brevetoxin B from the "red tide" dinoflagellate *Ptychodiscus brevis* (*Gymnodinium breve*). J. Am. Chem. Soc. 103:6773-6775.
11. Shimizu, Y., H.N. Chou, H.Bando, G.V. Duyne and J. Clardy. 1986. Structure of brevetoxin A (GB-1 toxin), the most potent toxin in the Florida red tide organism *Gymnodinium breve* (*Ptychodiscus brevis*). J. A. Chem. Soc. 108:514-515.

Extraction and Purification of Toxic Fractions from Barracuda (*Sphyraena barracuda*) Implicated in Ciguatera Poisoning

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Abstract

Samples of several barracuda fish (*Sphyraena barracuda*) implicated in ciguatera poisoning were analyzed for toxicity. Toxic fractions were isolated and purified using column and thin layer (TLC) chromatographic techniques. Crude extracts of fish samples were prepared by extracting with acetone and partition purification with hexane, methanol, and chloroform. Partially purified extracts were applied to a silica gel column and toxic fractions eluted with chloroform:methanol mixtures of 100:0, 95:5, and 50:50. The 5% methanol/chloroform was most efficient in separating toxic fractions. The fractions were purified further using silica gel TLC plates. Developing solvents used were chloroform:methanol at 95:5, 90:10, and 50:50. Visualization of separated compounds was accomplished by spraying the plate with sulfuric acid:acetic acid:methanol(1:1:9) mixture and heating the plate at 110°C for 3 minutes. Three separate toxic fractions were observed. Toxic potentials were confirmed in three fractions using intra peritoneal (i.p.) injection in mice and rectal temperature monitoring. All fractions were tested for the presence of decomposition products (histamine, putrescine, cadaverine) and okadaic acid using high performance liquid chromatography. Okadaic acid was detected in one of the toxic TLC fractions.

Introduction

Knowledge that certain fish and shellfish can become poisonous and cause death or illness when eaten has been known for centuries, but only more recently has the clinical nature and etiology of these foodborne intoxications become apparent[1]. *Gambierdiscus toxicus* has been identified as a source of

ciguatoxin and more recently several strains of *Prorocentrum* sp.[2,3]. The ability to culture selected organisms under large scale conditions has been accomplished (D.L. Park, unpublished data; R. Dickey, unpublished data)[4,5]. Over 400 species of bony fish have been reported to cause ciguatera[6,7]. It is believed that fish become ciguatoxic through the food chain; herbivorous fish feed on algae and detritus of coral reefs, then the larger reef carnivores prey on these herbivores[8,9]. There appears to be a wide distribution of the toxin in various fish tissues[10,11]. Although ciguatoxin has been crystallized following extraction from the livers of ciguatoxic eels, it has only recently been completely structurally identified[12,13]. There appear to be several toxins involved, i.e., ciguatoxin, maitotoxin, okadaic acid, and scaritoxin, associated with toxigenic dinoflagellates or fish[3,14-16]. The mouse assay has been used for ciguatera as well as the feeding of cats, chickens, mosquitoes and mongooses[17-21]. More recently several immunological methods have been developed[22,23]. This study focused on the analysis of barracuda fish (*Sphyraena barracuda*) implicated in a ciguatera poisoning outbreak which occurred in Puerto Rico.

Materials and Methods

Fish Samples

Barracuda fish (*Sphyraena barracuda*) implicated in a ciguatera poisoning outbreak were harvested 4 miles off the southwest coast of Puerto Rico. Samples of the suspect fish were obtained by the Food and Drug Administration, San Juan, Puerto Rico, from the Rosas Fish Market in Puerto Real, Cabo Rojo, PR 00623, on May 29, 1985. Following the poisoning outbreak, suspect fish were maintained at -18°C until ready for analysis.

Extraction and Purification of Barracuda Toxins

A modification of the method reported by McMillan and co-workers was used to extract the toxins (Figure 1)[24]. The partially purified extracts were dissolved in methanol (MeOH), then purified further on a silicic acid column (Mallinkrodt, 100 Mesh) previously activated at 100°C for 1 hour, as reported by Nukina and co-workers[25]. As solution solvents, binary systems of chloroform-methanol (100:0, 95:5 and 50:50) were tried in sequence.

Thin Layer Chromatography

Silica gel 60 plates (5 × 20 cm × 0.25 mm and 20 × 20 cm × 0.25 mm) were activated at 100°C for 10 minutes. The plates were developed in a closed non-equilibrated system with developing solvents of 5, 10, and 50% MeOH in chloroform. Visualization of separated compounds was accomplished by spraying with a H_2SO_4-acetic acid-MeOH (1:1:9) solution and heating at 110°C for 3 min. To heat the 20 × 20 cm plates, the extraction zone was protected (after development) with glass plates (8 × 20 cm), and the plates inverted to expose the developed side of the TLC plate. Application of the extract on the

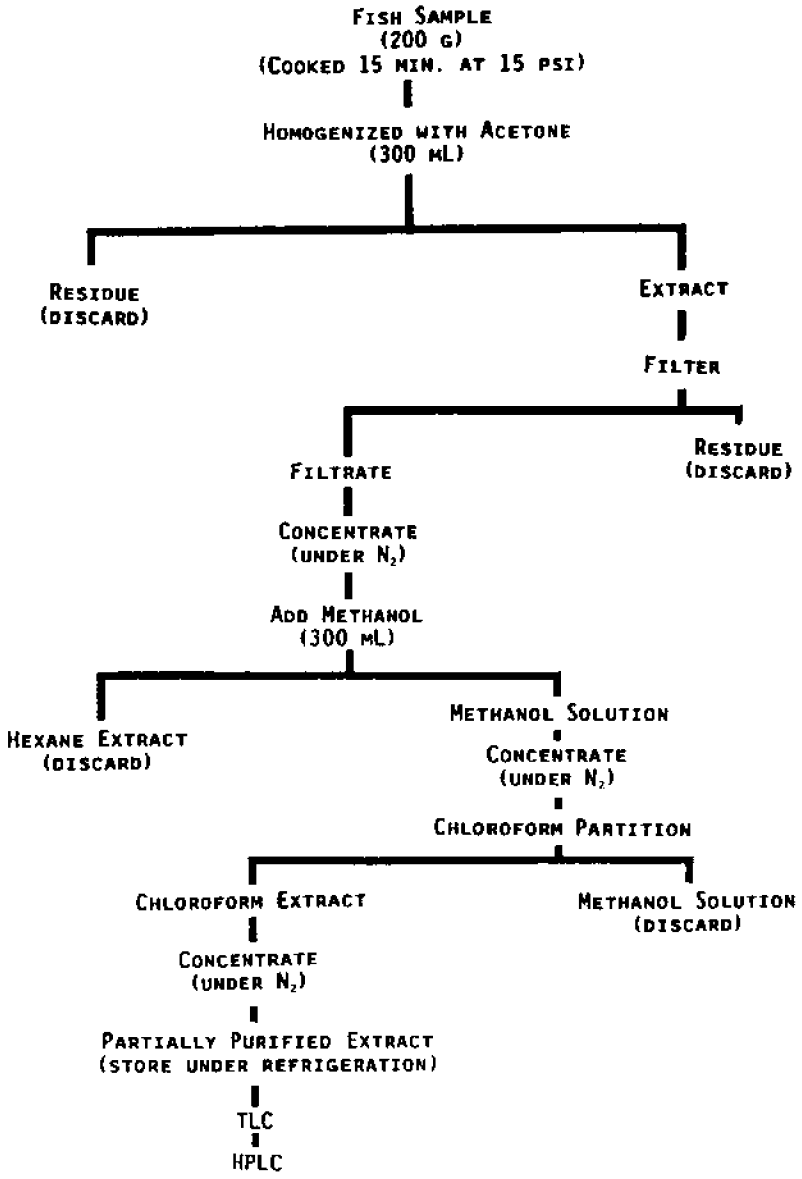

Figure 1. Extraction of Barracuda Ciguatoxin.
Extraction method used for isolating ciguatoxic fractions from barracuda (*Sphyraena barracuda*).

TLC plate (95 µl) was done with a Camag Linomat III continuous type dosemeter, equipped with a 100 µl syringe (Camag) using a pressure of 28 psi and a working speed of 9 sec/µl. The purified fractions were removed from the TLC plate by scraping off the silica gel and aspirating into a 10 ml syringe with a Millex-SR (Millipore Co, No. SLS2025 NS) filter attached to it.

Liquid Chromatography

Crude extracts of toxic and non-toxic fish were analyzed for histamine using the AOAC fluorometric method and for putrescine and cadaverine following the gas liquid chromatographic method of Staruszkiewiez and Bond[26]. Six purified fractions isolated by TLC were analyzed for okadaic acid (OA) using the method of Lee *et al.*[27]. Isolates were evaporated to dryness and redissolved in 500 µl chloroform, 100 µl of which were used for derivatization

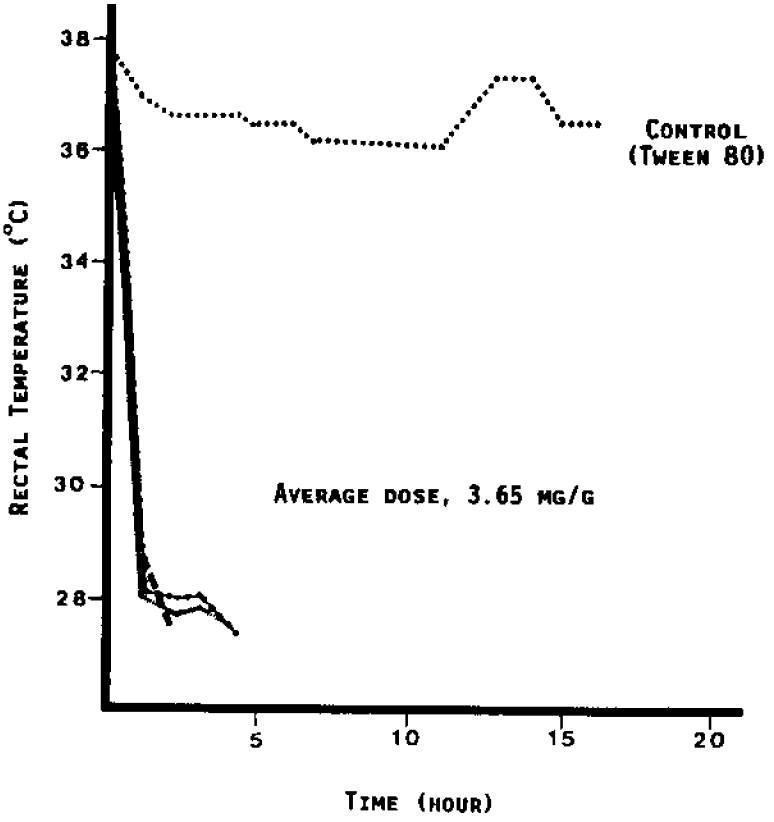

Figure 2. Effect of Ciguatoxic Barracuda Extracts on Rectal Temperature and Toxicity in Mice. Rectal temperature and toxic responses in mice dosed (i.p.) with crude extracts of ciguatoxic barracuda.

with the ADAM reagent. Before LC analysis, the derivatized isolate was purified further with a silica gel SEP-PAK column conditioned with hexane-chloroform. The column was washed with hexane-chloroform, then chloroform. Derivatized OA was eluted off the column with chloroform-methanol (95:5). Parameters for the LC system were; oven temperature, 35°C; columns, Lichrocart 250-4, Superspher 100 RP-18 (5 μm pore size, 250 mm length) and 4.4 Lichrocart pre-column RP-8 (5 μm pore size); eluting solvent, acetonitrile-water (80:20); flow rate, 1 ml/min. A fluorescence detection system (365 nm, excitation; 412 nm, emission) was used to monitor column fractions.

Mouse Assay

The mouse temperature depression toxicity test as reported by Sawyer *et al.* was employed using CD-1, female mice (Charles River) weighing between 19 and 21 grams[28]. One ml of extract, suspended in 5% Tween 80 solution (prepared with 0.9% NaCl solution), was injected intraperitoneally (i.p.) into the mouse and toxicity values monitored by taking rectal temperature measurements every hour for 16 hours with a digital thermometer (YSI type). Zero time temperature was taken just before dosing.

Results and Discussion

There was a rapid decrease in the rectal temperature values of almost 10°C when the mice were injected with crude toxic extracts of the contaminated fish sample (Figure 2). Experimental animals exhibiting this response usually did not survive more than 4 hours with the typical symptoms of convulsions followed by respiratory failure. Figure 3 shows the response of animals dosed with non-contaminated extracts. A decrease in temperature of around 4°C was observed during the first hour; however, the animal recovered and body temperature returned to normal. McMillan and co-workers reported that in mice treated either with control or contaminated extracts where the final solvent was chloroform this "knockdown" phenomenon in rectal temperature was observed[24]. Traces of chloroform incorporated in corn oil and injected to the mice elicited a similar phenomenon.

With respect to the polarity of the toxic fraction(s) isolated using a silica gel column, the toxins were eluted with a 5% MeOH-chloroform system. For the other elution solvents, 0 and 50% MeOH-chloroform, the animals exhibited no toxic response when exposed to an average dose representing 1.027 and 0.472 mg extract/g fish flesh, respectively. The average dose given the animals from the 5% MeOH-chloroform eluate was 1.37 mg extract/g fish flesh. This is in agreement with published results of Lewis and Endean and Nukina *et al.*, who have demonstrated isolation of the toxic fraction from crude extracts by slightly increasing the polarity of chloroform[11,25,29]. These results demonstrate that the toxic fraction is eluted with the methanol:chloroform (5:95)

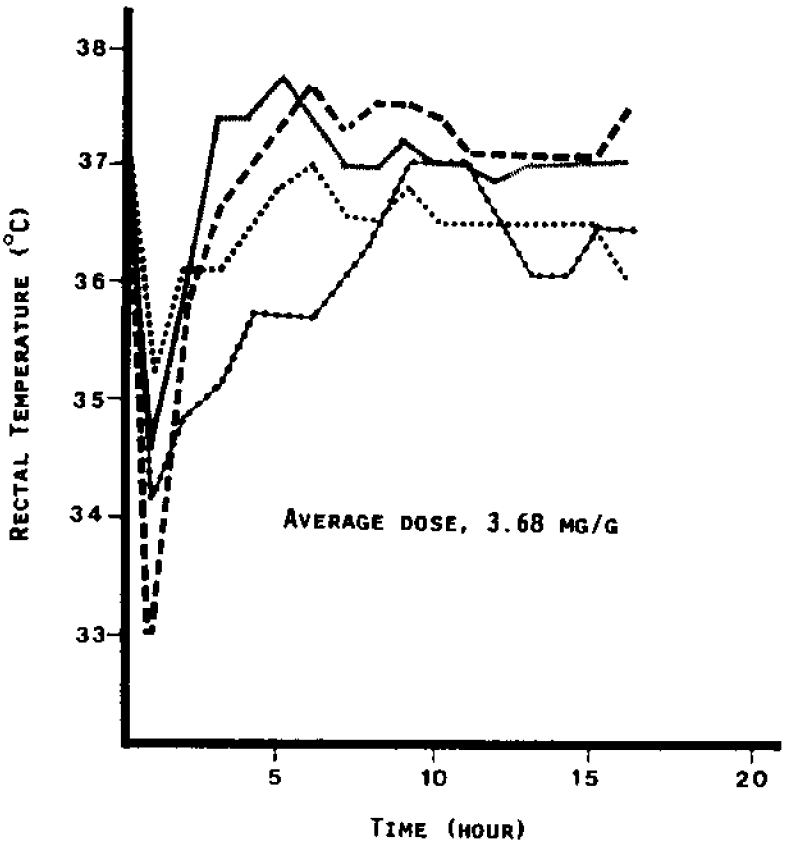

Figure 3. Effect of Control Extracts on Rectal Temperature.
Effect of non-ciguatoxic barracuda extracts on rectal temperature in mice. Rectal temperature response in mice dosed (i.p.) with crude extracts of non-ciguatoxic barracuda.

binary solvent system. An equally important factor to note is that the animal's death occurs following a rapid decrease in the rectal temperature in the order of 10°C.

The toxic fraction eluted with 5% MeOH/chloroform was further separated using silica gel TLC plates. Excellent resolution of 5 components of different polarity was obtained. The toxic responses in the mouse for each fraction are presented in Figures 4 and 5. It is important to note that to be considered toxic, the fractions must both cause a decrease in the rectal temperature as well as produce death at the end of the evaluation time (16 hours). Nukina and co-workers worked with a purified extract eluted with 10% methanol:chloroform from the silicic acid column[25]. They observed the existence of two toxic fractions. One acidic fraction (more polar) eluted with

methanol:H_20 (1:1) from an aluminum oxide column and another fraction considered neutral (less polar) which eluted with methanol:chloroform (1:11). Figures 4 and 5 show that, utilizing the TLC system, it is possible to have excellent resolution of at least five (5) fractions, three (3) of which caused death in mice at the concentrations tested. These results suggest that it is possible to distinguish between the hypothermic factor and the toxic factor. It also shows that more than one compound is involved with the ciguatera phenomenon. This would explain the array of multiple symptoms associated with human intoxication caused by the consumption of ciguatoxic fish.

Non-detectable levels of histamine, putrescine, and cadaverine were observed in the crude extracts of toxic and non-toxic fish analyzed. Five TLC

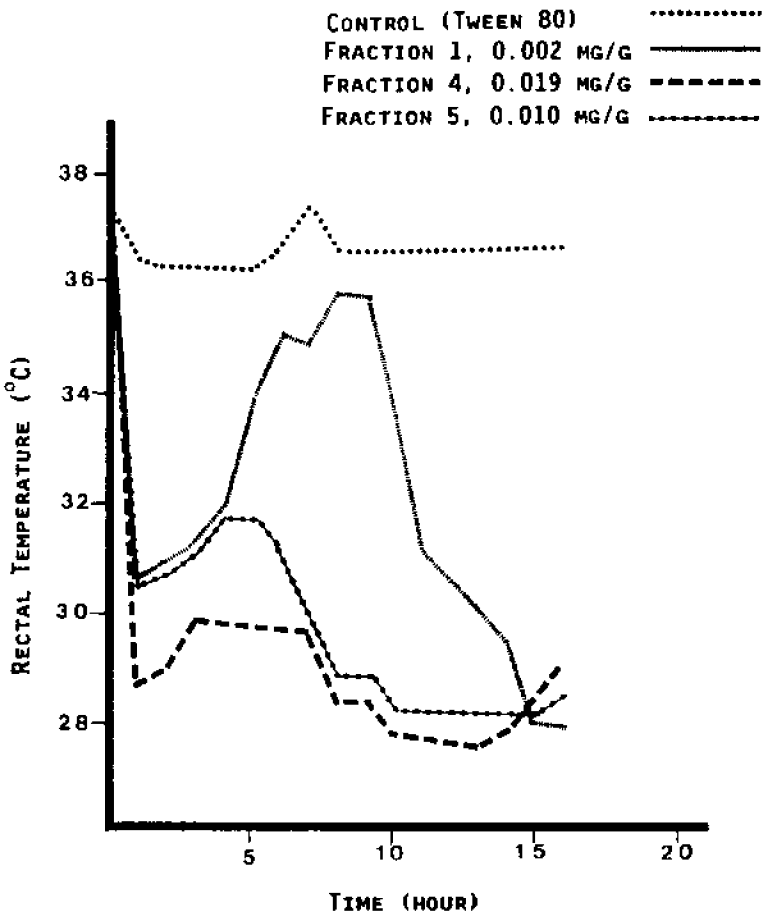

Figure 4. Purified Ciguatoxin Fractions: Rectal Temperature Response and Toxicity. Rectal temperature and toxic responses in mice dosed (ip) with isolated fractions following column and thin-layer purification of extracts from ciguatoxic fish (barracuda).

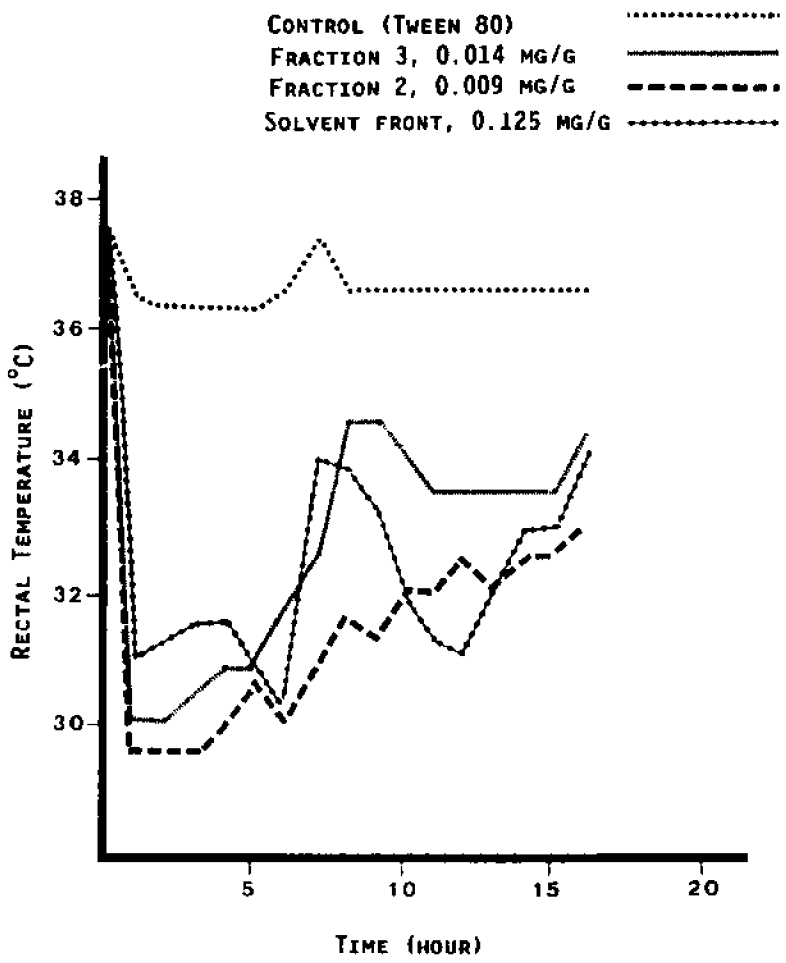

Figure 5. Purified Ciguatoxic Fractions: Rectal Temperature Response and Toxicity. Rectal temperature and non-toxic responses in mice dosed (ip) with isolated fractions following column and thin-layer purification of extracts from ciguatoxic fish (barracuda)

isolates from the toxic fish (Figures 4 and 5) were subjected to HPLC analyses for OA. One of the isolates toxic to the mouse (Fraction 1, Figure 4) tested positive for OA. Chromatograms of this isolate, standard OA, and a sample of mussel containing OA as a positive control, are shown in Figure 6. *Prorocentrum lima* and *P. concavum*, toxigenic dinoflagellates implicated as sources of ciguatera poisoning toxins have been reported to produce OA[3,14,30]. *P. lima* is widely distributed in the Caribbean Sea which allows for the possibility that OA is taken up by herbivores and passed up the food chain to carnivorous fish such as the barracuda. OA could contribute to

the diarrhea frequently seen in ciguatera fish poisoning patients. This is the first report of OA in fish tissue associated with a ciguatera poisoning outbreak. OA has also been implicated as one of the toxins responsible for diarrhoeic shellfish poisoning (DSP) outbreaks[1].

In 1983, Hoffman and co-workers published a study where they related the degree of response of the animal (mice) when the dose injected varied[17]. Among the most important symptomatic aspects, they pointed out a

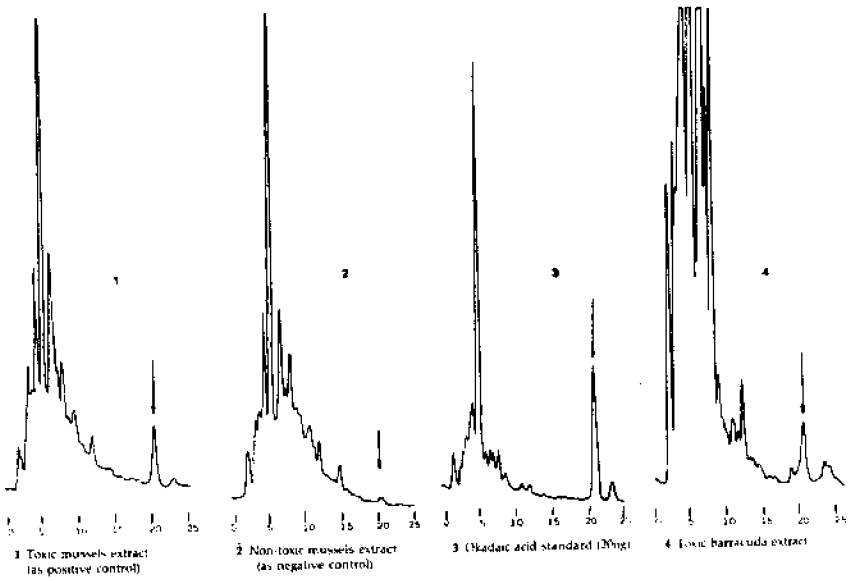

Figure 6. Chromatographic Comparison: Ciguatoxic Extracts, Shellfish Extracts and Okadaic Acid. Liquid Chromatograms of partially purified ciguatoxic fish (barracuda) extract,mussel extract containing okadaic, and okadaic standard.

typical behavioral trend, characterized primarily by decreases in rectal temperature, reflexes and motor activity. In this study, the authors established an outline of symptoms and categorized them to different degrees of toxicity. The first four categories were related directly to rectal temperature. Sawyer *et al.* studied the effect of the toxins associated with the ciguatera phenomenon and its relation with the changes in temperature of animals treated with partially purified extracts[28]. Their results demonstrated the inability of the mouse to control its corporal temperature when treated with ciguatoxic extracts. The authors proposed that ciguatoxins are a chemically related group of compounds with unique pharmacological properties and that the rectal temperature measurement represents a viable alternative to differentiate ciguatoxins from the other biotoxins of marine origin.

Figure 2 provides evidence on the toxic nature of the fish implicated in the ciguatera poisoning incident. Another fish specimen from the same lot was extracted and tested negative to the mouse bioassay (Figure 3). These results support the theory that for a given ciguatera poisoning outbreak, not all the fish would be contaminated. This highlights the complexity and difficulty in setting up an effective fish safety monitoring program for ciguatera.

Acknowledgements

Agricultural Experiment Station Journal Article No. 7296

LITERATURE CITED

1. World Health Organization. 1984. Aquatic (Marine and Freshwater) Biotoxins, Environmental Health Criteria 37, Geneva, Switzerland.
2. Bagnis, R., S. Chanteau and T. Yasumoto. 1977. Decouverte d'un agent etiologique vraisemblable de la ciguatera. C.R. Acad. Sci. (Paris). 28: 105-108.
3. Tindall, D.R., R.W. Dickey, R.D. Carlson and G. Morey-Gaines. 1984. Ciguatoxic dinoflagellates from the Caribbean Sea. In: Seafood Toxins. (E.P. Ragelis, ed.). ACS Symposium Series, #262, American Chemical Society, Washington, DC. pp. 225-240.
4. Babinchak, J., D. J. Jollow, M.S. Voegtline and T.B. Higerd. 1985. Toxin production by *Gambierdiscus toxicus* isolated from the Florida Keys. Mar. Fish. Rev. 48: 53-64.
5. Durand-Clement, M. 1986. A study of toxin production by *Gambierdiscus toxicus* in culture. Toxicon 24(11):1153-1157.
6. Halstead, B.W. 1978. Poisonous and Venomous Marine Animals of the World, Vol. 2. Vertebrates. U.S. Government Printing Offices, Washington, DC. 330 pp.
7. Banner, A.H. 1975. Ciguatera: A disease from coral reef fish. In: Biology and Geology of Coral Reefs, (O.W. Jones and R. Endean, eds.). Academic Press, New York. pp. 177-213.
8. Legrand, A-M., M. Fukai, P. Crachet, Y. Ishibashi and T. Yasumoto. 1992. Characterization of ciguatoxins from different fish species and wild *Gambierdiscus toxicus*. In: Proceedings of the Third International Conference on Ciguatera Fish Poisoning (T.R. Tosteson, ed.). Polyscience Publishers, Quebec, Canada. See pp. 27-36, these Proceedings.
9. Vernoux, J.P., L.P. Magras, S.A.E. Anadaloussi and N. Riyeche. 1986. A survey of comparative toxicity in the ciguatera food chain on Saint Barthelemy Island in the Caribbean. Bull. Soc. Path. Ex. 79:275-283.
10. Vernoux, J.P., N. Lahlou, S.A.E. Anadaloussi, N. Riyeche and L.P. Magras. 1985. A study of the distribution of ciguatoxin in individual Caribbean fish. Acta Tropica 42:225-233.

11. Lewis, R.J. and R. Endean. 1984. Ciguatoxin from the flesh and viscera of the barracuda, *Sphyraena jello*. Toxicon 22(5):805-810.
12. Yong-Goe, J. and P.J. Scheuer. 1986. The chemical nature of scaritoxin. Mar. Fish. Rev, 48:19-22.
13. Murata, M., A-M. Legrand, Y. Ishibashi and T. Yasumoto. 1989. Structure of ciguatoxin and its congener. J. Am. Chem. Soc. 111:8929-8931.
14. Gamboa, P.M. and D.L. Park. 1985. Fractionation of extracts of *Prorocentrum lima, Gambierdiscus toxicus* and ciguatoxic fish using counter current chromatography. Paper presented at 2nd Conference on Ciguatera, San Juan, Puerto Rico, April 23-25.
15. Vernoux, J.P. and S.A.E. Andaloussi. 1986. Heterogeneity of ciguatoxins extracted from fish caught at the coast of the French Antilles. Biochemie 68:287-291.
16. Yasumoto, T., M. Murata, Y. Ishibashi, M. Fukai and A-M. Legrand. 1992. Structure determination of ciguatoxins of Moray Eels and *Gambierdiscus toxicus* . In: Proceedings of the Third Interational Conference on Ciguatera Fish Poisoning (T.R. Tosteson ed.). Polyscience Publishers, Quebec, Canada. See pp. 3-12, these Proceedings.
17. Hoffman, P.A., H. R. Granade and J.P. McMillan. 1983. The mouse ciguatoxin assay: A dose-response curve and symptomology analysis. Toxicon 21: 363-369.
18. Pompon, A., E. Chungue, I. Chazelet and R. Bagnis. 1984. Ciguatera: Une methode rapide, simple et fiable de detection de la ciguatoxine. Bull. World Health Organ., 62(4):639-645.
19. Vernoux, J.P. and N. Lahlou. 1986. The chick ciguatoxin bioassay: A symptomology and sensitive analysis. Bull. Soc. Path. Ex. 79:140-146.
20. Chungue, E., R. Bagnis, and F. Parc. 1984. The use of mosquitoes (*Aedes aegypti*) to detect ciguatoxin in surgeon fishes (*Ctenochaetus striatus*). Toxicon 22: 161-164.
21. Bagnis, R, and G. Fevai. 1971. La ciguatera feline experimentale a Tahiti. Rev. Med. Vet., 122: 229-238.
22. Hokama, Y., L.H. Kimura, M.A. Abad, L. Yokochi, P.J. Scheuer, M. Nukina, T. Yashumoto, D.G. Baden, and Y. Shimizu, Y. 1984. An enzyme immunoassay for the detection of ciguatoxin and competitive inhibition by related natural polyether toxins. In: Seafood Toxins (E.P. Ragelis, ed.). ACS Symposium Series # 262, American Chemical Society, Washington, DC. pp. 307-320.
23. Emerson, D.L., R.M. Galbraith, J.P. McMillan and T.B. Higerd. 1983. Preliminary immunologic studies of ciguatera poisoning. Arch. Intern. Med. 143: 1931-1933.
24. McMillan, J., H. Ray and P. Hoffman. 1980. Ciguatera fish poisoning in the United States Virgin Islands: Preliminary studies. J. Coll. Virgin Is.

6:84-107.

25. Nukina, M., L. Koyanagi and P. Scheuer. 1984. Two interchangeable forms of ciguatoxin. Toxicon 22: 169-176.
26. Staruszkiewiez, W. and J. Bond. 1981. J. Assoc. Off. Anal. Chem. 64: 584-591.
27. Lee, J.S., T. Yanagi, R. Kenma and T. Yasumoto. 1987. Fluorimetric determination of diarrhetic shellfish toxins by high-performance liquid chromatography. Agric. Biol. Chem., 51: 877-881.
28. Sawyer, P., D. Jallow, P. Scheuer, R. York, J. McMillan, N. Withers, H. Fudenberg and T. Higerd. 1984. Effect of ciguatera-associated toxins on body temperature in mice. In: Seafood Toxins (E. Ragelis, ed.). ACS Symposium Series #262, American Chemical Society, Washington, DC. pp. 321-329.
29. Lewis, R.J. and R. Endean. 1983. Occurrence of a ciguatoxin-like substance in Spanish mackerel (*Scomberomorus cammersani*). Toxicon 21: 19-24.
30. Dickey, R.W., S.C. Bobzin, D.J. Faulkner, F.A. Benesath and D. Andrzejewski. 1990. Okadaic acid from *Prorocentrum concavum*. Toxicon 28: 371-377.

Characterization of Ciguatoxins from Different Fish Species and Wild *Gambierdiscus toxicus*

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Abstract

Subsequent to the isolation and structure elucidation of ciguatoxin (CTX) and its congener from the moray eel *Gymnothorax javanicus*, Bleeker, and the causative dinoflagellate *Gambierdiscus toxicus*, Adachi and Fukuyo, we carefully examined all the toxic components in moray eel, parrotfish and *G. toxicus*. As many as 21 toxins, including the above two toxins, were detected and characterized by high performance liquid chromatography (HPLC) and mass spectra. These toxins were separable into four groups according to the order of elution from a reversed phase column. Polarities of toxins in *G. toxicus* and fish suggested that toxins underwent oxidative modifications during the course of the food chain. CTX was the major toxin in the moray eel but was insignificant or absent in the parrotfish and *G. toxicus*. Detection of CTX in other carnivorous fish such as red snappers and groupers was facilitated by an HPLC method which detected fluorescence after derivatizing CTX with anthroylnitrile.

Introduction

Ciguatera is an important health and economic problem in the South Pacific area. In French Polynesia, the Tuamotu, Gambier and Marquesas Archipelagos are the most concerned areas. A number of fish of varying feeding habits are involved in the intoxication. Patients may show a wide range of symptoms including neurological, cardiovascular and gastrointestinal disorders [1]. Because of the diversity of the complex human symptoms, there has been a question as to whether the ciguatoxin (CTX) isolated from the moray eel, purified and designated by Scheuer's group was solely responsible for the intoxication [2]. Another question which remained unsolved was whether the ether-soluble toxin detected in the epiphytic dinoflagellate *Gambierdiscus toxicus*, Adachi and Fukuyo, the putative origin of ciguatoxin, was chemically related to ciguatoxin [3,4]. As *G. toxicus* produced little or no CTX under culture conditions, we isolated pure CTX from the moray eel viscera [5]. Ether-soluble

toxins of *G. toxicus* were also purified from wild specimens collected in the Gambier Islands. As described separately in these Proceedings, our previous studies revealed that CTX and a congener isolated from *G. toxicus* share the same ladder-shaped skeleton comprised of thirteen ether rings[6,7]. During the course of toxin purification, we noticed the presence of other toxins in addition to CTX and the congener[5,8]. The difference in the extent of oxygenation between the two toxins also suggested that a series of oxidative modifications of the toxins during food chain transmission might yield a number of congeners.

In the present study, detailed analyses of toxin compositions were carried out on *G. toxicus* and fishes at different stages of the food chain. As many as 21 toxins were detected and characterized by high performance liquid chromatography (HPLC) and mass spectral measurements. The results presented here support the hypothesis of oxidative modification of toxins in fishes.

Materials and Methods

The wild specimens of *G. toxicus* were collected in the Gambier Islands, French Polynesia, in 1979. Moray eels *Gymnothorax* (=Lycodontis) *javanicus* (840 specimens) were collected around the island of Tahiti and in the Tuamotu and Marquesas Archipelagos. The viscera (125 kg including 43 kg of livers) were used for isolation of CTX and minor toxins. Red snappers *Lutjanus bohar*, groupers *Plectropomus leopardus* and *Epinephelus fuscoguttatus*, and parrotfish *Scarus gibbus* were collected in French Polynesia, Micronesia (Saipan), Fiji (around Suva) and Japan (Okinawa).

Purification of CTX from the moray eels is illustrated in Figure 1. Details of the procedure were described previously[5]. Purification of toxins from other fishes or *G. toxicus* was carried out virtually in the same way. Comparison of retention times of toxins was achieved on a Develosil ODS-7 column (10x250 mm, Nomura Chemicals, Japan) by linear gradient elution starting from acetonitrile-water (60:40) to acetonitrile (0.5%/min, 1 mL/min) for moray eel toxins, and from acetonitrile-water (85:15) to acetonitrile (0.5%/min, 1 mL/min) for toxins in the parrot fish and in *G. toxicus*. The elution was continued at least for 60 min after the acetonitrile concentration had reached 100%. Elution of toxins was monitored by both mouse bioassays and by measuring the end absorption (210 nm) with a UV flow monitor. Toxins thus separated were further purified on the same column in the case of a relatively polar toxin group, or on either a Capcell pak C-8 column (10x250 mm, Shiseido Co., Japan) or on Asahipak ODP-50 column (10x250 mm, Asahi Kasei Co., Japan) with similar solvent systems. Mass spectra of the purified toxins were measured on a JEOL JMS DX-303HF spectrometer by using glycerol, thioglycerol or 4-nitrobenzylalcohol as a matrix, and ^{1}H NMR spectra on a JEOL GSX-400 (400 MHz) spectrometer. Fluorescence labelling of CTX with anthroylnitrile and subsequent HPLC determination of the fluorescent derivative were carried out

Moray eel viscera 125 kg

↓ Extracted with Acetone

↓ Partitioned Et_2O/H_2O

↓ Defatted with n-C_6H_{14}

Toxic extract

↓ $SiO_2/CHCl_3$-MeOH 97:3; 9:1

$CHCl_3$-Me_3OH 9:1

↓ Florisil/EtOAc; EtOAc-MeOH 9:1; 3:1

EtOAc-MeOH 9:1

↓ DEAE-Cellulose/$CHCl_3$; $CHCl_3$-MeOH 1:1

$CHCl_3$-MeOH 1:1

↓ Sephadex LH-20/$CHCl_3$-MeOH 6:4

Toxic fr.

↓ ODS cartridge /MeOH-H_2O 5:5; 6:4; 7:3; 8:2

MeOH-H_2O 8:2

↓ LiChroprep RP-18 /MeOH-H_2O 8:2 twice

/MeCN-H_2O 65:35 twice

CTX 350 μg (LD_{50} 350 ng/kg)

Figure 1. Extraction of Eel Ciguatoxin.
Purification procedure of ciguatoxin from moray eel viscera.

according to the method previously developed for measurements of pectenotoxin-1[9,10].

Results

As many as 21 toxins were detected from *G. toxicus*, parrot fish and moray eels. They were separable into four groups on the basis of their polarities. Each group was further divided into several components. Tentative

code names and mass unit of the protonated molecular ions of all the components detected are presented in Table 1. *G. toxicus* contained only group 3 and group 4 toxins but no ciguatoxin. The least polar toxin (4C), of which the molecular weight is still unknown, was the major toxin. In parrot fish, toxins were distributed among the groups 1, 3 and 4. Although a toxin very close to

Table 1. Tentative Coding of Ciguatoxins

ORDER OF ELUTION	CODE NO	MH+ (M/Z)	MORAY EEL (LIVER)	PARROT FISH (FLESH)	G. toxicus
POLAR					
	1A	1111	○		
	1B(CTX)		●	○	
		1023			
	2A	1117	○		
		1079	○		
	2B	1061	○		
		1117	○		
	2C	1061	○		
		1095	○		
	3A			○	
	3B	1077		●	
	3C	1085			○
		1023			○
	3D	941			○
		1095			○
		1023			○
		1061			○
		1023	○		
	*4A	1061		○	○
	*4B(GT-4B)	1061		○	○
	4C				●
LESS POLAR					

* : Stereo isomer ● : Major toxic fraction

Proposed coding of ciguatoxins from the moray eel, Parrotfish and G. *toxicus*. *Stereo isomer, solid circles are major toxic fractions.

CTX in the eluting position from the column was present in the parrot fish, it was distinct from CTX in the mass spectrum. The toxin 3B was the major toxin in the parrot fish. The toxins 4A and 4B isolated from the parrot fish were suggested to be identical with those of *G. toxicus* on the basis of their chromatograms (Figure 2) and mass spectral data. Toxins of the moray eels showed higher polarities than those of *G. toxicus* and the parrot fish. CTX was the principal toxin, but as many as nine toxins were isolated from the moray eels.

Trace amounts of CTX were identified by HPLC after derivatizing the toxin into an anthroyl ester. The minimum detection level for CTX was 1 ng per injection. Fluorometric analyses (Figure 3) show CTX as the major toxin in carnivorous fishes such as the moray eels, red snappers and groupers.

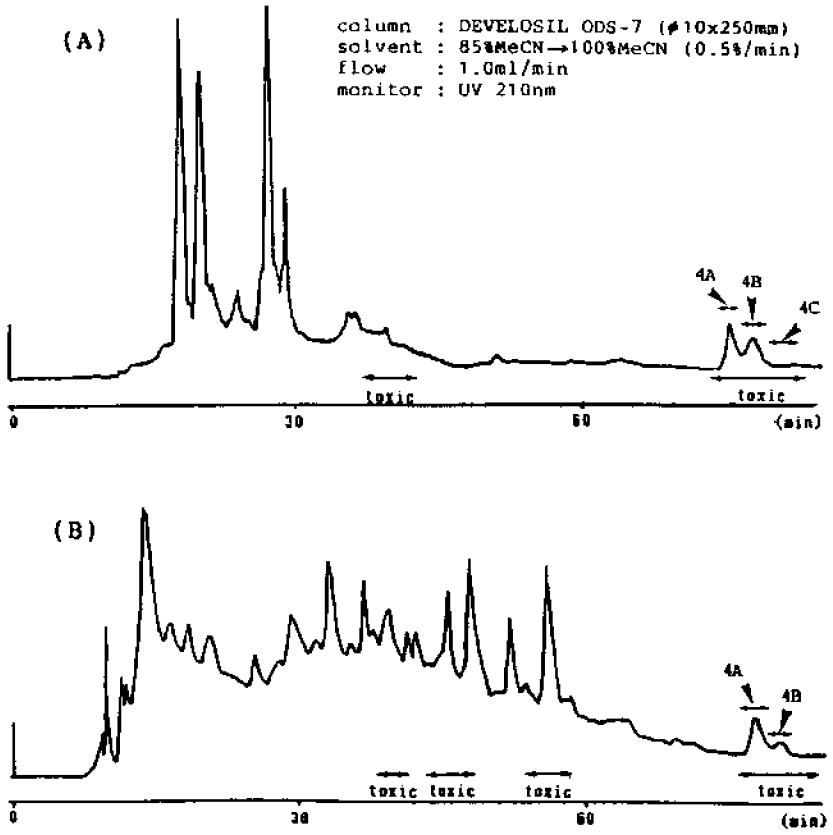

Figure 2. HPLC Analyses of *G. toxicus* and Parrotfish Toxins.
HPLC comparison between toxins of *G. toxicus* (A) and toxins of the parrotfish *Scarus gibbus* (B).

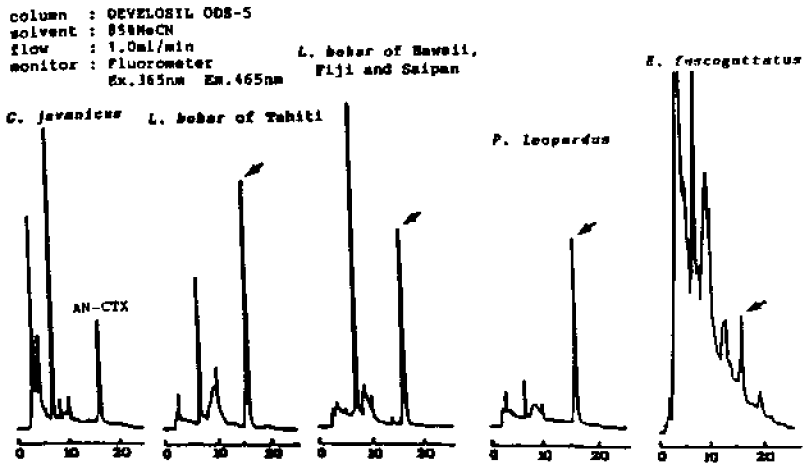

Figure 3. Fluorometric HPLC Detection of CTX.

Discussion

In previous studies we isolated CTX from moray eels and then determined its structure together with that of a congener isolated from the causative dinoflagellate *G. toxicus* [5-7]. Our hypothesis, drawn from the difference in the degree of oxygenation between the two toxins, that an oxidative modification of toxins must be taking place during the food chain was supported by results of the present study. The data shown in Table 1 clearly indicate that the polarity of toxins increases as they move up the food chain from *G. toxicus* to the parrot fish and then to the moray eel. The widely accepted previous assumption that CTX is the principal toxin in many ciguateric fish was proven to be applicable to carnivorous fishes but not to other fish species. The multiplicity of the toxins and different toxin compositions between the moray eel and the parrot fish may provide a basis to explain the diverse symptoms of ciguatera, especially to explain the difference between patients intoxicated by carnivores or by herbivores [1]. The fluorometric HPLC determination method for CTX was developed for the first time in this study. The method will be useful to identify the toxin in small samples, although difficulties may arise from the extremely low concentration of the toxin in fish. We have observed CTX concentrations as low as 0.5 ppb in fish flesh samples implicated in human intoxication in Okinawa. Developing a proper procedure for detecting such a low concentration of CTX will be an extremely difficult task. The structure of CTX suggests the possibility of obtaining a specific anti-CTX antibody by immunizing animals with a CTX-protein conjugate prepared via CTX-hemisuccinate. Developing a highly specific enzyme linked immunoas-

say method seems possible, if enough CTX is made available. The cross reactivity of such an antibody to the congeners isolated in this study remains to be tested in the future. In the present study we demonstrated no less than 21 toxins in ciguatera samples. It may be possible, however, that there are additional toxins in different species or fish from other regions. Structural elucidation of toxins other than CTX and 4B is under way.

Acknowledgements

This paper is dedicated to Professor J. Roux, Director of the Louis Malardé Institute, whose continued support to the collaboration between Tahitian and Japanese teams led us to many successful results including the structural determination of CTX and its congener. The authors thank Dr.R. Bagnis for his continued support to the ciguatera project, Messrs J. Bennett and M. Barsinas for collecting samples, and Mmes C. Lotte, J. Teore and T. Maubert for technical assistances. Some of the samples were provided by Professor Y. Hokama of the University of Hawaii and by Dr. U. Raj, a former staff member of the University of the South Pacific in Fiji. Special thanks go to Dr. M. Murata of Tohoku University, Japan for his valuable assistance in spectral measurements. This work was supported by the French Ministry of Research and Industry, Pasteur Institute of Paris, French Polynesia Government and by a Grant-in-Aid for Overseas Research of the Ministry of Education, Science and Culture, Japan.

Literature Cited

1. Bagnis, R. 1968. Clinical aspects of ciguatera (fish poisoning) in French Polynesia. Hawaii Med. J. 28:25-28.
2. Tachibana, K., M. Nukina, Y. Joh and P.J. Scheuer. 1987. Recent developments in the molecular structure of ciguatoxin. Biol. Bull. 172:122-127.
3. Yasumoto, T., I. Nakajima, R. Bagnis and R. Adachi. 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. Bull. Jpn. Soc. Sci. Fish. 43:1021-1026.
4. Bagnis, R., S. Chanteau and T. Yasumoto. 1977. Un agent étiologique vraisemblable de la ciguatéra. C.R. Acad Sc. Paris, 285 Série D: 671-674
5. Legrand, A-M., M. Litaudon, J-N. Genthon, R. Bagnis and T. Yasumoto. 1989a. Isolation and some properties of ciguatoxin. J. Appl. Phycol. 1:183-188.
6. Murata, J., A-M. Legrand, Y. Ishibashi and T. Yasumoto. 1989. Structure of ciguatoxin and its congener. J. Am. Chem. Soc. 111:8929-8931.
7. Murata, M., A-M. Legrand, Y. Ishibashi, M. Fukui and T. Yasumoto. 1990. Structures and configurations of ciguatoxin from the moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. J. Am. Chem. Soc. in press.

8. Legrand, A-M., P. Cruchet, R. Bagnis, M. Murata, Y. Ishibashi and T. Yasumoto. 1990. Characterization and spectral evidence for the presence of multiple ciguatoxins. In: Toxic Marine Phytoplankton (E. Graneli, B.G. Sundstrom, L. Elder and D.M. Anderson, eds.). Elsevier, New York. pp. 374-378.
9. Goto, J., N. Goto, F. Shamsa, M. Saito, S. Komatsu, K. Suzaki and T. Nambara. 1983. New sensitive derivatization of hydroxysteroids for high performance liquid chromatography with fluorescence detection. Anal. Chimica Acta. 147:397-400.
10. Lee, J.S., M. Murata and T. Yasumoto. 1989. Analytical methods for determination of diarrhetic shellfish toxins. In: Mycotoxins and Phycotoxins'88, (Natori, Hashimoto and Ueno, eds.). Elsevier, Amsterdam. pp. 327-334.

Brevetoxins and Related Polymeric Ether Toxins: Structures and Biosynthesis

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ABSTRACT

Several novel toxins have been isolated from the dinoflagellate *Gymnodinium breve* Davis (*Ptychodiscus brevis*), and their structures have been determined. All of them are characterized by the presence of large 7-membered, 8-membered and 9-membered rings. NMR and X-ray analysis of the conformation of the molecules have revealed the enormous flexibility of this linear polycyclic system, which may be a crucial factor in the interaction of these toxins with membranes.

The feeding studies of the biosynthesis of the brevetoxins using stable isotope precursors have shown the unusual nature of the dinoflagellate polyketides. A new series of toxins, hemibrevetoxins has been isolated from *G. breve*. They are an abridged form of brevetoxins, and their structures are expected to provide information on the structure-activity relationship and biosynthesis of brevetoxins.

INTRODUCTION

Chemical studies of the secondary metabolites of the Florida red tide causing organism, *Gymnodinium breve* Davis have shown that it produces a number of compounds with neurotoxic properties. Chemically these neurotoxins are classified as polyethers which have previously been encountered in fungi and bacteria of various species. The polyether toxins of *Gymnodinium breve* are, however, unique in the sense that they have a large ring system including 8- and 9-membered rings and thus, inherently have greater conformational flexibility. Mechanistically, these toxins have been shown to interact with sodium channels and cause a depolarization of the excitable membranes[1]. The conformational flexibility, in combination with the linear ring system, may play an important role in the penetration of these molecules into the membrane and binding. Thus, the elucidation of the toxin conformation will be essential to our understanding of the molecular mechanism of the

action of these important toxins. Recent chemical investigation of ciguatoxins has revealed that they also have a polyether cyclic system closely resembling brevetoxins, which implies a similarity in molecular properties, mechanisms of action and biosynthetic pathways of these two classes of toxins.

Materials and Methods

G. breve was cultured in NH-15 medium either in Fernbach flasks (2 l each) or in 5 gallon glass bottles (12 l) under 4000-5000 Lux illumination (12 hr. DL cycle) for 3 weeks. The cells were extracted with methylene chloride, and the extract was fractionated between petroleum ether and 10 % aqueous methanol. The residue from the methanolic extract was purified by flash chromatography on SiO_2, first with methylene chloride: benzene: methanol (40: 5: 1), and then with methylene chloride: ethyl acetate: methanol (50: 30: 1). High pressure liquid chromatography [normal phase, SiO_2, isooctane: isopropanol (4:1)] of the residue from the desired fraction resulted in the purification of brevetoxins and hemibrevetoxins. NMR spectra were recorded on Brucker 300 and 500 MHz instruments.

Results And Discussion

Although a number of chemical characteristics of *G. breve* toxin were reported before 1972 the first paper dealing with an insight into the chemical structure (NMR and MS data of brevetoxin) was reported in 1974 by Shimizu, *et al.* at the Food and Drugs from the Sea Conference held in Puerto Rico[3,4]. In that report the full NMR, IR, and MS spectra of GB-2 toxin (brevetoxin B) were presented, and its polyether structure was speculated[5]. Subsequently, Baden *et al.* and Risk *et al.* also reported the isolation of a toxin identical with GB-2[6,7].

The structure of brevetoxin B was established by X-ray crystallography in 1981[8]. The unprecedented polyether structure aroused enormous interest in the structure of other toxins, especially brevetoxin A (GB-1 toxin), which was known to be the most toxic of all brevetoxins and to have a considerably different structure from that of brevetoxin B. Thus, after the structure of brevetoxin B had been clarified, our efforts were focused on the structure elucidation of brevetoxin A.

Extensive NMR studies, including COSY and numerous decoupling experiments led to partial structures (Figure 1a). However, both proton and carbon signals for certain portions of the structure were undetectable or badly defused, and we were unable to complete the structure from the NMR data. Meanwhile, Nakanishi's group proposed a strucure (Figure 1b) for brevetoxin A on the bases of NMR spectra and mass fragmentation patterns[9,10]. The structure differed significantly from subsequent analyses but presented the correct structures of the B, D and E-rings. The connectivities

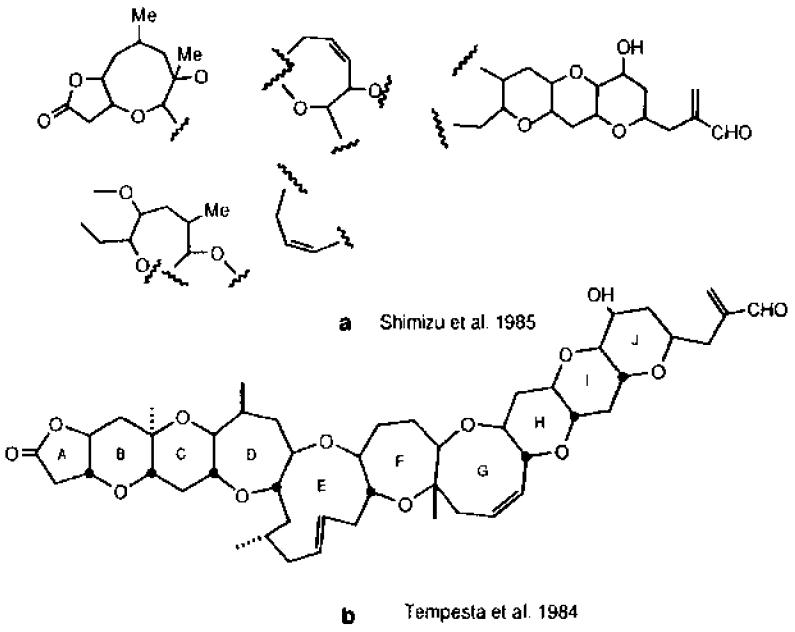
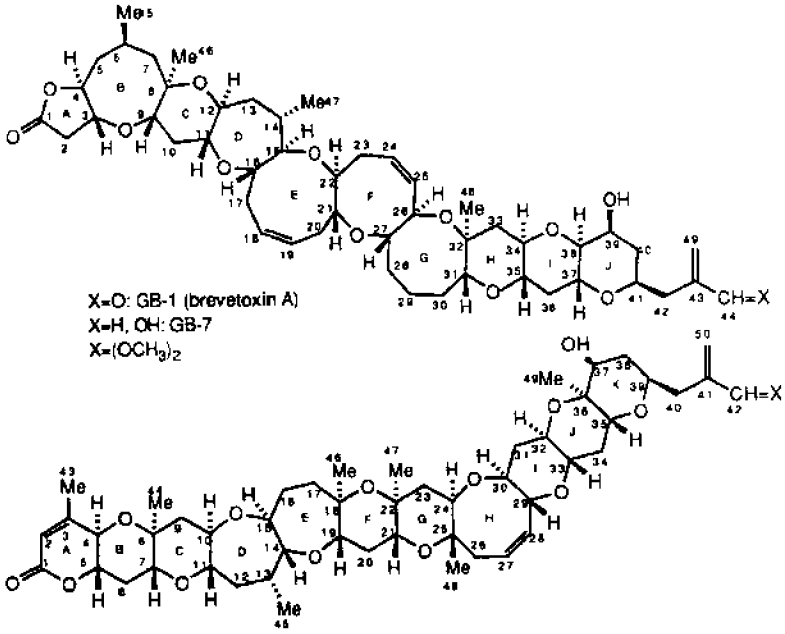


Figure 1. Structure of Brevetoxin A.
a) Structural fragments of brevetoxin A as determined by NMR data.
b) Structure of brevetoxin A.

around the E, F and G-rings were uncertain, F- and G- rings were apparently switched and the angular methyl group misplaced. The locations of double bonds, which were also deduced by the mass fragmentation pattern of the methylated $NaBH_4$ reduction product of an ozonolysis product, were also misplaced. Later, the NMR and mass spectral data were re-interpreted into a new structure by the same group[11].

Brevetoxin A was obtained as crystals even in an early stage of the isolation work. However, the silky needles obtained from methanol in the refrigerator were unfit for X-ray crystallography. Later, it was discovered that brevetoxin A formed different crystals at a higher temperature (~60°C). These crystals, which were grown to beautiful prisms of ca. 2 mm width, gave a good X-ray diffraction map, however, the computer analysis of the data was initially unsuccessful. Successful analysis of the structure (Figure 2) was first accomplished with the dimethyl acetal derivative of brevetoxin A, which was originally isolated as an artifact from silica gel chromatography of a toxin mixture using methanol containing solvents, and later by the action of acid catalysis on brevetoxin A in methanol[12]. Subsequently, the structure of another natural product, GB-7 was determined by X-ray to be the alcohol

derivative of brevetoxin A[13]. Surprisingly, both X-ray determined structures showed that the G ring existed in a boat-chair form, and the molecules were twisted 90° at the ring. Assignment of NMR signals to the new structure showed that some of the missing NMR signals belonged to the G ring, raising the possibility that the ring existed in slowly interconverting conformational isomers in solution. The crystallographic structure of brevetoxin A from crystallization at 60°C was eventually solved[13]. The unit cell was packed with two conformers differing at ring E and the aldehyde side chain (Figure 3). The NMR signals were not clearly observed in the E ring.

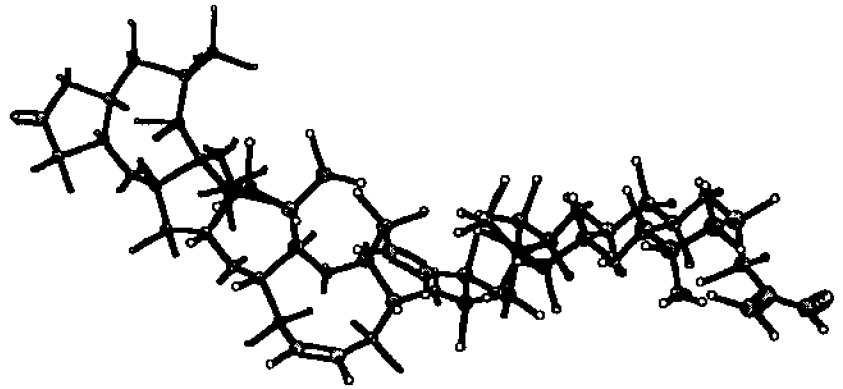


X=O: GB-1 (brevetoxin A)
X=H, OH: GB-7
X=$(OCH_3)_2$

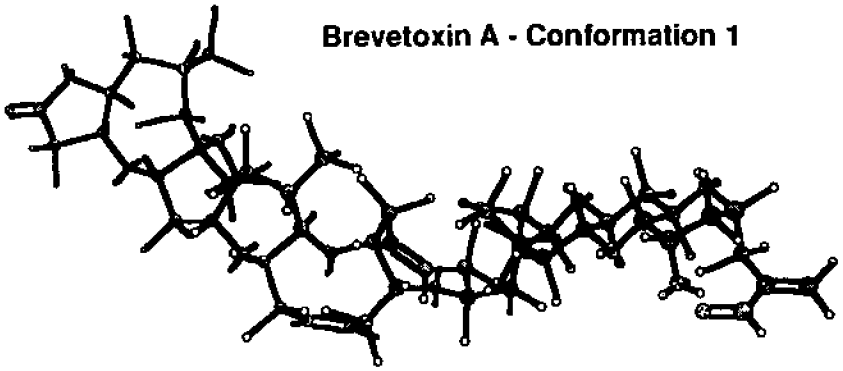
X=O: GB-2 (brevetoxin B)
X=H, OH: GB-3
X=O, 37-OAc: GB-5
X=O, 27, 28β-epoxide: GB-6

Figure 2: Structure of Brevetoxins.

In the revised NMR assignment of the structure of brevetoxin A, Nakanishi's group reported that measurement of NMR spectrum in benzene made it possible to assign the missing part of the signals (Tempesta, private communication)[11]. The experiment was repeated by our group, but improvement of the spectrum was limited. Chemical shift and coupling pattern differences due to solvent and temperature changes made it difficult for us to compare these results with the data collected in CD_2Cl_2 and $CDCl_3$. In an



Brevetoxin A - Conformation 1



Brevetoxin A - Conformation 2

Figure 3: Two Different Conformers of Brevetoxin A Crystals.

effort to resolve this problem otherwise, we decided to look at the low temperature NMR of brevetoxin A in CD_2Cl_2. The experiment was prompted by the report that low temperature NMR of ciguatoxin had overcome a similar problem which was due to conformational perturbations[2].

Normally, a measurement at low temperture is apt to cause a freeze in conformations or slower conformational interconversions, resulting in more complex or broadened NMR signal. In fact, spectra measured at -30°C and -50°C showed considerable broadenings of most signals as well as chemical shift changes, thereby making the assignment of the signals impossible. However, the COSY spectrum at 253°K exhibited some significant differences to the COSY spectrum at room temperature (Figures 4 and 5). A number of cross peaks that were not observed at room temperature (298°K), now appeared in the spectrum at 253°K.

There is a new set of cross peaks at δ 3.45, 3.16 ppm, which seems to be connected to the cross peak that represents protons 26 and 27. This cross peak *i* is, in turn, related to cross peak *v*, which is connected to cross peak *vi*. Cross

peak *i* is also related to *ii* and *iii*, and *ii* is connected to *iv*, and *iii* is also related to *iv*. Cross peaks *ii* to *vi* are all new cross peaks. Since they all seem to be related to the cross peak that represents protons 26 and 27, this pattern may represent one of the missing connectivities in the structure of brevetoxin A in solution, namely the protons on C-27 to C-31 of ring G (Figure 4).

The new cross peak *vii*, seems to have a connection with cross peak *ii* and to be connected to the system that contains *ii* and *iv*. The position of cross

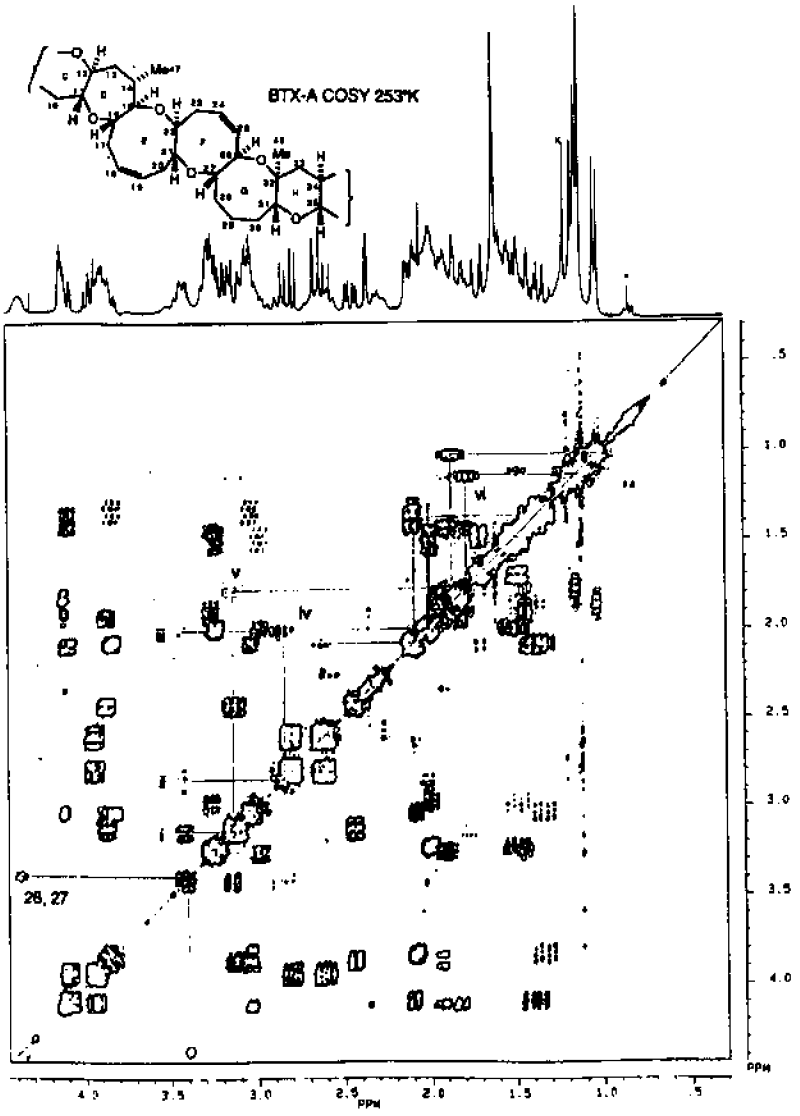


Figure 4: NMR Spectrum of Brevetoxin A. CD_2Cl_2 at 253°K (0.0 to 4.5 ppm region).

peak *vii* on the COSY spectrum suggests that it may be related to the olefinic portion of ring E , that is represented either by a cross peak between protons 17 and 18 or protons 19 and 20 (Figure 5). As mentioned earlier, E is a moiety in which some form of conformational interchange might be taking place. Resonances had been assigned to the olefinic protons of the ring in a

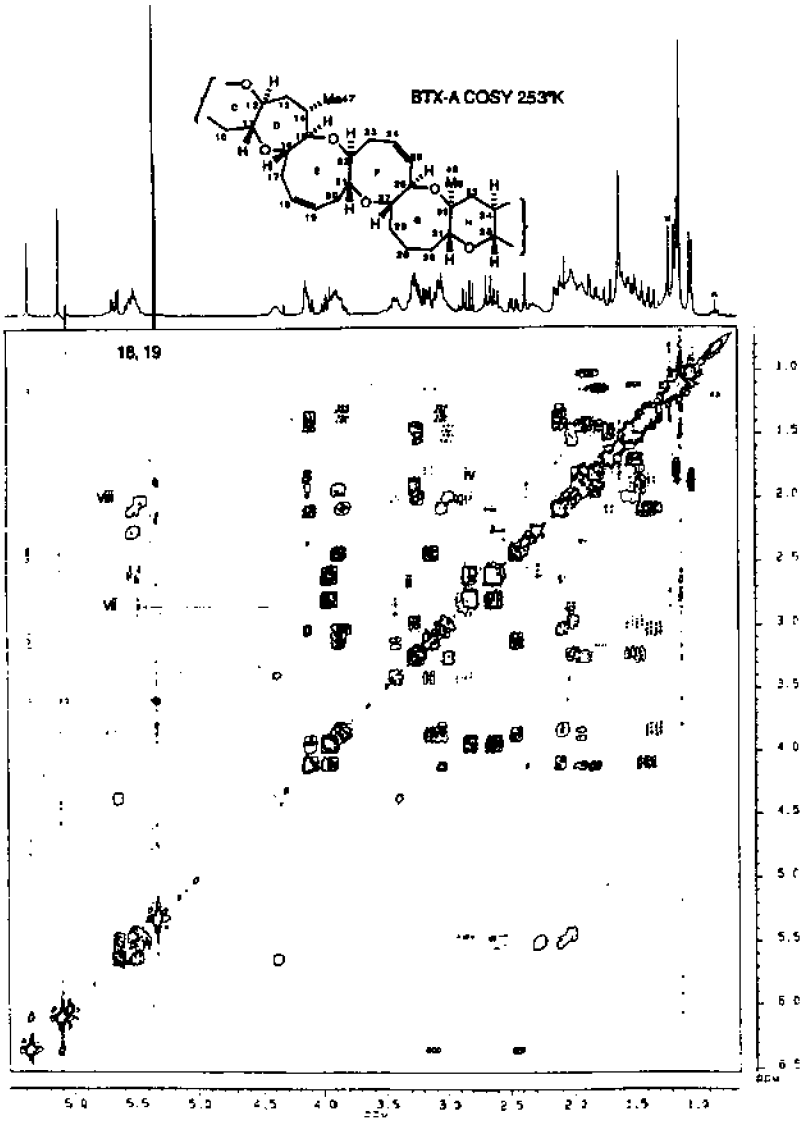


Figure 5: NMR Spectrum of Brevetoxin A. CD_2Cl_2 at 298°K (0.0-6.5 ppm region).

previous study, but it was difficult to assign signals in the spin system connected to the olefin. The subsequent solution of the X-ray structure of brevetoxin A indicated that there were two conformations that the double bond in the E ring could adopt[13]. In conformation 1, the olefin is up to the ring, while in conformation 2, it is twisted down as shown in Figure 3. In the crystals formed at a high temperature, the unit cell is packed with these two conformers. Thus, it may be reasonable to assume that these conformers undergo a slow interchange in solution.

The peak at *viii* seems to be connected to the peak at *vii*. Because of the chemical shifts of peaks *viii* and *vii*, one may make a preliminary conclusion that the two cross peaks are connected to the olefinic portion of ring E. An examination of the spectrum, however, will clearly indicate that some of the proton couplings of that connectivity are still missing. The proton spectra of brevetoxin A at various low temperatures shows that it exists in more than one conformation in several portions of the structure that are slowly interconverting. It has been shown that the molecule in the solid state consists of two perpendicularly linked polycyclic sheets linked at ring G. Rings A-F and H-J make up the two sheets. In solution, ring G slowly interconverts from the boat-chair form to the crown form. The appearance of the low temperature ^1H NMR spectra clearly lends support to this speculation. Molecular mechanics calculations have indicated that the boat-chair form is the most stable of the two conformations, but only by ~2.9 Kcal/mol. Thus one should expect relatively easy conversion between the two forms, and as a result it would be very difficult to see very sharply defined resonances of certain protons, especially close around ring G which is directly involved in the conformational change. Despite these factors, some assignments of the resonances around rings E and G have been made. Our NMR experiments indicate that some resonances around these two rings are still missing and it is our contention that more detailed NMR experiments are required before the assignments can be made with any degree of certainity.

In addition to brevetoxins, *G. breve* also produces several smaller compounds, which cause characteristic rounding of the mouse neuroblastoma cells, similar to brevetoxins, as well as showing cytotoxicity at 5 μmoles level[14]. These compounds have been recently re-named as hemibrevetoxins. The structure (Figure 6) of the major hemibrevetoxin, hemibrevetoxin B was established on the basis of NMR studies (including COSY, NOESY and LRCOSY) and was found to be essentially the right half of the brevetoxin A molecule[15]. Like brevetoxins, hemibrevetoxin must be biosynthesized by a cyclization cascade initiated from the right hand of the chain by the opening of the cis-epoxide, followed by a hydride ion transfer and consecutive trans-epoxide openings. The presence of alkene tails in hemibrevetoxins not only affirms the polyene origin of brevetoxins, but also shows its relationship with ciguatoxins, which have been shown to have a

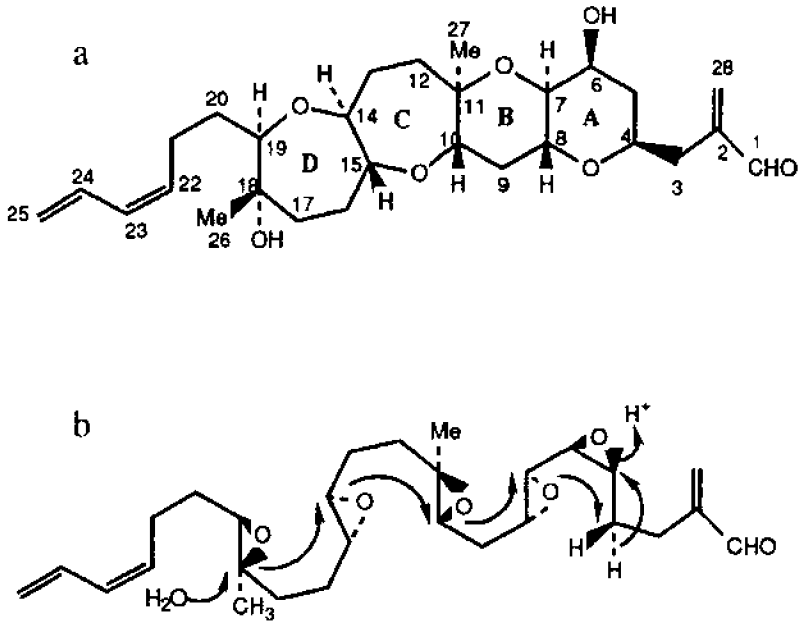


Figure 6. Hemebrevetoxins: Structure and Biosynthesis.
a) Hemebrevetoxin B.
b) Cyclization cascade involved in the biosynthesis of hemebrevetoxin B.

diene or diol side chain. Work on the structures of brevetoxin A and C is in progress.

Considerable progress has been made in the understanding of the biosynthesis of brevetoxins. Results of the feeding experiments, utilizing 1-^{13}C, 2-^{13}C and 1,2-^{13}C acetates, have shown that the biosynthesis of brevetoxins does not follow the ordinary polyketide pathway known in fungi and bacteria [16,17,18]. In an earlier paper, we proposed a new biosynthetic mechanism which involved the condensation of dicarboxylic acids such as succinate, hydroxylmethylglutarate, and α-ketoglutarate[12]. Later, Lee *et al.* concurred with our hypothesis, however, differed in certain proposed precursors[19]. Our recent analysis of various feeding studies seems to implicate a deep involvement of amino acid metabolism in the formation of the precursors of the brevetoxins (Figure 7).

Acknowledgements

The work written in this paper was supported by NIH grants GM 28754 and GM 24425, which is greatly appreciated.

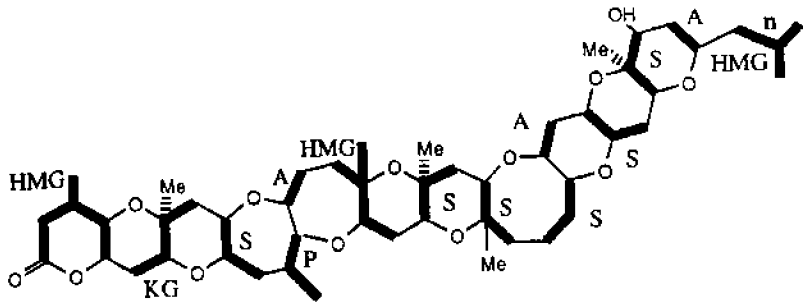


Figure 7. Biosynthesis of Brevetoxin A.

Literature Cited

1. Steindinger, K.A. and D.G. Baden. 1984. Toxic Marine Dinoflagellates. In: Dinoflagellates (D.L. Spector, ed.). Academic Press, New York. pp. 201-261.
2. Murata, M., A. M. Legrand, Y. Ishibashi and T. Yasumoto. 1989. Structures of ciguatoxin and its congener. J. Am. Chem. Soc. 111: 8929 - 8931.
3. Alam, M. 1975. Marine biotoxins. Texas Rep. Biol. Med. 33: 183 - 199.
4. Shimizu, Y., M. Alam and W. E. Fallon. 1974. Red Tide Toxins. In: Food and Drugs from the Sea Proceedings (H. H. Weber and G. D. Ruggieri, eds.). Marine Technology Society, Washington, D. C. pp. 238 -251
5. Shimizu, Y. 1978. Dinoflagellate Toxins. In: Marine Natural Products, Chemical and Biochemical Perpectives. Vol 1. (P. J. Scheuer, ed.). Academic Press, New York. pp. 1-42.
6. Baden, D. G., T. J. Mende and R. E. Block. 1979. Two Similar Toxins Isolated from *Gymnodinium breve*. In: Toxic Dinoflagellate Blooms. (D. L. Taylor and H. Seliger, eds.). Elsevier, New York. pp. 327 - 334.
7. Risk, M., Y. Y. Lin, R. D. McFarlane, M. V. S. Ramnujam, L. L. Smith and N. M. Trieff. 1979. Purification and Chemical Studies on a Major Toxin from *Gymnodinium breve*. In: Toxic Dinoflagellate Blooms (D. L. Taylor and H. H. Seliger, ed.). Elsevier, New York. pp. 335 - 344.
8. Lin, Y. Y., M. Risk, S. M. Ray, D. Van Engen, J. Clardy, J. Golik, J. C. James and K. Nakanishi. 1981. Isolation and structure of brevetoxin B from the "Red Tide" dinoflagellate *Ptychodiscus brevis* (*Gymnodinium breve*). J. Am. Chem. Soc. 103: 6773 - 6775.

9. Tempesta, M., J. Golik, J. C. James, K. Nakanishi, J. Pawlak, T. Iwashita, M. L. Gross and K. B. Tomer. 1984. The structure elucidation of brevetoxin A (*Gymnodinium breve*). The International Chemical Congress of Pacific Basin Societies. Honolulu, HI. Abs. 10E45.
10. Nakanishi, K. 1985. The chemistry of brevetoxins: A review. Toxicon 23: 473 - 479.
11. Pawlak, J., M. S. Tempesta, J. Golik, M. G. Zagroski, M. S. Lee, K. Nakanishi, T. Iwashita, M. L. Gross and K. B. Tomer. 1987. Structure of brevetoxin A as constructed from NMR and MS data. J. Am. Chem. Soc. 109: 1144 - 1150.
12. Shimizu, Y., H-N. Chou, H. Bando, G. Van Duyne and J. C. Clardy. 1986. Structure of brevetoxin A (GB-1), the most potent toxin in the Florida red tide organism *Gymnodinium breve* (*Ptychodiscus brevis*). J. Am. Chem. Soc. 108: 514 - 515
13. Van Duyne, G. D. 1990. X-Ray Crystallographic Studies of Marine Toxins. In: Marine Toxins (S. Hall and G. Strichartz, eds.). American Chemical Society, Washington, D. C. pp. 144-163.
14. Shimizu, Y. 1982. Recent progress in marine toxin research. Pure & Appl. Chem. 54: 1973 - 1980.
15. Prasad, A. V. K. and Y. Shimizu. 1989. The structure of hemibrevetoxin-B: A new type of toxin in the Gulf of Mexico red tide organism. J. Am. Chem. Soc. 111: 6476 - 6477.
16. Lee, M. S., D. S. Repeta, K. Nakanishi and M. G. Zagroski. 1986. Biosynthetic origin and assignments of ^{13}C NMR peaks of brevetoxin B. J. Am. Chem. Soc. 108: 7855 - 7856.
17. Chou, H. N. and Y. Shimizu. 1987. Biosynthesis of brevetoxins. Evidence of the mixed origin of the backbone carbon chain and the possible involvement of dicarboxylic acids. J. Am. Chem. Soc. 109: 2184 - 2185.
18. Shimizu, Y., S. Gupta and A. V. K. Prasad. 1990. Biosynthesis of Dinoflagellate Toxins. In: Toxic Marine Phytoplankton (E. Graneli, B. G. Sundstrom, L. Elder and D. M. Anderson, eds.). Elsevier, New York. pp. 62 - 73.
19. Lee, M. S., G-W. Qin, K. Nakanishi and M. G. Zagroski. 1989. Biosynthetic studies of brevetoxins, potent neurotoxins produced by the dinoflagellate *Gymnodinium breve*. J. Am. Chem Soc. 111: 6234 - 6241

Pharmacology and Toxicology

Effects of Toxins on the Voltage-Gated Na Channel

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Abstract

The voltage-gated sodium channel is a transmembrane glycoprotein which presents a variety of binding sites for a large number of neurotoxins which modify its normal behavior. In this introductory paper I wish to review the effects of various toxins, including ciguatera fish toxin, which seems to act at a site on sodium channels unique to this class of polyethers.

Introduction

Reports of fish poisoning by coastal marine fish resembling ciguatera fish poisoning date back to the early 15th century [1]. However, despite the long history of ciguatera, we are just beginning to learn about the chemical characteristics and structure of the ciguatera toxins. It is known, however, that there are at least three toxic components in ciguatera: the principal toxin, called ciguatoxin, whose structure has just become available; maitotoxin, which is more polar than ciguatoxin and has a molecular weight of around 3300, with no apparent chemical similarities between maitotoxin and ciguatoxin; and third, okadaic acid, a polyether fatty acid derivative which has been isolated from *Prorocentrum lima*, a dinoflagellate included in the benthic ciguatera community [2-4].

During the past 5 years or so it has been shown that some of the toxic components of the ciguatoxins act as neurotoxins on the Na^+ channel, whereas others act on Ca^{2+} channels. This property will help as an assay in the further physico-chemical characterization of the toxins, while at the same time making these toxins potential tools in the study of electrically propagated impulses (Na^+ channels) as well as in the study of signal transduction (Ca^{2+} channels). As an introduction to the pharmacology of ciguatoxin, which will be discussed in the papers that follow, I will review briefly the current knowledge of the structure of the voltage-gated Na^+ ion channel. I will then show how toxins modify these properties, trying to indicate, whenever possible the potential sites in the molecule where particular toxins might act. For more in-depth reviews on each of these subjects the reader is referred to the book by B. Hille as well as the reviews by C. Armstrong, W. Catterall and G. Strichartz[5-8].

General Aspects of Channels

Excitation and electrical signaling in the nervous system involve the movement of ions through ionic channels which are specifically responsive to some stimulus: membrane potential change, neurotransmitter or other chemical stimulus, mechanical deformation, etc. The proteins which underlie these responses might be thought of as enzymes, in that they catalyze the movement of ions across otherwise impermeable membranes[9]. These polypeptides have been shown to have specific binding sites for molecules such as the marine toxin tetrodotoxin, which modify their properties. The use of this specific binding greatly facilitated the isolation, purification and characterization of the voltage-activated Na^+ channel.

Sodium channels are large, 200-300 kilodalton (kD) glycopeptides which are anchored in the membrane by hydrophobic, electrostatic and covalent bonding. The subunit structure of the Na^+ channel was found to vary according to the tissue from which it had been isolated. Thus, the Na^+ channel isolated from mammalian brain was found to be a complex of three subunits: the α subunit 260kD, $ß_1$ (36 kD) and $ß_2$ (33 kD). In skeletal muscle, the $ß_2$ subunit is absent and in the channel purified from electric eel only the α-subunit was found[7]. These results imply that the large α subunit is the main functional component of the sodium channel.

The primary structure of these channels was deduced by cloning and sequencing the DNA complementary to its mRNA[11]. It was first noted by Noda that there are four homologous domains in the electroplax sodium channel, with six regions of probable α–helical structure long enough to be membrane-spanning segments, designated S1 through S6[11]. S1-S3 and S5-S6 are mainly hydrophobic, whereas S4 is amphipathic, highly charged and thought to be involved in voltage-gating. It has been found recently that a synthetic peptide with the sequence of S4 incorporates spontaneously into lipid bilayers and promotes the formation of voltage-gated, cationic channels[12].

Structure and Function of Sodium Channels

When the protein (channel) is stimulated, there is a structural rearrangement, the details of which currently escape us, which leads to the flow of ions down their electrochemical potential gradient. This response of the channel is called gating, an opening or closing of the pore. When the pore is open, it allows passive flow of a restricted class of ions down their electrochemical potential gradient. These ionic currents can also produce the opening of other voltage-sensitive channels in adjacent membranes, such as the voltage-sensitive Ca^{2+} channels in the sarcoplasmic reticulum, an event which produces Ca^{2+} release in the sarcomere of muscle cells.

Figure 1 shows the electrical current which flows through Na^+ channels when the membrane potential is clamped at different values. The traces show the salient features of the voltage-gated Na^+ channel: the activation of the current to a peak followed by a decrease in the current (at a fixed voltage), or inactivation. The simplest kinetic scheme which reflects these features calls for three conformations or states of the Na^+ (Ca^{2+}) channel: a nonconducting, resting state, a conducting, open state and a different non-conducting, inactivated state. The true channel kinetics are in all probabilities more complicated. In fact, in order to fit the data, it is necessary to postulate the existence of intermediate nonconducting states between the resting and the

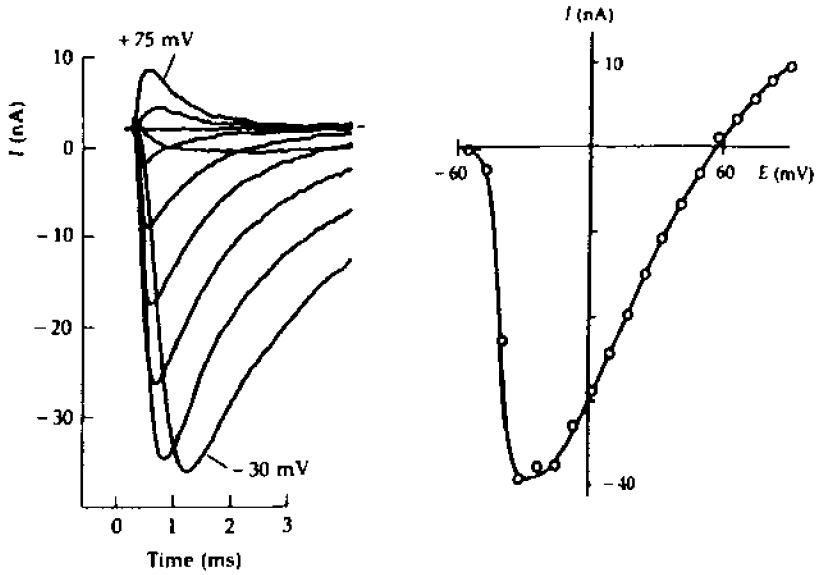

Figure 1. The Voltage-Gated Na Channel.
The electrical current flow (left panel) and conductance (right panel) of Na channels at different (fixed) membrane potentials.

open states. In addition more than one conducting state and multiple pathways to the inactivated state have been proposed[13]. From Figure 1, it is possible to see that the current-voltage relation for the peak Na^+ current indicates that the channels are largely closed at rest (negative potentials) and that they open upon depolarization (cf. right panel).

With the development of the patch clamp technique, it has been possible to expand our knowledge of the workings of the channel protein through measurements of the properties of one single channel (out of 15-2000 channels μm^2) rather than those of many individual channels at one point in time. This, in turn, has resulted in a more in-depth knowledge of the function of the Na^+

channel and of the mode of action of the antagonists of the protein, such as the neurotoxins.

In the past few years there has been a rapid advance in the elucidation of the mechanism of action of naturally occurring neurotoxins. The quickening pace has been driven by an interest in the toxic molecules produced by marine organisms and has been aided by the application of highly sophisticated biochemical and pharmacological techniques. These studies have led to an increased understanding of the variable states of the sodium channel as it goes through the modifications which eventually lead to the action potential. Furthermore, it has been possible to determine the heterogeneity of channel structure through differential effects of toxins and, finally, it has been possible to establish the interaction between the different toxin binding sites, which has helped in the determination of the role that the different parts of the Na^+ channel protein play in channel activation.

Toxins and Their Actions

In the light of these advances, I wish to outline the mode of action of toxins which are known to act at various states of the Na^+ channel, with particular emphasis on those states which interact with the toxins which we are concerned with in this Conference. Table 1 shows a list of the toxins I will consider. The first group listed is composed of those toxins which block the sodium channel of many excitable membranes with high-affinity ($K_D \sim 10^{-9}$M)

Table 1. Na Channel Inhibitors: Binding Site and Action.

Binding Site	Ligand	Action
External	organic cations: TTX STX Peptides: μ-conotoxins	inhibition of Na transport, reversible binding
Within Channel	lipid soluble: BTX veratridine	persistent activation, irreversible binding, TTX sensitive
External	polypeptide toxins: sea anemone toxin α-scorpion toxin	inhibit activation, reversible binding
External	β-scorpion toxins	modify activation

and selectivity. Tetrodotoxin (TTX) was first used as a label to count the number of Na^+ channels on membranes and later to follow the progress of the channel protein during purification[14]. The mechanism of action of TTX and saxitoxin (STX) involves a guanidinium ion present in both compounds. This group blocks the open channel, and this blockage is antagonized by Na^+, Ca^{2+} and H^+ ions. These toxins act only on the side of the channel protein accessible from the external surface of the cell membrane[15-18]. Thus, the studies with TTX, STX and the μ-conotoxins has shown that Na^+ channels differ in structure between their internal and external surfaces, that they have binding sites for mono and divalent cations and that their functioning can be ascribed to conformational changes within the molecule. The characteristic of the next group of toxins, the alkaloids, is that they can depolarize the membranes of nerve and muscle cells and increase the resting Na^+ permeability of excitable cells[19]. Batrachotoxin (BTX) is the most studied compound in this class. The binding of this class of toxins leads to the stabilization of the channels in the open state without affecting the activation process. Studies with these toxins reveal that their effect, which is elicited when they are added either to the inside or to the outside of the membrane, is to increase the population of open channels over a broad range of membrane potentials. This results in slow depolarization of the membrane leading to a transient period of rapid, spontaneous impulse firing. Binding to closed channels is a slow process, which has been interpreted to indicate that the toxins bind to a site in a hydrophobic environment[20]. Although the properties of the alkaloid-modified Na^+ channels are qualitatively the same, differences between the various toxins are apparent, indicating that they may not alter the same region in the channel molecule [21,22].

The next group of toxins, act only when added to the outside of cells. These toxins prolong the duration of the action potential by slowing down inactivation. The binding of α-toxin to the channel seems to be voltage dependent and the dissociation of the toxin from its binding site is relatively fast at large potentials[23,24].

The polypeptide toxins which act when added to the outside of the cell (groups 3 and 4 in Table I) are divided in two classes due to the difference in their electrophysiological actions. The ones in group 3, sea anemone toxin and the α-scorpion toxins slow down the inactivation of Na^+ channels in a great variety of excitable cells[24,25]. The interaction of B-toxins with Na^+ channels results in a more rapid activation and a slowing down of both deactivation and inactivation. The end result is a set of conducting channels that close slowly at rest and show repetitive impulse firing in response to minimal stimulation[26,27].

A group of lipid soluble toxins not listed in the table but which have been shown to depolarize nerve and muscle membrane in a dose-dependent, TTX-antagonized manner are ciguatoxin (CTX) and the brevetoxins (PbTX) which seem to be part of a separate group of toxins. The depolarization of nerve

terminals produced by these lipophilic toxins causes massive release of transmitter, which accounts for their actions on a variety of organ systems[28,29]. The action of these toxins is just beginning to be explored in detail, as the availability of purified material increases. When PbTX or ciguatoxin are applied to muscle or to nerve, the peak sodium current is reduced, but the sodium current can be activated at potentials when the channel is normally closed[30,31]. It has also been found that; a) activation is 1000x slower, b) sodium channel inactivation is inhibited and c) of the two toxins, ciguatoxin is about 50 times more potent than brevetoxin. Studies on the binding competition with other toxins known to act on the Na^+ channel protein have revealed that both toxins enhance the binding PbTx to its own receptor site and that both can displace the binding of labelled brevetoxin to its receptor. Other competition experiments of this sort have shown that neither CTX nor PbTx can displace toxins which bind to sites 1-4 (see Table 1) in the Na^+ channel protein[32,33]. Thus these experiments suggest that PbTx and CTX share a 5th receptor site on the Na^+ channel protein, and that it is possible that this receptor site is located on a region of the Na^+ channel involved in voltage-dependent gating.

In conclusion, toxins alter the state of the Na^+ channel in several ways. Different toxins interact with one another, further indicating that the structural changes which underlie the workings of the Na^+ channel protein move through vast domains of the protein during normal physiological gating. Clearly more work remains to be done both structurally and physiologically in order to understand the mode of action of ciguatoxin and brevetoxins.

Literature Cited

1. Anderson, D.M. and P.S.Lobel. 1987. The continuing enigma of ciguatera. Biol. Bull. 172: 89-107.
2. Murata, M., A.M. Legrand, Y. Ishibashi and T. Yasumoto. 1989. Structure of ciguatoxin and its congener. J. Am. Chem. Soc. 111: 8929-8931.
3. Yasumoto, T. 1978. Large scale toxin production by *Diolopsalis sp.*-biochemical study of maitotoxin and ciguatoxin. So. Pac. Comm. Rep. 785: 7-8.
4. Murakami, Y., Y. Oshima and T. Yasumoto. 1982. Identification of okadaic acid as a toxic component of a marine dinoflagellate *Prorocentrum lima*. Bull. Jpn. Soc. Sci. Fish. 48: 69-72.
5. Hille, B. 1984. Ionic Channels of Excitable Membranes. Sinauer Associates Inc., Sunderland, MA.
6. Armstrong, C.M. 1981. Sodium channels and gating currents. Physiol. Rev. 61: 644-683.

7. Catterall, W.A. 1986. Molecular properties of voltage-sensitive sodium channels. Ann.Rev.Biochem. 55: 953-985.
8. Strichartz, G. 1989. The molecular pharmacology of toxins that modify voltage-gated sodium channels. In: Natural Toxins. Characterization pharmacology and therapeutics, (C.L.Ownby and G.V.Odell eds). Pergamon Press, New York. pp. 43-53.
9. Moczydlowski, E. 1986. Single channel enzymology. In: Ion channel reconstitution, (C.Miller, ed). Plenum Press, New York and London. pp. 75-113.
10. Catterall, W.A., J.W. Schmidt, D.J. Messner and D.J. Feller. 1986. Structure and biosynthesis of neuronal sodium channels. Ann. NY. Acad. Sci. 479:186-203.
11. Noda, M., S. Shimizu, T. Tanabe, T. Takai, T. Kayano, T. Ikeda, H. Takahashi, H. Nakayama, Y. Kanaoka, N. Minamino, K. Kangawa, H. Matsuo, M.A. Raftery, T. Hirose, S. Inayama, H. Hayashida, T. Miyata and S. Numa. 1984. Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. Nature 312: 121-127.
12. Tosteson, M.T., D.S.Auld and D.C.Tosteson. 1989. Voltage gated channels formed in lipid bilayers by a positively charged segment of the Na-channel polypeptide. Proc. Natl. Acad. Sci. 86: 707-710.
13. Armstrong, C.M. and Wm.F. Gilly. 1979. Fast and slow steps in the activation of sodium channels. J. Gen. Physiol. 74: 691-711.
14. Haffemann, D.R. 1972. Binding of radioactive tetrodotoxin to nerve membrane preparations. Biochim. Biophys. Acta. 266: 548-556.
15. Narahashi, T., J.W. Moore and W.R. Scott. 1964. TTX blockage of sodium conductance increase in lobster giant axons. J. Gen. Physiol. 47: 964-974.
16. Henderson, R., J.M. Ritchie and G.R. Strichartz. 1974. Evidence that TTX and STX act at a metal cation binding site in the sodium channels of nerve membranes. Proc. Natl. Acad. Sci. 71: 3936-3940.
17. Kao, C.Y. 1986. Structure-activity relations of TTX, STX and analogues. Ann. NY. Acad. Sci. 479: 52-67.
18. Weiss, R.E. and R. Horn. 1986. Single-channel studies of TTX-sensitive and TTX-resistant sodium channels in developing rat muscle reveal different open channel properties. Ann. NY. Acad. Sci. 479: 152-161.
19. Albuquerque, E.X. and J.W. Daly. 1976. Batrachotoxin, a selective probe for channels modulating sodium conductances in electrogenic membranes. In: The specificity and action of animal, bacterial and plant toxins, (P.Cuatrecasas, ed). Chapman and Hall, London. pp. 297-338.
20. Angelides, D., S. Rerakawa and G.B. Brown. 1986. Spatial relations of the neurotoxin binding sites on the sodium channel. Ann. NY. Acad. Sci. 479: 221-237.
21. Khodorov, B.I. and S.V. Revenko. 1979. Further analyses of the mechanism of action of batrachotoxin on the membrane of myelinated nerve.

Neuroscience 4: 1315-1330.
22. Leibowitz, M.D., J.B. Sutro and B. Hille. 1986. Voltage-dependent gating in veratridine-modified Na^+ channels. J. Gen. Physiol. 87: 25-46.
23. Wang, G.K. and G. Strichartz. 1982. Simultaneous modifications of sodium channel gating by two scorpion toxins. Biophys. J. 40: 175-179.
24. Catterall, W.A. 1977. Membrane potential-dependent binding of scorpion toxin to the action potential Na^+ ionophore. Studies with a toxin derivative prepared by lactoperoxidase-catalyzed iodination. J. Biol. Chem. 252: 8660-8668.
25. Frelin, C, P. Vigne, H. Schweitz and M. Lazdunski. 1984. The interaction of sea anemone and scorpion neurotoxins with tetrodotoxin-resistant Na channels in rat myoblasts. Molec. Pharmacol. 26: 70-74.
26. Wang, G.K. and G.R. Strichartz. 1985. The actions of an isolated B-toxin on Na channel activation. Biophys. J. 47: 438a.
27. Wheeler, K.P., D.D. Watt and M. Lazdunski. 1983. Classification of Na channel receptors specific for various scorpion toxins. Pflügers. Arch. 397: 164-165.
28. Gallagher, J.P. and P.Shinnick-Gallagher. 1985. Effects of crude brevetoxin on membrane potential and spontaneous or evoked end-plate potentials in rat hemidiaphragm. Toxicon 23: 489- 496.
29. Ohizumi, Y. 1987. Pharmacological actions of the marine toxins ciguatoxin and maitotoxin isolated from poisonous fish. Biol. Bull. 172: 132-136
30. Huang, J.M.C., C.H. Wu and D.G. Baden. 1984. Depolarizing action of a red-tide dinoflagellate brevetoxin on axonal membranes. J. Pharmacol. Exp. Therap. 229: 615-621.
31. Benoit, E., A.M. Legrand and J.M. Dubois. 1986. Effects of ciguatoxin on current and voltage clamped frog myelinated nerve fibre. Toxicon 24: 357-364.
32. Poli, M.A., T.J. Mende and D.G. Baden. 1986. Brevetoxins, unique activators of voltage- sensitive sodium channels, bind to specific sites in rat brain synaptosomes. J. Am. Soc. Pharmacol. Exptl. Ther. 38: 129-135.
33. Lombet, A., J-N. Bidard and M. Lazdunski. 1987. Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na channel. FEBS Lett. 219: 355-359.

Pathomorphological Studies on Experimental Maitotoxicosis and Ciguatoxicosis in Mice

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Abstract

Pathomorphological findings induced by maitotoxin (MTX) in mice were compared with those of ciguatoxin (CTX) poisoning. The target organs of MTX were the heart, digestive tract, cortex of adrenal glands and lymphoid tissues including the thymus, spleen, lymph nodes and Peyer's patches. MTX first stimulated calcium influx into the zona fasciculata of the adrenal glands, which then caused the release of cortisol into the blood. The excess amount of cortisol in the blood produced acute involution of the thymus and other lymphoid tissues.

The most fatal morphological changes after the administration of CTX occurred in the heart muscle. Either intra peritoneal (i. p.) (0.7 μg/kg) or oral administration (0.7 μg/kg) resulted in marked swelling and focal necrosis of cardiac muscle cells and effusion in the interstitial space of the heart. The injuries to the myocardium caused acute heart failure. Even after 5 months, intermuscular fibrosis of the injured heart persisted. Although severe diarrhea was brought about by CTX, no morphological alterations were seen in the mucosa of the small intestine. In contrast, unmyelinated nerves and synapses in the smooth muscle layer of the intestine and vas deferens were degenerated after the administration of CTX. About 15% of the mice given CTX at a dose of 0.7 μg/kg showed a continuous penile erection.

Introduction

A number of investigators have isolated several causative agents of ciguatera from the viscera of toxic coral reef fishes or from dinoflagellates in culture. Among them, MTX and CTX are the most potent marine toxins known. The biological activities of MTX have been extensively studied by many investigators, and it is now widely accepted as being a potent activator of Ca^{2+} channels in various cell types[1-11]. It has been reported that MTX results in the Ca^{2+} dependent release of certain hormones from cultured cell lines, as well as the Ca^{2+} dependent contraction of either skeletal, cardiac or

smooth muscle cells. The mode of action of CTX has also been studied by a number of researchers, and it has been confirmed that CTX causes an increased Na^+ permeability across the tetrodotoxin-sensitive Na^+ channels of smooth and/or cardiac muscle cells[12-16]. In contrast to those pharmacological studies, there has been only limited information about the *in vivo* pathological changes induced by either MTX or CTX. In the present study we demonstrate the pathomorphological changes induced by MTX or those of CTX and compare the differences between the two experimental poisonings.

Materials and Methods

The MTX used in the present studies was extracted from a cultured dinoflagellate, *Gambierdiscus toxicus*, and the CTX was isolated mainly from reef snappers *Lutjanus bohar* collected in Micronesia and Okinawa, Japan. Both phycotoxins were purified as previously described[17,18]. For the experimental animals, 4-week old male ICR mice weighing 20 to 23 g were obtained from Charles River Japan, Inc. Specimens for morphological examinations were prepared as previously described[11]. For electron microscopy, specimens were observed with a Hitachi H 700 H transmission electron microscope.

Results

The Effects of MTX and CTX on the Heart

One of the target organs of both MTX and CTX is the heart. Figure 1 shows the ultrastructure of the heart of a mouse 30 min after receiving 200 ng/kg of MTX. A marked swelling of the endothelial cells lining the capillaries in the cardiac tissue was often seen. The lumen of the capillaries was extremely narrow or even closed. Cardiac muscle cells around the capillaries became degenerated and necrotic. The cells were condensed and the arrangement of almost all of the mitochondrial cristae was irregular.

Various doses of CTX resulted in multiple single cell necroses in the myocardium of the left and right ventricles and the septum of the heart (Figure 2a). Marked swelling of the cardiac muscle cells was seen with the electron microscope (Figure 2b). The mitochondria became rounded and most organelles including myofibrils, sarcoplasmic reticulum and T-systems were separated from each other. Myofibrils in several cells had partially disappeared. Occasionally, an accumulation of blood platelets was seen in the capillaries among the cardiac muscle cells. Marked effusion of a serum-like substance was found around the capillaries and interstitial space of the heart. Usually a severe dyspnoea and lung edema then followed. These changes in the surviving mice persisted for over 3 days.

Almost all mice given over 1 μg/kg CTX died within several hours after the injection. About 1 month later, the surviving mice initially given 0.7 μg/kg

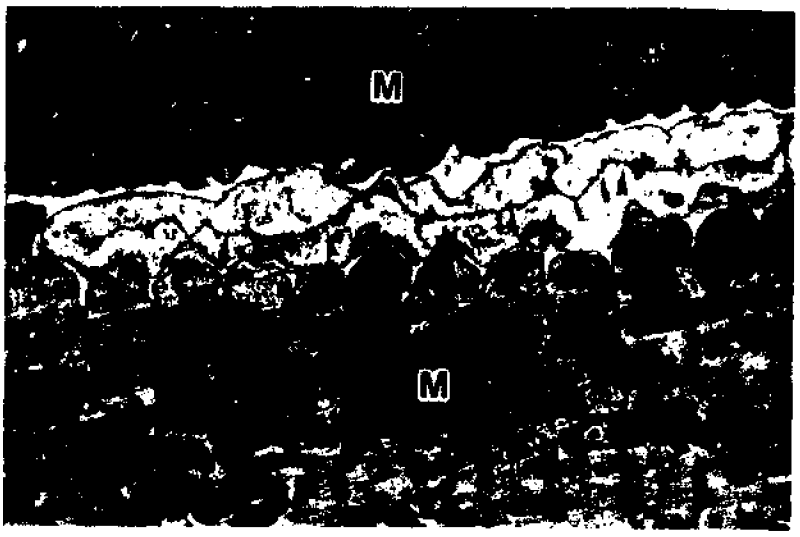

Figure 1. Effect of MTX on Mouse Myocardium.
Electron micrograph of the myocardium of a mouse given 200 ng/kg of MTX 30 minutes before sacrifice. Note marked narrowing of lumen of the capillary (arrow heads). (M) myocardium. ×5,100

seemed to be completely well clinically. At autopsy, the hearts of these animals were enlarged. The degenerated microorganelles as seen with the electron microscope had regenerated completely. However, the tubular invagination of the sarcolemma and sarcoplasmic reticulum were unusually dilated and the effusion was replaced by thick bundles of collagen fibers. Fibrosis of the heart was seen even 5 months after a single injection of 0.5 μg/kg CTX (Figure 2c). With a single dose of 0.3 μg/kg or less, no visible changes were detected in the cardiac tissue. However, 15 repeated injections of CTX 0.1 μg/kg resulted in marked dilation of the right and left ventricles and also showed severe histopathological changes similar to a single dose of 0.7 μg/kg (Figure 2d).

Many antagonistic drugs for cholinergic and adrenergic autonerves such as atropine, guanethidine and 5-hydroxy dopamine had no effect on the severity of CTX induced lesions in the heart, whereas reserpine even aggravated the pathological findings. It is of interest that the cardiac injuries induced by CTX were not altered by pretreatment with atropine, although this agent prevented the CTX induced diarrhea completely.

From these findings it may be assumed that CTX attacks cardiac muscle cells directly, while MTX preferentially attacks endothelial lining cells of the capillaries in the cardiac tissue.

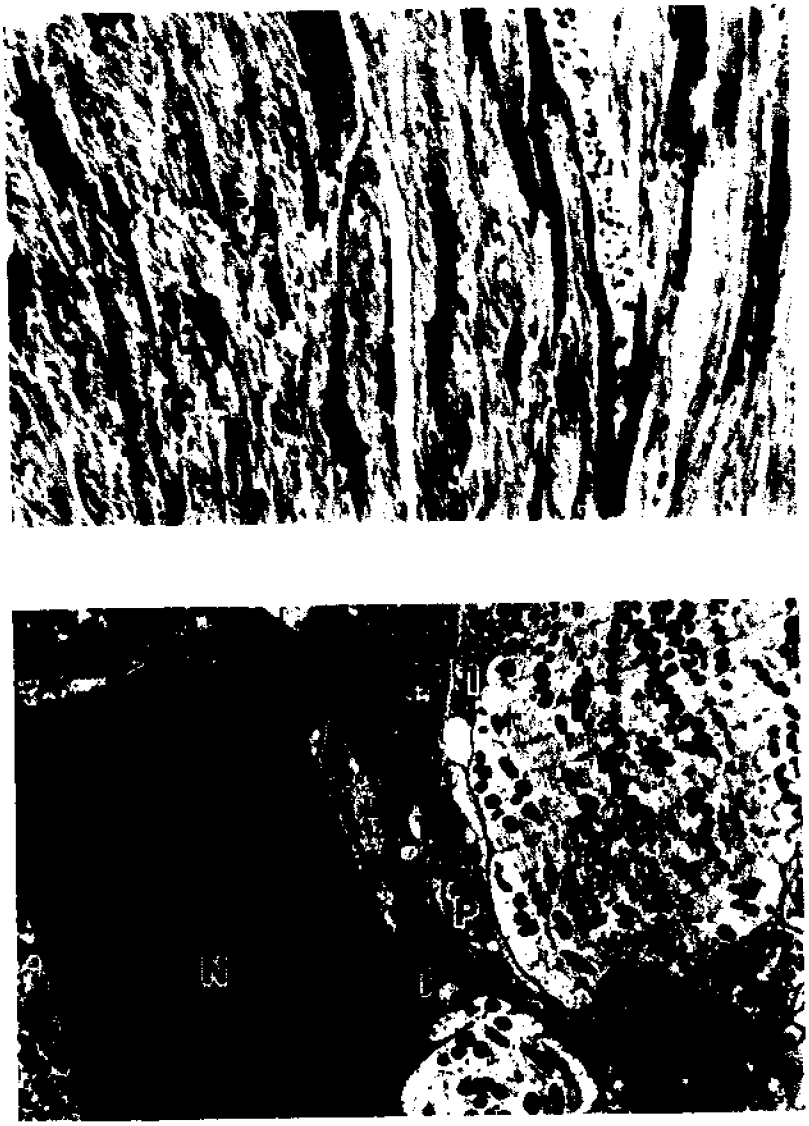

Figure 2. Effect of CTX on Mouse Cardiac Tissue.
Micrographs of the cardiac tissue of a mouse given 0.7 μg/kg of CTX. *Upper panel* (2a) Light micrograph of the left ventricle. There are many single cell necroses. × 60 *Lower Panel* (2b) Electron micrograph of the cardiac cells of a mouse 50 min. after the injection. Swelling of a cardiac cell is prominent. An accumulation of blood platelets (P) is seen in the capillary. Around the capillary, effusion is present in the interstitial space (I). (N) Necrotizing cell. × 2,600

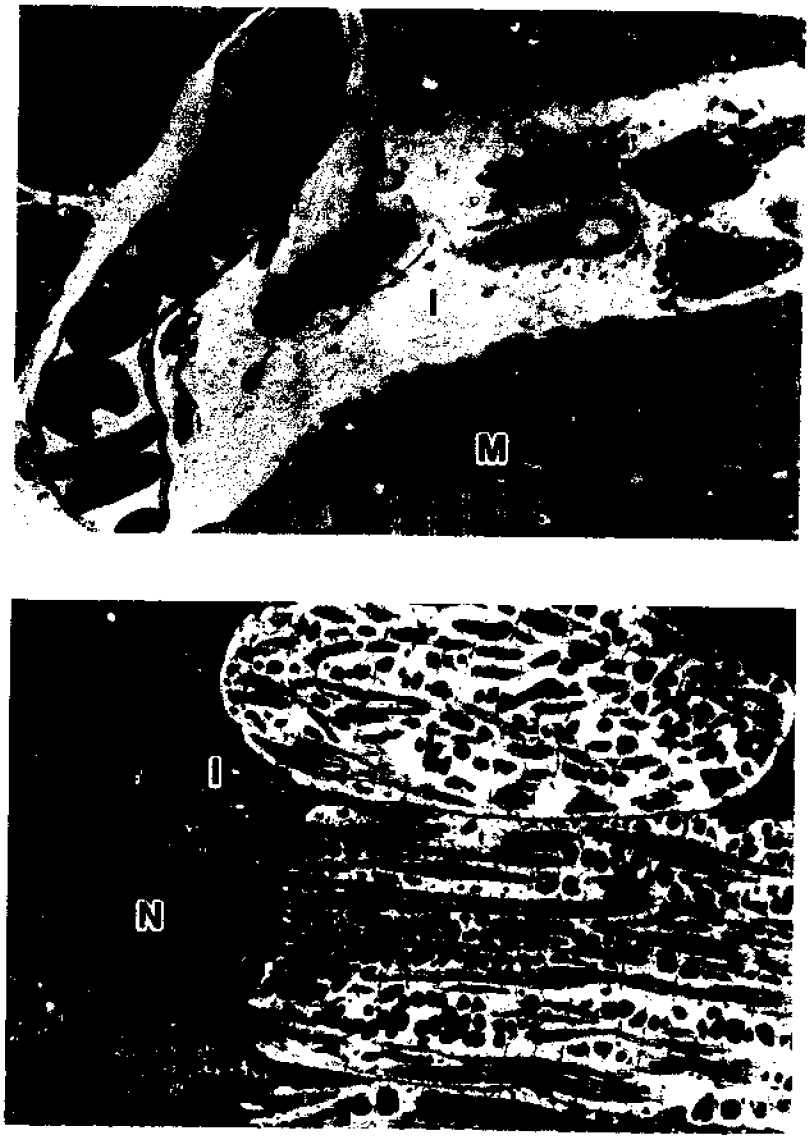

Figure 2. Effects of CTX on Mouse Cardiac Tissue.
Micrographs of the cardiac tissue of a mouse given 0.7 μg/kg of CTX. *Upper Panel* (2c) Electron micrograph of the cardiac cells about 4 weeks after a single injection of CTX. Around the capillary there are bundles of collagen fibers (I) with many macrophages. (M) Cardiac muscle. ×2,600 *Lower panel* (2d) Electron micrograph of the heart of a mouse given 15 repeated injections of 0.1 μg/kg of CTX. Almost all muscle cells are swollen. Intermuscular spaces (I) are impregnated by a dense serum-like substance. (N) necrotized cardiac cell. × 2,600

The Effects of MTX and CTX on Digestive Organs

Diarrhea always occurred shortly after the administration of CTX but was not encountered with MTX. Interestingly, the pathomorphological findings showed no discernible changes in the mucosa of the intestine in CTX treated animals. Only swelling of the unmyelinated nerves and synapses in the smooth muscle of the intestine was observed (Figures 3a and b). In contrast to the CTX poisoning, maitotoxicosis was always accompanied by multiple erosions and ulcers in the stomach. No abnormal changes were seen in the unmyelinated nerves of the gastrointestinal organs after the administration of MTX.

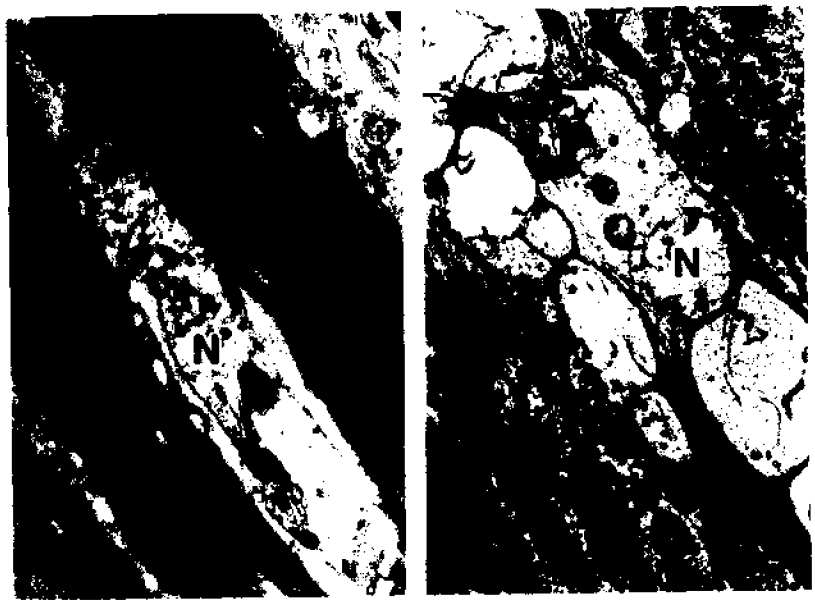

Figure 3. Effect of CTX on Mouse Intestine.
Electron micrographs of nerve fibers (N) in Auerbach's plexus of the intestine. *Left panel* (3a) Control × 14,000. *Right panel* (3b) Six hours after the administration of 0.7 μg/kg of CTX. Nerve fibers and synapses are markedly swollen. Almost all synaptic vesicles disappeared. × 14,000

The Effects of MTX and CTX on Adrenal Glands

Both MTX and CTX injured the adrenal glands. However, the target of MTX was the zona fasciculata of the adrenal cortex and that of CTX were cells in the adrenal medulla. Histological examinations revealed the presence of many vacuoles in the adrenocortex of the MTX treated animals. Ultrastructurally, these vacuoles were identified as autophagosomes containing remnants of microorganelles (Figure 4a).

It is well known that MTX is a potent activator of calcium channels in

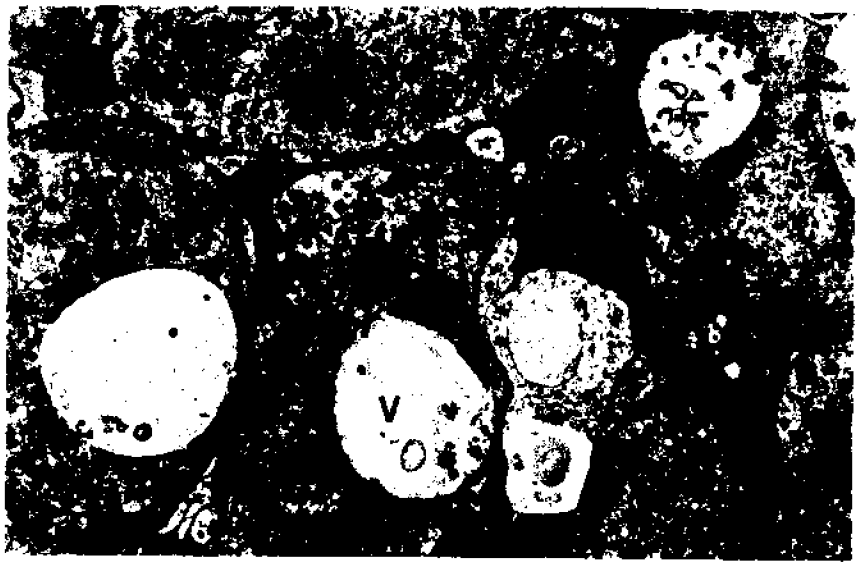

Figure 4. Effect of MTX on Mouse Adrenal Glands.
(4a) Electron micrographs of the adrenal glands of a mouse given 400 ng/kg of MTX. Cells in zona fasciculata 24 hr after injection. Numerous autophagosomes (V) are seen in the cells. ×2600.

various cell types, and it has been reported that this toxin results in Ca^{2+}-dependent release of certain hormones. Therefore, we determined the total calcium content of the adrenal glands and compared that of the thymus and spleen of MTX-injected mice pretreated with $CoCl_2$ (Table 1). Homogenates of these organs were extracted with 0.2N perchloric acid and analyzed with the Zeeman atomic spectrophotometer. In mice injected with MTX only, the total calcium of adrenal glands was increased significantly when compared with

Table 1. Effect of MTX on Organ Calcium

Treatment	Thymus	Spleen	Adrenal Glands
Control	1.71±0.20	0.95±0.09	2.18±0.09
MTX	1.33±0.25	1.00±0.10	3.09±0.15†
MTX + CoCl2	1.31±0.31	1.08±0.12	2.29±0.11

Total calcium content (μg/mg protein) was measured 1 hr after i.p. injections of 200 ng/kg of maitotoxin. † Effect of the treatment is significant ($p<0.05$).

that of the control ($p<0.05$), while that of adrenal glands of mice pretreated with $CoCl_2$, showed no significant increase after the injection of MTX. Moreover, a histochemical analysis of calcium distribution demonstrated that the density of calcium antimonate in mitochondria and SER of the mice given MTX was far more intensive than that of control mice (Figure 4b and c)[19].

Because the target of MTX is limited to the zona fasciculata, the amount of serum cortisol was determined by the sensitive radioimmunoassay method of Okamoto and Sone[11]. Figure 5 shows the time course of the plasma concentration of cortisol after the administration of MTX (200 ng/kg). The plasma cortisol level attained a maximum within the first 30 min and then decreased. The administration of CTX resulted in the degeneration of adrenomedullar cells. Marked vacuole formation of ganglion cells was seen in the medulla (Figure 4d). In contrast, cells in the cortical layers were resistant to CTX.

The Effects of MTX on Lymphoid Tissues

Lymphoid tissues were the target organs of MTX but not CTX. Figure 6a shows a micrograph of the thymus of a mouse given MTX (400 ng/kg) 24

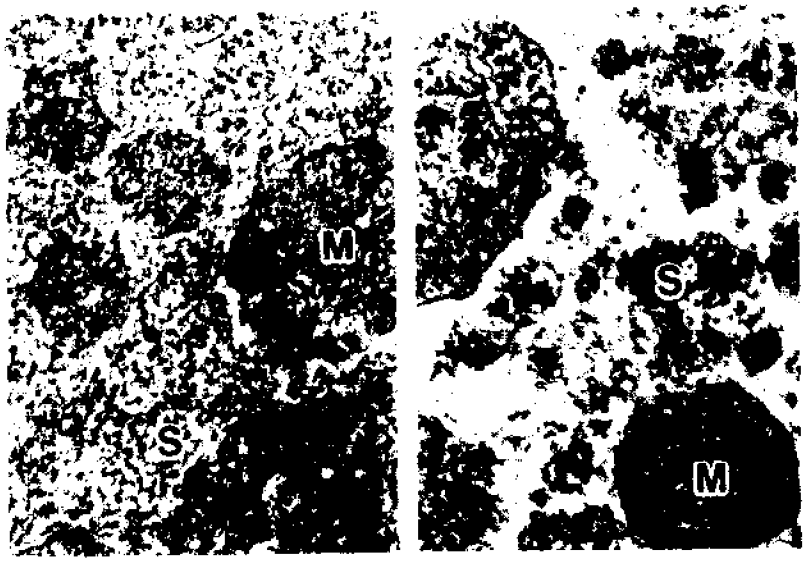

Figure 4. Effect of MTX and CTX on Mouse Adrenal Glands.
Left panel (4b) Electron micrographs of the adrenal glands of a mouse given 400 ng/kg of MTX. Histochemical detection of calcium in the adrenocortical cells. Control animal. ×45,000 *Right panel* (4c) Histochemical detection of calcium in the adrenocortical cells. A mouse treated with MTX. Dense reaction products located on the mitochondria (M) and smooth endoplasmic reticulum (S). ×45,000

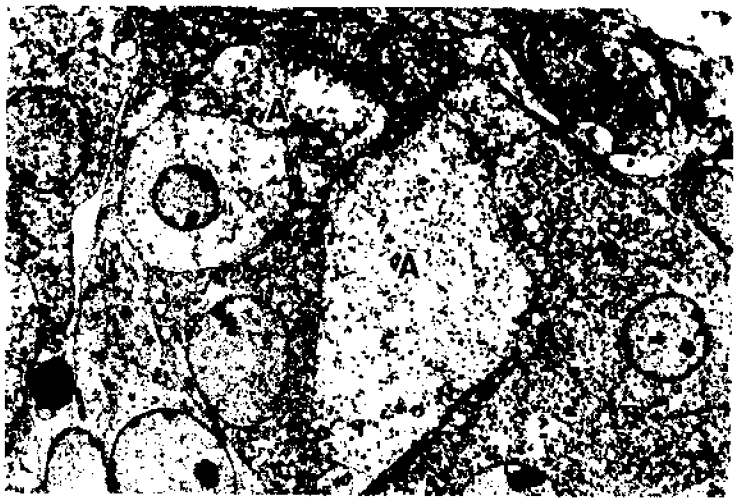

Figure 4. Effect of MTX and CTX on Mouse Adrenal Glands.
(4d)Electron micrographs of the adrenal glands of a mouse given 0.7 μg/kg of CTX. Norepinephrin secreting cells in the medulla of the adrenal gland after treatment with CTX. Vacuoles (A) were seen in these cells. ×2,600

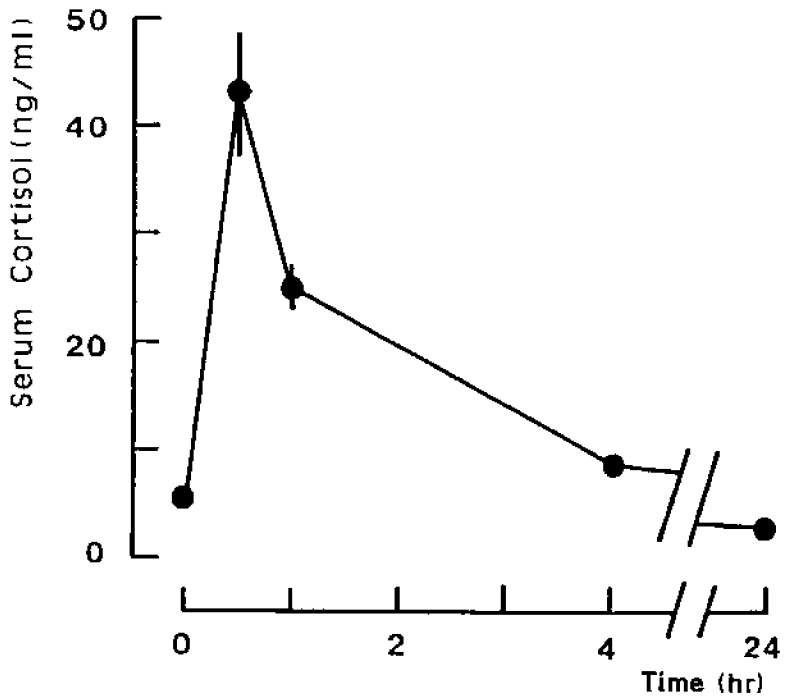

Figure 5. Effect of MTX on Plasma Cortisol in Mice.
Time course of effects of i.p. injections of MTX (200 ng/kg) of plasma cortisol in mice[11].

hours prior to sacrifice. Almost all lymphocytes in the cortical layer of the thymus became necrotic. Numerous macrophages with lymphocytic debris were observed in the cortex. However, with a single dose of only 50 ng/kg or less, no discernible changes occurred. Yet, when injections of 45 ng/kg of MTX were repeated 13 times, marked atrophy of the thymus was seen. Histologically, lymphocytes in the cortex disappeared and cortical tissue was replaced by many fibroblasts and collagen fibers (Figures 6b and c).

The spleen also decreased markedly in volume in MTX treated animals. Histologically, periarterial lymphoid sheaths in white pulp were incompletely formed (Figure 6d). Electron microscopy revealed that the endothelial cells lining the venous sinus increased in height and contained many electron-dense granules (Figure 6e). The volume of the sinuses decreased and the number of blood cells and macrophages present was diminished. T-cell dependent areas in various lymphoid tissues, including lymph nodes and Peyer's patches in the small intestine, became atrophic. Lymphocytes in the circulating blood also decreased in number from 65 % (before injection) to 13%, 13 days after the administration of MTX (Figure 7). Mice pretreated with $CoCl_2$, a calcium channel inhibitor, and bilateral adrenalectomized mice

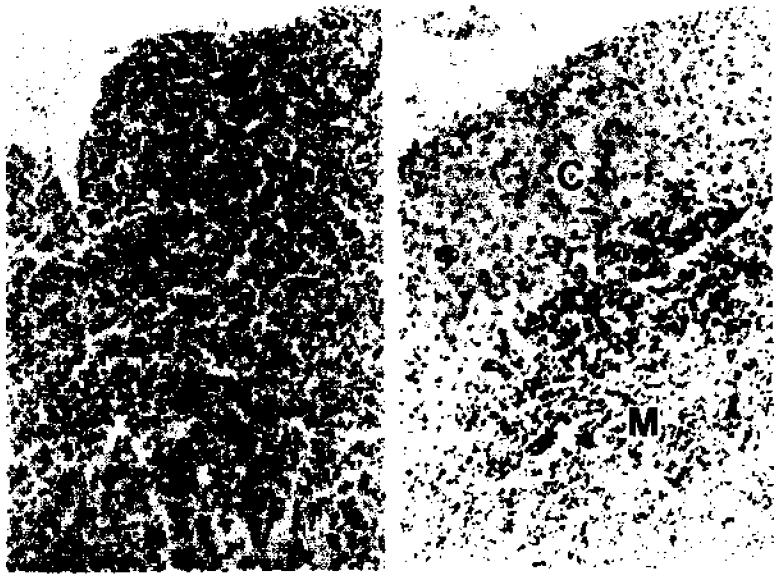

Figure 6. Effect of MTX on Mouse Lymphoid Tissue.
Micrographs of the lymphoid tissues. *Left panel* (6a) A photomicrograph of the thymus of a mouse treated with 400 ng/kg of MTX 24 hr after the injection. Marked lymphocytic debris was seen in the cortex of the organ. ×15
Right panel (6b) A photomicrograph of the thymus of a mouse after 13 repeated injections of 45 ng/kg MTX. At the cortical layer (C) no lymphocytes were present. (M) Medulla. ×30

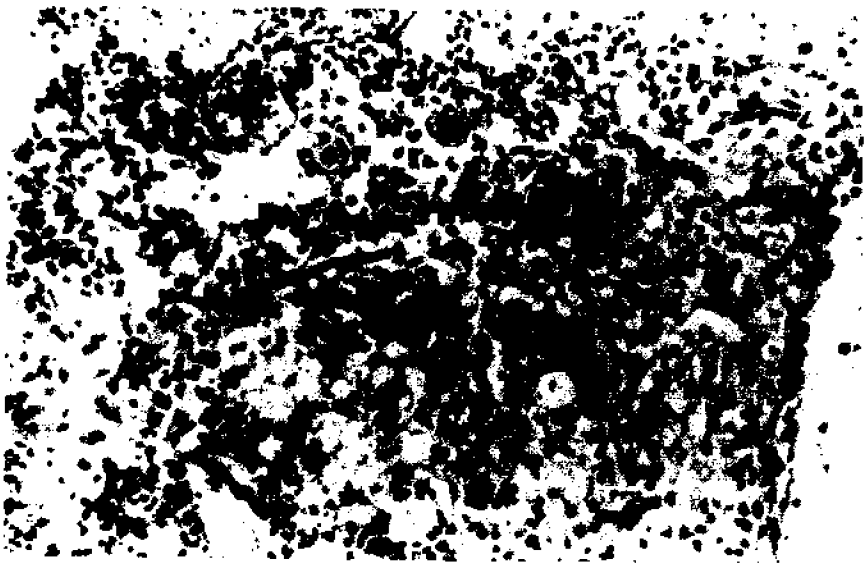

Figure 6. Effect of MTX on Mouse Lymphoid Tissue.
Micrographs of the lymphoid tissues. *Upper panel* (6c) An electron micrograph of the same animal (Figure 6b, opposite page). Numerous fibroblasts (F) and bundles of collagen fiber were seen in the cortical layer. × 3,000
Lower panel (6d) A photomicrograph of the spleen of a mouse treated with MTX. Note marked atrophy of T-cell dependent areas. × 50

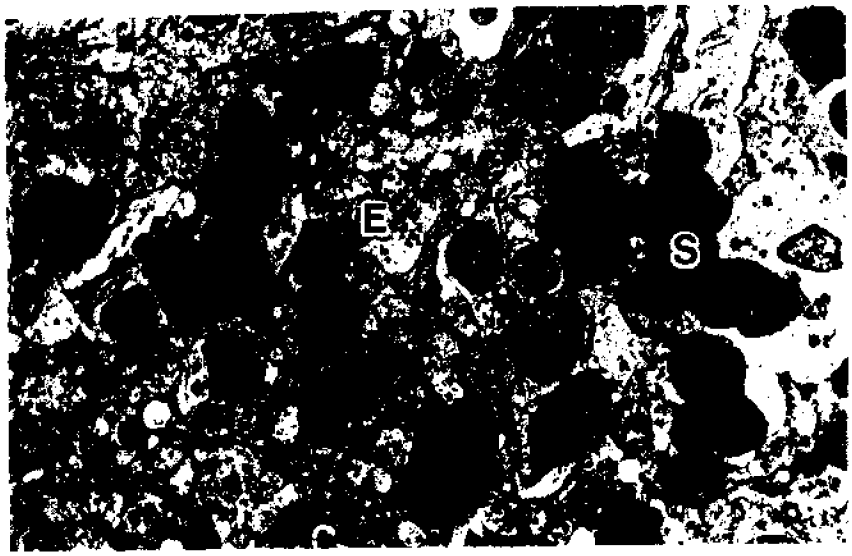

Figure 6. Effect of MTX on Mouse Lymphoid Tissues.
(6e) Micrographs of the lymphoid tissues. An electron micrograph of the same spleen (Figure 6d, previous page). Endothelial lining cells (E) of venous sinuses increased in height and contained many electron dense granules. Venous sinuses (S) were narrowed. × 2,600

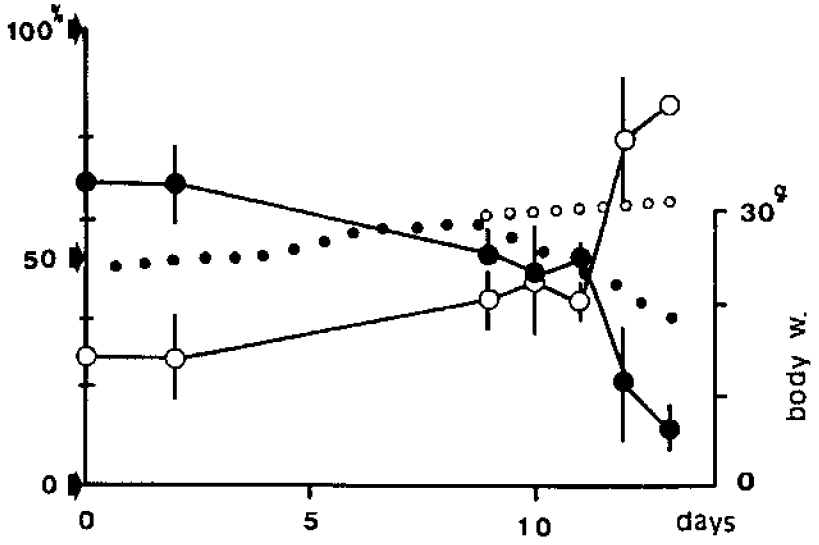

Figure 7. Effect of MTX on Mouse Blood Lymphocytes.
The time course of % of lymphocytes in the mouse blood, large solid circles; % of lymphocyte, large open circles; % of polymorph leukocytes, small circles; Body weights (solid, experimental; open, control).

Figure 8. Effect of CTX on Mouse Penis.
Photomicrographs of the penises. *Upper panel* (8a) Control flaccid state. ×7
Lower panel (8b) Continuous erected penis from a mouse treated with CTX. ×7

showed no discernible changes in lymphoid tissues.

The Effects of CTX on Penis and Vas Deferens

One of the most peculiar phenomena observed after the administration of CTX was the continuous erection of the penis. About half of the surviving mice given 0.7 μg/kg showed continuous erection of the penis even after death. Histologically, the corpus cavernosum of the penis was heavily engorged, with the length of the corpus cavernosum being about twice that of the normal flaccid state (Figure 8a and b). The enlarged corpus compressed the penile bone, so that the glans penis was erected and the length of the prepuce shortened. There were many small thrombi in the engorged cavernosum.

A number of investigators have studied the effects of MTX on the vas deferens electrophysiologically. They confirmed the Ca^{2+}-dependent contraction of smooth muscle cells of the vas deferens. When viewed with the electron microscope, synapses and unmyelinated nerve fibers were markedly swollen. However, no prominent changes were seen in the smooth muscle cells themselves.

Discussion

Table 2 summarizes the pathomorphological findings of MTX and CTX poisonings in various target organs. Maitotoxicosis apparently differed from

Table 2. Comparison of Pathology of Maitotoxicosis and Ciguatoxicosis in Mice.

Target Organs	Maitotoxicosis	Ciguatoxicosis
Heart	Single cell necroses Narrowing of capillaries	Marked swelling of cardiac muscle cells Effusion into intermuscular space
Stomach and Intestine	Multiple gastric erosions and ulcers	No discernible changes in mucosa Swelling of autonerve fibers and synapses in smooth muscle
Adrenal Glands	Multiple autophagosomes in cells of zona fasciculata	Vacuoles in the medulla
Lymphoid Tissues	Massive necroses of lymphocytes in the thymic cortex Atrophy of T-cell dependent areas in the spleen and lymph nodes	—
Penis	—	Continuous erection Thrombosis

ciguatoxicosis in the pathomorphological responses of mice. The trigger of maitotoxicosis was the MTX-induced increase in the permeability of the plasma membrane of cells in the zona fasciculata of the adrenal glands to Ca^{2+}. The stimulated Ca^{2+} influx caused the release of cortisol into the blood. This excess amount of cortisol produced acute involution of the thymic lymphocytes. Reduced formation of T-cell dependent areas in various lymphoid tissues followed. This view was also strongly supported by the findings that pretreatment with $CoCl_2$, a potent inhibitor of the Ca^{2+} channel, as well as unilateral or bilateral adrenalectomy prevented the lymphoid lesions. Gastric ulcers that occurred shortly after the administration of MTX may be closely related to the excess of cortisol in the blood.

In contrast to the MTX-poisoning, CTX-intoxication may be mainly caused by a direct effect of this toxin on various target cells. CTX stimulated an increased Na+ permeability across the tetrodotoxin-sensitive Na^+ channels of cardiac muscle cells and unmyelinated nerve cells in the small intestine and vas deferens. Pretreatment with atropine or bilateral adrenalectomy had no particular effect on the heart injuries induced by CTX. Therefore, whether or not norepinephrine played an important role in the cardiac lesions is unclear.

Results of the repeated injection experiments indicated that both MTX and CTX accumulated in the body of mice, for both toxins resulted in severe injuries of the target organs when administrated at successive low doses, whereas a single low dose produced no significant changes.

Literature Cited

1. Takahashi, M., Y. Ohizumi and T. Yasumoto. 1982. Maitotoxin, a Ca^{2+} channel activator candidate. J. Biol. Chem. 257, 7287-7289.
2. Takahashi, M., M. Tatsumi, Y. Ohizumi and T. Yasumoto. 1983. Ca^{2+} channel activating function of maitotoxin, the most potent marine toxin known, in clonal rat pheochromocytoma cells. J. Biol. Chem. 258: 10,944-10,949.
3. Ohizumi, Y. and T. Yasumoto. 1983a. Contractile response of the rabbit aorta to maitotoxin, the most potent marine toxin. J. Physiol. London, 337: 711-721.
4. Ohizumi, Y and T. Yasumoto. 1983b. Contraction and increase in tissue calcium content induced by maitotoxin, the most potent known marine toxin, in intestinal smooth muscle. Br. J. Pharmac. 79: 3-5.
5. Legrand, A-M. and R. Bagnis. 1984a. Effects of ciguatoxin and maitotoxin on isolated rat and rabbit duodenum. Toxicon 22: 471-475.
6. Legrand, A-M. and R. Bagnis. 1984b. Effects of highly purified maitotoxin extracted from dinoflagellate *Gambierdiscus toxicus* on action potential of isolated rat heart. J. Molec. Cell Cardiol. 16: 663-666.
7. Kobayashi, M., S. Kondo, T. Yasumoto and Y. Ohizumi. 1986. Cardiotoxic effects of maitotoxin, a principal toxin of seafood poisoning on guinea pig and rat cardiac muscle. J. Pharmac. Exp.Ther. 238: 1077-1083.
8. Gusovsky, F., T. Yasumoto and J. W. Daly. 1987. Maitotoxin stimulates phosphoinositide breakdown in neuroblastoma hybrid NCS-20 cells. Cell. Molec. Neurobiol. 7: 317-322.
9. Gusovsky, F., J.W. Daly, T. Yasumoto and E. Rojas. 1988. Differential effects of maitotoxin on ATP secretion and on phosphoinositide breakdown in rat pheochromocytoma cells. FEBS Let. 233: 139-142.
10. Terao, K., E. Ito, Y. Sakamaki, K. Igarashi, A. Yokoyama and T. Yasumoto. 1988. Histopathological studies of experimental marine

toxin poisoning. II. The acute effects of maitotoxin on the stomach, heart and lymphoid tissues in mice and rats. Toxicon 26: 395-402.

11. Terao, K., E. Ito, Y. Kakinuma, K. Igarashi, M. Kobayashi, Y. Ohizumi and T. Yasumoto. 1989. Histopathological studies on experimental marine toxin poisoning. 4. Pathogenesis of experimental maitotoxin poisoning. Toxicon 27: 979-988.
12. Rayner, M. D. and T.I. Kosaki. 1970. Ciguatoxin: Effects on Na+ fluxes in frog muscle. Fed. Proc. 29: 548.
13. Setliff, P. J., M.D. Rayner and S.K. Hong. 1971. Effect of ciguatoxin on sodium transport across the frog skin. Tox. Appl. Pharmacol. 18: 676-684.
14. Ohizumi, Y., S. Shibata and K. Tachibana. 1981. Mode of the excitatory and inhibitory actions of ciguatoxin in the guinea-pig vas deferens. J. Pharmacol. Exp. Therap. 217: 475-480.
15. Ohizumi, Y., Y. Ishida and S. Shibata. 1982. Mode of the ciguatoxin-induced supersensitivity in the guinea-pig vas deferens. J. Pharmacol. Exp. Therap. 221: 745-752.
16. Bidard, J-N., H.P.M. Vijverberg, C. Frelin, E. Chungue, A-M. Legrand, R. Bagnis and M. Lazdunski. 1984. Ciguatoxin is a novel type of Na+ channel toxin. J. Biol. Chem. 259: 8353-8357.
17. Yasumoto, T. 1985. Recent progress in the chemistry of dinoflagellate toxins. In: Toxic Dinoflagellates, (D.M. Anderson, A. W. White and D.G. Baden, eds.). Elsevier, New York. pp 259-262.
18. Legrand, A-M., M. Litaudon, J.N. Genthon, R. Bagnis and T. Yasumoto. 1989. Isolation and some properties of ciguatoxin. J. Appl. Phycol. 1: 183-188.
19. Aguas, A.P. and P.A. Nickerson. 1981. Subcellular distribution of calcium in zona fasciculata cells of the rat adrenal cortex. Tissue and Cell. 13: 491-499.

Mechanisms of Pharmacological and Toxicological Action of Ciguatoxin and Maitotoxin

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Abstract

Ciguatoxin (CTX) caused an increase in the contractile force of isolated guinea pig atria. Tetrodotoxin markedly inhibited the inotropic action of CTX. CTX shifted the current-voltage curve of Na+ inward currents in a negative direction. These results suggest that CTX activates Na^+ channels by modifying the voltage dependence of channel activation to increase Na^+ inward currents, thus producing cardiotonic actions. Furthermore, CTX at concentrations above 3 $\mu g\ ml^{-1}$ led to cardiac arrest and mitochondrial swelling.

Maitotoxin (MTX) caused a sustained inward Ca^{2+} current in single cardiac cells. The I-V relation of MTX-activated current was linear. The mean open time was about 10 msec. These data suggest that MTX activates Ca^{2+} channels with novel properties. MTX at 10 $ng\ ml^{-1}$ or more produced a gradual rise in diastolic tension. Myocardial cells were characterized ultrastructurally by severely overcontracted sarcomeres and swollen mitochondria. These morphological changes were abolished in the Ca^{2+} free solution, suggesting that MTX increases the Ca^{2+} influx through a new class of Ca^{2+} channels, to create the Ca^{2+} overloaded state and causing the observed cardiotoxic effects.

Introduction

Ciguatera is a disease caused primarily by the ingestion of a variety of fishes inhabiting tropical and subtropical seas. It is well known that the principal toxin in ciguatera fish is ciguatoxin (CTX), a highly toxic substance (LD_{50} in mice, 0.45 $\mu g/kg$). CTX has been reported to induce a depolarization of the cell membrane of the pedal ganglion, muscle and neuroblastoma N1E115[1-3,5]. CTX causes a marked release of neurotransmitter from autonomic nerve endings and an inotropic action[4,6-9]. Maitotoxin (MTX), a water-soluble substance, has been isolated from the viscera of a surgeonfish, *Ctenochaetus striatus*, and from the toxic dinoflagellate *Gambierdiscus toxicus*. MTX induces a Ca^{2+}-dependent release of noradrenaline from PC12 cells and Ca^{2+}-dependent contraction of smooth muscle[10-13]. MTX has been

shown to cause cardiotonic and cardiotoxic effects on heart muscle[14-18]. Since Ca^{2+} plays an important role in regulation of cellular functions, MTX has been widely used as a quantitative probe by numerous investigators[19-23]. Recently, MTX has been shown to increase formation of inositol phosphates from phosphoinositides, and cause the release of arachidonic acid from phospholipids in PC12 cells[24,25].

Materials and Methods

Mechanical response

Guinea pig left atria were excised and mounted vertically in an organ bath containing a Krebs-Ringer bicarbonate solution. Left atria were electrically stimulated at a frequency of 2 Hz by rectangular pulses of 5 msec at supramaximal intensities. Isometric contractions were measured with a force-displacement transducer. Guinea pig myocardial cells were prepared and the beating activity of single cells measured as reported previously[18,26].

Eletrophysiological experiments.

Guinea pig myocardial cells were superfused at 37°C with Tyrode's solution. Electrical properties of the myocytes were examined by patch-clamp methods using firepolished pipettes[27]. The current was measured by means of a patch-clamp amplifier, data stored on tape through a digital PCM data recording system, and analyzed with a computer.

Cytoplasmic-free Ca^{2+} concentration.

Isolated rat cardiac myocytes were prepared as described above. The $[Ca^{2+}]_i$ in rat myocardial cells was measured according to the method of Powell *et al.* with slight modification[28]. The cells were suspended in Tyrode's solution containing 0.5 mM $CaCl_2$ and incubated at 30°C for 1 hr in the presence of 50 μM Quin2 acetoxymethyl ester. The Quin2-loaded cells were then washed twice, suspended in Tyrode's solution containing 1 mM $CaCl_2$ and kept at room temperature.

Electron micrograph experiments

After treatment of the atria with MTX or CTX, the atria were rinsed quickly with a fixative solution (30°C) containing 2.5% glutaraldehyde in each respective incubation media, followed by immersion in the same fixative solution for a further hour at room temperature. Subsequently, tissues were rinsed repeatedly in 100 mM phosphate buffer (pH 7.4) and postfixed with buffered 1% osmic acid for 1 hr. Following standard dehydrating procedures using ethanol, the tissues were embedded in Epon and sliced thinly with a diamond knife. These sections were doubly stained with uranyl acetate and lead citrate, and then observed in a JEM 100-B electron microscope[17].

RESULTS

Mechanical response

CTX caused a concentration-dependent inotropic effect on the guinea pig isolated left atria in the range of 0.1 to 10ng ml^{-1}. The inotropic effect of CTX was markedly inhibited by treatment with tetrodotoxin (TTX, 5×10^{-7} M), and at concentrations of 3×10^{-5} M, TTX abolished the positive inotropic action of CTX. The inotropic action of CTX at lower concentrations (0.1-0.3 ng ml^{-1}) was completely inhibited by practolol (10^{-5} M) or reserpine (2 mg kg^{-1} daily, for 3 days), whereas at higher concentrations (0.6-10 ng ml^{-1}) CTX action was only partially inhibited by both drugs (Table 1). At concentrations above 3 μg ml^{-1}, CTX caused a biphasic inotropic change and finally led to cardiac arrest.

Table 1. Antagonists of the Inotropic Effect of CTX on Guinea Pig Atria.

Drug	Dose	Before CTX %	After CTX %	Increase in tension of CTX %	ED_{50} (ng/ml)
None (4)	—	0	100.0 ± 22.4	100.0 ± 22.4	0.5 ± 0.2
TTX (4)	5×10^{-7} M	-47.1 ± 5.5	21.5 ± 0.1*	68.6 ± 5.5*	8.9 ± 3.8*
Practolol (4)	10^{-5} M	-18.4 ± 5.9	22.4 ± 5.7*	40.8 ± 5.0*	0.6 ± 0.1
Reserpine (4)	2 mg kg^{-1} day^{-1}	0	80.1 ± 1.5*	80.1 ± 1.5*	0.6 ± 0.1

Effects of tetrodotoxin (TTX), practolol and reserpine on the positive inotropic effect of ciguatoxin (CTX) in the guinea-pig left atria. The maximum response to CTX in control is expressed as 100%. Each value indicates the mean ± S.E. mean and numbers in parentheses are the number of experiments. *Significantly different from the maximum response in the absence of CTX at $P < 0.05$.

In the isolated guinea pig left atria, MTX (0.1 to 4 ng ml^{-1}) caused a concentration-dependent inotropic effect. The MTX-induced inotropic effect was nearly abolished by Co^{2+} (2mM), but was little affected by propranolol (10^{-6}M), reserpine (2mg kg^{-1}, twice), or TTX (5×10^{-7} M). MTX at concentrations above 5 ng ml^{-1} caused a biphasic inotropic change and a gradual rise in diastolic tension of the atria, and finally led to cardiac arrest. The increase in the resting tension with concentrations in the range of 5 to 10 ng ml^{-1} occurred in a concentration dependent manner. The MTX (5 ng ml^{-1})-induced increase in tension was abolished by Co^{2+} (2 mM) or Ca^{2+}-free solution.

In the isolated atrial cells, CTX (3 ng ml^{-1}) induced a time-dependent increase in the degree and rate of longitudinal contractions. After the application of CTX, the degree of contractions was increased by 110%. The cellular motion was changed into irregular beating and myocytes then gradually shortened. MTX (0.1-10 ng ml^{-1}) caused an increase in the degree and the rate of contractions of myocytes. The cellular motion was changed into irregular beating and then shortened with a fibrillatory movement. The proportion of myocytes showing arrhythmic motions increased with time, attained a maximum, and then slowly declined with a concomitant increase in the percentage of damaged round cells. In the presence of verapamil (10^{-6} M), the change of normally beating myocytes into cells showing arrhythmic movements was delayed, and regularly beating cells still remained in the preparation.

Electrophysiological experiments

In the whole-cell patch-clamp experiments, CTX shifted the voltage dependence of Na^+ channel activation toward more hyperpolarized potentials without affecting the time course of inactivation and the peak current amplitude. In the presence of CTX (20 ng ml^{-1}) Na^+ currents were inactivated after the depolarizing pulse and then a small sustained inward current was observed. The holding current at -80 mV shifted in the inward direction in the presence of MTX (0.3 ng ml^{-1}). This inward current was enhanced by adrenalin and abolished by Cd^{2+}. The I-V relation of MTX-activated currents was linear and the reversal potential was -23 mV. In the cell-attached patch-clamp experiments, MTX induced long bursts of channel opening events. The unitary conductance was 12 pS and the mean open time of MTX-activated channels was 10 ms.

Cytoplasmic Ca^{2+} concentration

In Quin2 loaded cardiac myocytes, the $[Ca^{2+}]_i$ increased from a resting level of 116 ± 9 nM to a maximum of 750 ± 46 nM after treatment with MTX (5 ng ml^{-1} or more).

Ultrastructure

In the guinea pig left atria treated with CTX at 3 μg ml^{-1} for 60 min, the mitochondria were swollen and the sarcoplasm contained few glycogen granules. Marked ultrastructural changes were observed 30 min after application of MTX (30 ng ml^{-1}). The sarcomeres were severely contracted. In some cases, a localized overcontraction proceeded to the extent that the stretched areas between clumped myofibrils were partly empty of contractile material, and broken segments of myofibrils were scattered between the condensed contraction clumps. Mitochondria were heavily swollen and showed an increase in the matrix space with disorganized cristae. The chromatin of the nucleus was aggregated peripherally and the sarcoplasm was clear and contained few

glycogen granules. In Ca^{2+}-free medium no apparent ultrastructural change was observed, suggesting that these morphological changes were dependent on external Ca^{2+}.

Discussion

In the guinea pig left atria, CTX caused a cardiotonic action[14,4,9]. This effect was markedly inhibited by TTX. The inotropic effect of CTX at lower concentrations was abolished by practolol and reserpine, while that of CTX at high concentrations was partially inhibited by both the drugs. In the single atrial cells CTX produced an increase in the degree of longitudinal contraction, suggesting direct action on the cell membrane. Furthermore, CTX shifted the voltage-dependence of Na^+ channel activation to more negative membrane potentials, suggesting that CTX modified the activation process of fast Na^+ currents[9]. CTX caused prolongation of the action potential duration and this effect was also reversed by TTX. These results suggest that CTX-induced prolongation of the action potential duration was attributed to an increase in the Na^+ current. It is also suggested that CTX activated Na^+ channels by modifying the voltage-dependence of channel activation to increase Na^+ inward currents, thus producing an increased Ca^{2+} availability in the cardiac muscle cell[9]. It is possible that the sustained inward current induced by CTX at high concentrations contributed to depolarization, thus producing cardiotoxic actions.

MTX produced a positive inotropic effect on cardiac muscle[14-17]. MTX at high concentrations induced a rise in diastolic tension followed by atrial arrest. In myocardial cells MTX caused an increase in the degree of contraction and subsequent arrhythmogenic actions. These cardiotonic and cardiotoxic effects were inhibited by Ca^{2+} antagonists and were abolished by Ca^{2+}-free medium. The intracellular free Ca^{2+} concentration of myocytes was increased markedly by MTX. Furthermore, the MTX-induced ultrastructural changes were not observed in Ca^{2+}-free medium. These results suggest that MTX increases the Ca^{2+} permeability of some Ca^{2+} channels to elevate the intracellular Ca^{2+} concentration, thus producing cardiotonic and cardiotoxic effects[17].

In order to clarify the mechanism of Ca^{2+}-dependent cardiotoxic and cardiotonic actions of MTX, patch-clamp techniques were used to analyse eletrophysiological properties of a sustained inward current induced by MTX[18]. This current was predominantly carried by Ca^{2+} or Ba^{2+}. Quite importantly, the current-voltage relationship and unitary conductance of MTX-activated channels were clearly different from those of voltage-dependent Ca^{2+} channels. MTX-activated channels had a mean open time that was ten times longer than that of voltage-dependent Ca^{2+} channels. These data

suggest that MTX activates a new class of voltage-independent Ca^{2+} channels to increase the Ca^{2+} permeability, which may account for the mechanism of Ca^{2+}- dependent effects[18].

Acknowledgements

I am grateful to Professor Takeshi Yasumoto of Tohoku University for generously supplying ciguatoxin and maitotoxin.

Literature Cited

1. Boyarsky, L.L. and M.D. Rayner. 1970. The effect of ciguatera toxin on *Aplysia* neurons. Proc. Soc. Exp. Biol. Med. 134: 332-335.
2. Rayner, M.D. and T.I. Kosaki. 1970. Ciguatoxin: Effects on Na+ fluxes in frog muscle. Fed. Proc. 29: 548.
3. Rayner, M.D. 1972. Mode of action of ciguatoxin. Fed. Proc. 31: 1139-1145.
4. Lewis, R.J. and R. Endean. 1986. Direct and indirect effects of ciguatoxin on the guinea-pig atria and papillary muscles. Naunyn-Schmiedebergs Arch. Phamacol. 334: 313-322.
5. Bidard, J.N., H.P.M, Vijverberg, C. Frelin, E. Chungue, A.M. Legrand, R. Bagnis and M. Lazdunski. 1984. Ciguatoxin is a novel type of Na+ channel toxin. J. Biol. Chem. 259: 8353-8357.
6. Ohizumi, Y., S. Shibata and K. Tachibana. 1981. Mode of the excitatory and inhibitory actions of ciguatoxin in the guinea-pig vas deferens. J. Pharmacol. Exp. 217:475-480.
7. Ohizumi, Y., Y. Ishida and S. Shibata. 1982. Mode of ciguatoxin-induced supersensitivity in the guinea-pig vas deferens. J. Pharmacol. Exp. Ther. 221:748-752.
8. Lewis, R.J. and R. Endean. 1984. Mode of action of ciguatoxin from the Spanish mackerel, *Scomberomorus commersoni*, on the guinea-pig ileum and vas deferens. J. Pharmacol. Exp. Ther. 228: 756-760.
9. Seino, A., M. Kobayashi, K. Momose, T. Yasumoto and Y. Ohizumi. 1988. The mode inotropic action of ciguatoxin on guinea-pig cardiac muscle. Br. J. Pharmacol. 95: 876-882.
10. Takahashi, M., Y. Ohizumi and T. Yasumoto. 1982. Maitotoxin, a Ca^{2+} channel activator candidate. J. Biol. Chem. 257: 7287-7289.
11. Takahashi, M., M. Tatsumi, Y. Ohizumi and T. Yasumoto. 1983. Ca^{2+} channel activating function of maitotoxin, the most potent marine toxin known, in clonal rat pheochronocytoma cells. J. Biol. Chem. 258: 10944-10949.
12. Ohizumi, Y., A. Kajiwara and T. Yasumoto. 1983. Excitatory effects of the most potent marine toxin, maitotoxin, on the guinea-pig vas deferens. J. Pharmacol. Exp. Ther. 227: 199-204.

13. Ohizumi, Y. and T. Yasumoto. 1983. Contractile response of the rabbit aorta to maitotoxin, the most potent marine toxin. J. Physiol. 337: 711-721.
14. Legrand, A.M. and R. Bagnis. 1984. Effects of ciguatoxin and maitotoxin on isolated rat atria and rabbit duodenum. Toxicon 22: 471-475.
15. Kobayashi, M., G. Miyakoda, T. Nakamura and Y. Ohizumi. 1985a. Ca^{2+}-dependent arrythmogenic effects of maitotoxin, the most potent marine toxin known, on isolated rat cardiac muscles. Eur. J. Pharmacol. 111: 121-123.
16. Kobayashi, M., Y. Ohizumi and T. Yasumoto. 1985b. The mechanism of action of maitotoxin in relation to Ca^{2+} movements in guinea-pig and rat cardiac muscles. Br. J. Pharmacol. 86: 385-391.
17. Kobayashi, M., S. Kondo, T. Yasumoto and Y. Ohizumi. 1986. Cardiotoxic effects of maitotoxin, a principal toxin of seafood poisoning, on guinea-pig and rat cardiac muscle. J. Pharmacol. Exp. Ther. 238:1077-1083.
18. Kobayashi M., R. Ochi and Y. Ohizumi. 1987. Maitotoxin-activated single calcium channels in guinea-pig cardiac cells. Br. J. Pharmacol. 92: 665-671.
19. Freedman, S.B., R.J. Miller, D.M. Miller and D.R. Tindall. 1984. Interactions of maitotoxin with voltage-sensitive calcium channels in cultured neuronal cells. Proc. Natl. Acad. Sci. 81:4582-4585.
20. Schettini, G., K. Koike, I.S. Login, A.M. Judd, M.J. Cronin, T. Yasumoto and R.M. Macleod. 1984. Maitotoxin stimulates hormonal release and calcium flux in rat anterior pituitary cells in vitro. Am. J. Physiol. 247:E520-E525.
21. Kim, Y.I., I.S Login and T. Yasumoto. 1985. Maitotoxin activates quantal transmitter release at the neuromuscular junction: evidence for elevated intraterminal Ca^{2+} in the motor nerve terminal. Brain Res. 346: 357-362.
22. Ueda, H., S. Tamura, N. Fukushima and H. Takagi. 1986. Pertussis toxin (IAP) enhances maitotoxin (a putative Ca^{2+} channel agonist)-induced Ca^{2+} entry into synaptosomes. Eur. J. Pharmacol. 122: 379-380.
23. Niki, I., T. Tamagawa, H. Niki, A. Niki, T. Koide and N. Sakamoto. 1986. Stimulation of insulin release by maitotoxin, an activator of voltage-dependent calcium channels. Biomed. Res. 7: 107-112.
24. Gusovsky, F., T. Yasumoto and J.W. Daly. 1989. Calcium-dependent effects of maitotoxin on phosphoinositide breakdown and cyclic AMP accumulation in PC12 and NCB-20 cells. Mol. Pharmacol. 36: 44-53.
25. Choi, O.H., W. L. Padgett, Y. Nishizawa, F. Gusovsky, T. Yasumoto and J.W. Daly. 1990. Maitotoxin: Effects on calcium channels, phosphoinositide breakdown, and arachidonate release in pheochromocytoma PC12 cells. Mol. Pharmacol. 37: 222-230.
26. Ohizumi, Y., M. Kobayashi, A. Muroyama, H. Nakamura and J. Kobayashi. 1988. The mechanism of the inotropic action of striatoxin, a

novel polypeptide toxin from a marine snail, in isolated cardiac muscle. Br. J. Pharmacol. 95: 867-875.

27. Hamill, O.P., A. Marty, E. Neher, B. Sahmann and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch. 391: 85-100.
28. Powell, T., P.E.R. Twist and V.W. Twist. 1984. Cytoplasmic free calcium measured by Quin2 fluoresence in isolated myocytes at rest and during potassium-depolarization. Biochem. Biophys. Res. Com. 122:1012-1020.

Inhibition of Skeletal Muscle Response to Acetylcholine by Dinoflagellate and Ciguatoxic Fish Extracts

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Abstract

The effect of toxic dinoflagellate and ciguatoxic fish extracts on the contractility of fast skeletal muscle fibers was studied recording the amplitude and time course of twitches elicited in isolated frog sartorius muscles by either bath applied acetylcholine (ACh) or high concentrations of extracellular potassium. Toxic extracts were obtained by methanol extraction, from the dinoflagellate *Ostreopsis lenticularis* and from ciguatoxic barracuda (*Sphyraena barracuda*), harvested along the southwest coast of Puerto Rico. In the presence of 20 µg/mL of the ciguatoxic barracuda extract, the amplitude of the twitches produced by either ACh or potassium was reduced to approximately 10 to 20% of the control. A similar inhibitory effect was observed after addition of 500 µg/ml of *O. lenticularis* extracts. Veratridine increased by approximately 100% the amplitude and duration of ACh-induced muscular twitches. These potentiated responses were reversibly inhibited by the ciguatoxic fish extracts. Muscle twitches elicited by potassium were also blocked by the ACh receptor blocker alpha bungarotoxin and by low calcium, high magnesium solution. Taken together these pharmacological observations suggest that the inhibitory component present in these toxic extracts is an antagonist of the subsynaptic nicotinic cholinergic receptor.

Introduction

Ciguatera is a complex human toxic syndrome caused by the ingestion of fish associated with tropical coral reefs. It is manifested by early and generally severe gastrointestinal symptoms followed by sensory and motor abnormalities, that appear somewhat later in the toxic episode of longer duration[1-3] Besides nausea, vomiting and diarrhea, long lasting paresthesias of the lower extremities and generalized motor weakness are the most frequently reported clinical manifestations[4,5].

Toxins produced by benthic dinoflagellates, in particular *Gambierdiscus toxicus* , its ecological associate *Ostreopsis lenticularis* , have been implicated as ciguatera-causing toxins[6,7]. Based on their natural abundance and toxicity the dinoflagellates *Prorocentrum lima, P. concavum* and *O. siamensis* are also likely sources of ciguatera related toxins[8]. Recently, the structure of the ciguatoxin isolated from the moray eel *Gymnothorax javanicus* and of one of the toxins present in *G. toxicus* were elucidated[9]. These authors proposed that the *G. toxicus* toxin was a precursor of the moray eel ciguatoxin. However, the total number and structures of ciguatera toxins remain unclear.

Among the fish associated with ciguatera syndrome, barracuda (*Sphyraena barracuda*) is one of the most frequently reported in the Caribbean[5]. Tosteson *et al.* found that extracts obtained from the tissues, particularly the head and viscera, of one third of the barracuda specimens caught along the southwest coast of Puerto Rico were ciguatoxic as determined by the mouse bioassay method[10,11]. Toxicity of these barracuda tissues exhibited seasonal variations, a characteristic that may reflect yearly fluctuations in the abundance of toxic, benthic dinoflagellates. Ballantine *et al.* observed seasonal fluctuations in toxicity and population density in natural populations of *O. lenticularis* harvested in this coastal area[12].

Methanol extracts of clonal cultures of both *G. toxicus* and *O. lenticularis* isolated from the coastal waters of southwest Puerto Rico are toxic to mice[13] In this work, we found that toxic methanol extracts from ciguatoxic barracuda and *O. lenticularis* inhibited contractions of frog sartorious muscles elicited by applied and nerve-released acetylcholine. These results suggest that a component among those present in these crude, complex extracts, acts as an antagonist of the nicotinic cholinergic receptor.

Materials and Methods

Biological preparation

Small (2") specimens of the frog *Rana pipiens* were sacrificed by rapid destruction of the brain and spinal cord and dissected under the microscope to isolate both sartorius muscles joined by the symphysis of the innominate bones. The isolated muscle pairs were kept in normal frog Ringer's with the following ionic composition: 120 mM NaCl, 2.1 mM KCl, and 1.87 mM $CaCl_2$ buffered to pH 7.2 with 5 mM of N-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid and NaOH. Neostigmine (3-6 μM) was routinely added to this solution to inhibit muscle acetylcholinesterase activity.

Recording of muscle contraction

In a contraction chamber, muscles were fastened by the joined pelvic ends to a fixed support and by the tibial ligaments to a force transducer (Gould Statham UCB) which was on line with a pre-amplifier coupled to a chart

recorder (Beckman R511A) operating at a sensitivity of 20 mm/g of force and a chart velocity of 2.5 mm/sec. Muscles were stretched slowly to a resting tension of 250-500 mg. Application of a standard concentration (see below) of acetylcholine (ACh) to the paired sartorius muscles in the bath under these partially isometric conditions, resulted in a series of summed muscular twitches or fasciculations that decayed with time as the muscle fatigued. The amplitude of these twitches was recorded as pen displacements in the chart and measured (in mm) every four seconds. These amplitude values were averaged and plotted using the Sigma Plot computer program (Jandel Scientific, CA.)

Reagents and drugs

Muscle contractions were activated in some experiments by depolarizing the preparations with ACh as the chloride salt (Sigma Chemical Co.), dissolved in the bath at a final concentration of 5.5×10^{-6} M. In other experiments muscles were depolarized adding KCl to the bath solution in quantities sufficient to increase the extracellular K^+ concentration to 10 mM. In some experiments veratridine and alpha-bungarotoxin (obtained from Sigma Chemical Co.) were added to the frog Ringer's.

Toxic extracts of the dinoflagellate *Ostreopsis lenticularis* and of ciguatoxic barracuda (*Sphyraena barracuda*), prepared as described by Tosteson *et al.* were

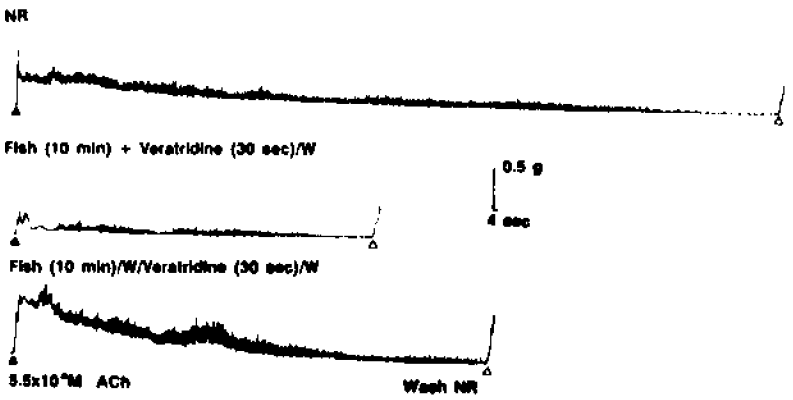

Figure 1. Effect of Dinoflagellate Extracts on ACh Induced Muscle Contractions. Representative records of the inhibitory effect of *O. lenticularis* extracts on twitches induced by applying ACh to frog sartorius muscles. Control responses to ACh were obtained in normal frog Ringer's (NR) before (top record) and after (bottom record) exposure of the muscles to 500 µg/ml of the extract (middle record). Filled arrow heads indicate application of ACh while open arrow heads signal beginning of wash out period with NR.

dissolved in small volumes of distilled water (50 mg/ml of *O. lenticularis* extracts, 2 mg/ml of *S. barracuda* residue) for their addition to the solution bathing the muscle [10,13]. The oily ciguatoxic barracuda extract was previously solubilized in µl volumes of purified formamide (Fisher Scientific, PA.).

RESULTS

Application of ACh (5.5×10^{-6} M) to muscles previously exposed for 10 min to frog Ringer's containing 20 µg/mL of the ciguatoxic barracuda extract or 500 µg/mL of the *O. lenticularis* extracts, resulted only in very small and feeble muscle twitches (Figure 1). This inhibition of the ACh-induced muscle twitches in the presence of either extract was reversed by washing the preparation several times with normal Ringer's solution.

A five fold increase in the frog Ringer's potassium concentration (to 10 mM) produced forceful and multiple muscle twitches which decayed very

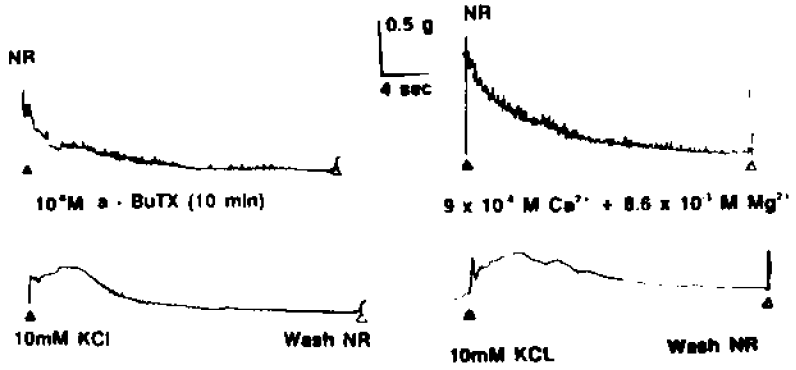

Figure 2. Effect of Increased Concentrations of Extracellular K+ on Muscle Contraction.
Records illustrating the response of two sartorius muscle pairs to an increase in $[K^+]_0$ to 10 nM. Top records show the rapidly decaying fast contractions produced by high K^+ in normal frog Ringer's. Treatment of the preparations with either the ACh receptor blocker alpha bungarotoxin (bottom left) or a low Ca^{2+}, high Mg^{2+} Ringer's solution (bottom right) abolished the fast components of the responses and resulted in slow, tonic contractures. Filled arrow heads signal application of high external potassium and open arrow heads indicate beginning of wash out period with NR.

rapidly (see Figure 2, top records). These K^+ induced twitches were inhibited by blocking the motor end plate nicotinic cholinergic receptor with alpha-bungarotoxin, or inhibiting the release of ACh from the motor nerve terminal by equilibrating the preparation in a solution of low Ca^{2+} and high Mg^{2+} concentrations. Under these conditions only these sustained or tonic muscle

contractures were observed (see Figure 2, bottom records), demonstrating that the fast muscle twitches were elicited by ACh released from motor nerve terminals depolarized by high extracellular K^+. As seen in Figure 3, these fast contractions were inhibited in the presence of fish and dinoflagellate extracts. However, upon return to normal Ringer's, recovery of the fast response to high $[K]_0$ was not complete in these preparations reflecting, most probably, a decrease in the amount of ACh released from nerve terminals with repeated depolarizations. Increasing $[K]_0$ to 40 nM caused only slow, tonic

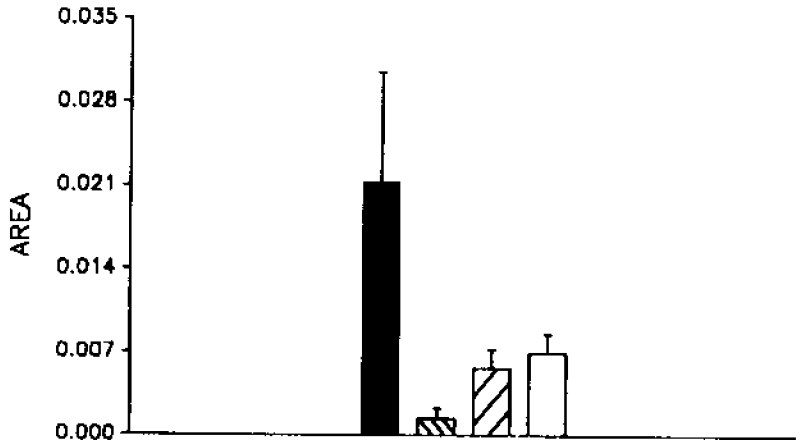

Figure 3. Effect of Ciguatoxic Barracuda and Dinoflagellate Extracts on the Amplitude of K+ Induced Muscle Contraction.
Amplitude of muscle twitches in frog sartorius muscles depolarized by increasing $[K^+]_0$ to 10 mM. The area under the curve was measured to determine magnitude of the response (expressed in arbitrary units) both in the presence of 20 µg/ml of the ciguatoxic barracuda extract (right diagonals, n=2) and 500 mg/ml of dinoflagellate extract (left diagonals, n=2) and in the absence of any of these extracts. Solid bar indicates muscles response in normal frog Ringer's (n=7) while open bar depicts partial recovery of the response after washing out the toxic extracts with normal Ringer's (n=6). Vertical lines indicate ± S.E.

muscle contractures that were not affected by the ciguatoxic fish extract.

The observed inhibitory effect of these toxic extracts on muscle contractions induced by either externally applied ACh or by K^+ stimulation of ACh release from nerve terminals suggested that a component acting as an antagonist of the nicotinic cholinergic receptor was present. However, using chromatographically pure moray eel ciguatoxin, Molgó *et al.* demonstrated that this toxin exerts an excitatory action on amphibian muscle, mediated by an increase in the membrane Na^+ permeability[14]. To explore a possible relationship between the action of these crude extracts and the muscle membrane Na^+ conductance, we exposed the muscles to veratridine. This plant alkaloid

is known to shift the activation of the voltage-gated Na^+ channels to more negative membrane potentials and to block channel inactivation[15]. These two actions result in prolonged activation of Na^+ channels at resting membrane potentials and would, thus, amplify any direct depolarizing action by similar components present in the extracts tested here.

Short (30 sec) pulses of 10^{-8} M veratridine applied to muscles in normal frog Ringer's had a direct excitatory action. Treatment with veratridine did enhance, by approximately 100%, the amplitude and duration of the response

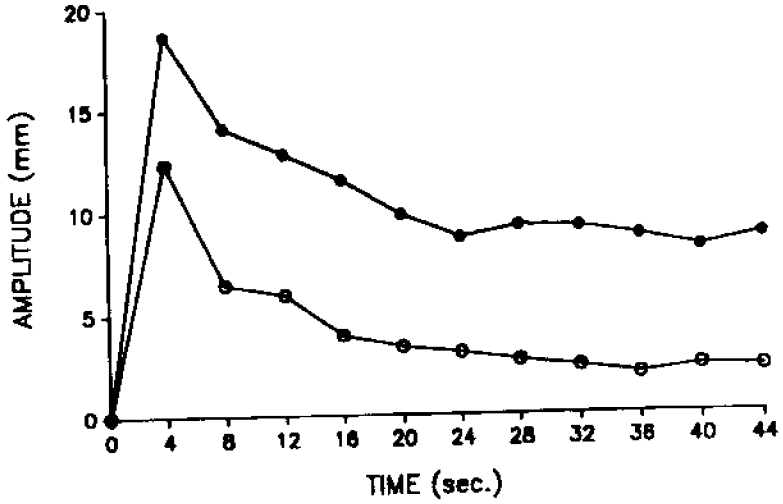

Figure 4. Effect of Veratridine on ACh Induced Muscle Contractions.
Potentiating effect of a 30 sec pulse of 10^{-8} M veratridine on the fast contraction elicited applying 5.5 x 10^{-6} M ACh to frog sartorius muscles (solid circles, n=5). The response to 5.5 x 10^{-6} M ACh obtained in normal frog Ringer's (open circles, n=5) was regarded as control.

of these muscles to externally applied ACh (see Figure 4). No excitatory response was observed in muscles treated with veratridine in the presence of ciguatoxic barracuda extract and addition of this alkaloid did not antagonize the inhibitory effect of the fish extract on ACh-induced muscle twitches. Similar to control preparations a veratridine pulse applied to a muscle washed with normal Ringer's after treatment with the barracuda extract, did result in an enhanced ACh response (Figure 5).

As seen in Figure 6, muscles whose response to ACh had been potentiated by veratridine were still sensitive to the inhibitory action of the ciguatoxic barracuda extract. This inhibition was easily reversed by washing the preparation with normal Ringer's. In contrast, the potentiating effect of veratridine

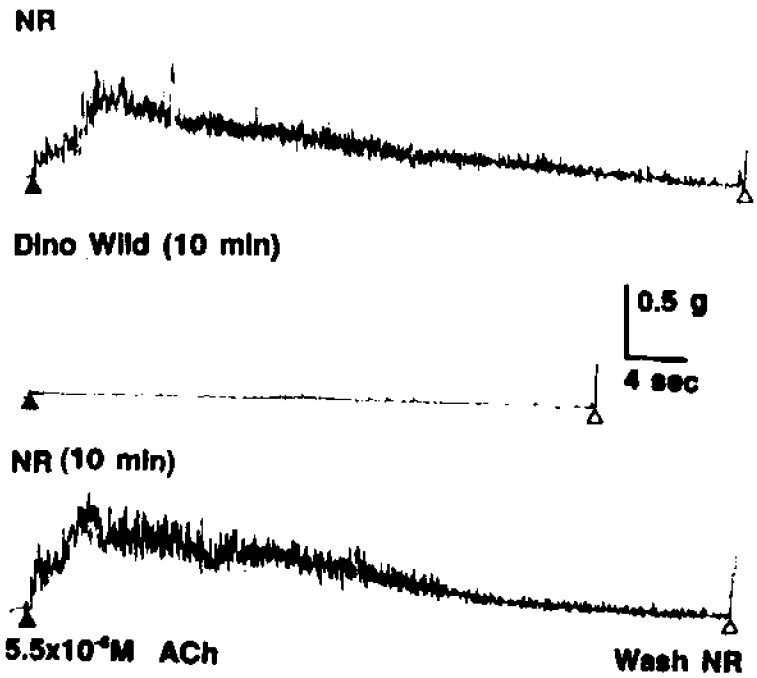

Figure 5. Effect of Ciguatoxic Barracuda Extracts on Veratridine Potentiation of ACh Induced Muscle Contractions.
Effect of a 30 sec pulse of 10^{-8} M veratridine on the fast contractions elicited by 5.5 x 10^{-6} M ACh to frog sartorius muscles. Middle record shows the muscle response to ACh during a 10 min incubation period with ciguatoxic barracuda extract (20 µg/ml). Bottom record was obtained after washing out the preparation with normal frog Ringer's (NR). Solid arrow heads indicate application of ACh while open arrows heads signal beginning of wash out period with NR.

was very long lasting and was observed again when ACh was applied to muscles that had been washed with normal Ringer's. These results further indicated that inhibition of muscle contractility by these ciguatoxic extracts was mediated by an antagonism of ACh action.

Discussion

Results reported here provide evidence that toxic methanol extracts obtained from tissues of ciguatoxic barracuda and from the benthic dinoflagellate *O. lenticularis* contain a component that antagonized the stimulatory effect of ACh on the amphibian skeletal muscle fiber. Pharmacologically this component appears to be acting as a reversible blocker of the nicotinic cholinergic receptor. Fast contractions or twitches of frog sartorius muscle fibers acti-

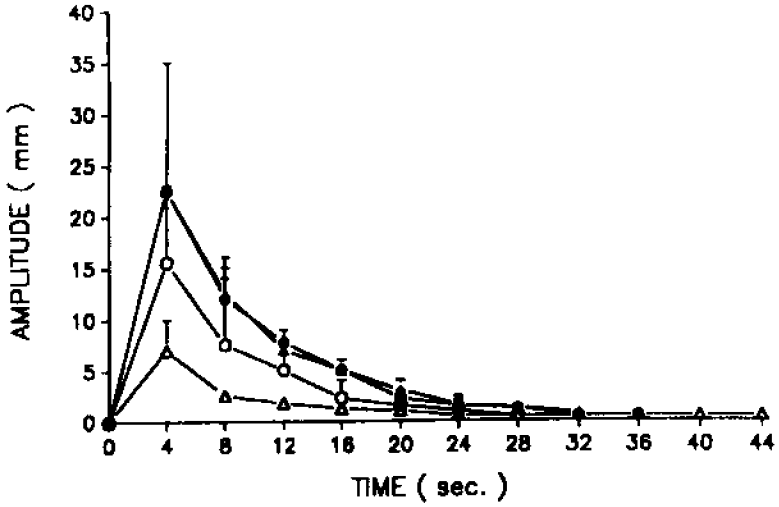

Figure 6. Effect of Ciguatoxic Barracuda Extracts on the Amplitude of Veratridine Potentiated ACh Induced Muscle Contractions.
Potentiation of the contractions produced applying 5.5 x 10^{-6} M ACh to frog sartorius muscles by (10^{-8}) veratridine (solid triangles, n=2) was reversibly abolished in the presence of 20µg/ml ciguatoxic barracuda extract (open triangles, n=2). When washed with normal frog Ringer's, the amplitude of the ACh-elicited twitches recovered to previous, potentiated levels (solid triangles and circles, n=2).

vated by ACh, either externally applied or released from the motor nerve terminals by a K^+ depolarization, were similarly inhibited by barracuda and dinoflagellate extracts. Muscles exposed for a short time to a low concentration on veratridine exhibited a long lasting, nearly irreversible, enhanced response to ACh. These veratridine-potentiated responses were inhibited in the presence of ciguatoxic barracuda extract but recovered fully washing the preparation with normal Ringer's, further suggesting an antagonism of ACh receptors by these extracts.

In contrast with our observations, recent reports concerning the action of chromatographically pure moray eel ciguatoxin on frog neuromuscular transmission, demonstrate that this toxin facilitates ACh release from motor nerve terminals and induces postsynaptic membrane depolarizations that activate spontaneous contractile activity in cutaneous pectoris muscle fibers[14]. However, it is not yet clear that this ciguatoxin is the single causative agent involved in ciguatera intoxication. The broad spectrum of clinical symptoms experienced by ciguatera victims as well as the large number and variety of toxins found in the various dinoflagellate species implicated in this poisoning

suggest that more than one component is involved in this toxic syndrome[16].

Ciguatera intoxication produces a paralysis of the lower extremities in some patients. This has been ascribed to a curarizing neuromuscular block as it is alleviated by the administration of acetylcholinesterase blocking agents[17]. This clinical observation suggests that at least one toxic component associated with the ciguatera syndrome may be an antagonist of the nicotinic cholinergic receptor. Chromatographic purification of the ciguatoxic barracuda extracts used in the present study has produced several different toxic fractions[18]. Further experiments are needed to determine if one of these fractions contains the proposed cholinergic blocker.

Literature Cited

1. Bagnis, R., T. Kuberski and S. Laugier. 1979. Clinical observations on 3,009 cases of ciguatera (fish poisoning) in the South Pacific. Am. J. Trop. Med. Hyg. 28: 1067-1073.
2. Lawrence, D.N., B.M. Enrique, R.M. Lumisk and A. Maceo. 1980. Ciguatera fish poisoining in Miami. J. Am. Med. Assoc. 244:254-280.
3. Casanova, M.F. 1982. Ciguatera poisoning: Medical letter. Arch. Neurol. 39:387.
4. Maharaj, S.R., P. Kulkarni and M. A. Pinnock. 1986. Ciguatera fish poisoning in a Jamaican family. W.I. Med. J. 35: 321-323.
5. Escalona de Motta, G., J. Feliú and A. Izquierdo. 1986. Identification and epidemiological analysis of Ciguatera cases in Puerto Rico. Mar. Fish. Rev. 48: 14-18.
6. Yasumoto, T., I. Nakajima, R. Bagnis and R. Adachi. 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. Bull. Jpn. Soc. Sci. Fish. 43: 1021-1026.
7. Yasumoto, T., I. Nakajima, Y. Oshima and R. Bagnis. 1979. A new toxic dinoflagellate found in association with ciguatera. In: Toxic Dinoflagellate Blooms, (F. J. R. Taylor and H. Seliger, eds.). Elsevier , New York. pp. 65-70.
8. Anderson, D.M. and P.S. Lobel. 1987. The continuing enigma of ciguatera. Biol. Bull. 172: 89-107.
9. Murata, M., A.M. Legrand, Y. Ishibashi, M. Fukui and T. Yasumoto. 1990. Structures and configurations of ciguatoxin from moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate *Gambierdiscus toxicus*. J. Am. Chem. Soc. 112: 4380-4386.
10. Tosteson, T.R., D.L. Ballantine and H.D. Durst. 1988 Seasonal frequency of ciguatoxic barracuda in southwest Puerto Rico. Toxicon. 26: 795-801.
11. Hoffman, P.A., H.R. Granade and J.R. McMillan. 1983. The mouse ciguatoxin bioassay: a dose-response curve and symptomatology analy-

sis. Toxicon. 21: 363-369.

12. Ballantine, D.L., T.R. Tosteson and A.T. Bardales. 1988. Population dynamics and toxicity of natural populations of benthic dinoflagellates in southwestern Puerto Rico. J. Exp. Mar. Biol. Ecol. 119: 201-212.
13. Tosteson, T.R., D.L. Ballantine, C.G. Tosteson, V. Hensley and A. T. Bardales. 1989. Associated bacterial flora, growth and toxicity of cultured benthic dinoflagellates *Ostreopsis lenticularis* and *Gambierdiscus toxicus*. Appl. Environ. Microbiol. 55: 137-141.
14. Mołgó, J., J.X. Comella, A.M. Legrand. 1990. Ciguatoxin enhances quantal transmitter release from frog motor nerve terminals. Br. J. Pharmacol. 99: 695-700.
15. Catterall, W.A. 1985. The voltage sensitive sodium channel: a receptor for multiple neurotoxins. In: Toxic Dinoflagellates, (D. M. Anderson, A. W. White, D. G. Baden, eds.). Elsevier, New York. pp. 329-342.
16. Tindall, D.R., D.M. Miller, and J.W. Bomber. 1989. Culture and toxicity of dinoflagellates from ciguatera endemic regions of the world. Toxicon. 27: 83.
17. Donnet, A., M. Habib, R.A. Vigouroux, A. Bourgeade and R. Khalil. 1987. Manifestations myasthéniformes persistantes aprés intoxication ciguatérique: Effet favorable des anticholinestérasiques. La Presse Médicale. 16: 734.
18. Tosteson, T.R., R.A. Edwards and D.G. Baden. 1992. Caribbean ciguatera and polyether dinoflagellate toxins: Correlation of barracuda ciguatoxins with standard toxins. In: Proceedings of the Third International Conference on Ciguatera Fish Poisoning (T.R. Tosteson ed.). Polyscience Publishers, Quebec, Canada. See pp. 101-116.

Caribbean Ciguatera and Polyether Dinoflagellate Toxins: Correlation of Barracuda Ciguatoxins with Standard Toxins.

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Abstract

Tissue samples of ciguatoxic barracuda (*Sphyraena barracuda*) caught along the south coast of Puerto Rico were extracted and the crude extracts chromatographically purified. These partially purified toxic fractions were further fractionated using reverse phase high performance liquid chromatography. Ciguatoxic barracuda fractions showed different retention times on C-18 columns and were consistently more hydrophobic than the polyether dinoflagellate toxins, brevetoxin and okadaic acid. Oral and i.p. mouse assays were analyzed to identify ciguatoxic fractions. Immunoassays and sodium channel receptor assays were employed to compare HPLC fractions of the barracuda ciguatoxins with brevetoxin. Based on relative toxicity and comparative assays with brevetoxin there appear to be four distinct toxins in the ciguatoxic barracuda extracts. The two most hydrophobic, toxic fractions were distinct in that one showed high i.p. toxicity and negligible similarity to brevetoxin PbTx-3, while the other was of high oral toxicity and showed comparable activity to PbTx-3 in both immuno and sodium channel binding assays. These data suggest that diverse, multiple toxins cause ciguatera fish poisoning in the barracuda.

Introduction

Ciguatera fish poisoning is a human health problem that affects the people of Puerto Rico, and all persons living near tropical seas for whom marine fish represent a significant source of food[1-5]. This type of poisoning has been recognized as a public health problem in the United States and its territories[6,7,8]. Ciguatera traditionally has been limited to tropical regions; however, increased commercialization of tropical reef fishes and tourism have increased the frequency of this type of fish poisoning among persons living in temperate portions of the United States and Canada[4,9].

Barracuda is a frequently implicated fish species in ciguatera fish poisoning in the Caribbean[3,10]. Ciguatoxicity of barracuda (*Sphyraena barracuda*) head, viscera and flesh tissues has been determined in specimens caught along the southwest coast of Puerto Rico[11]. Monthly frequencies of ciguatoxic barracuda showed an apparent seasonal variability, suggesting that the toxins, at least in their active form, were not accumulated in barracuda tissues for extended periods of time. Variability in barracuda ciguatoxicity may reflect fluctuations in the toxicity of smaller reef fish prey, seasonal fluctuations in toxic vectors (benthic dinoflagellates and bacteria) and/or changes in the ability of the barracuda to detoxify ingested poisons or their precursors. Benthic bacterial and/or algal micro-organisms were originally suggested as possible vectors of ciguatera toxin(s) by Randall[12]. Toxins associated with these microbes eaten by herbivorous fish would be passed through the "food chain" when these animals are consumed by larger carnivorous fish[13]. The toxic benthic dinoflagellate *Gambierdiscus toxicus*, initially discovered in French Polynesia (Gambier Islands), appears to be directly related to abundance and occurrence of ciguateric fish in the Pacific[14-16]. The structure of ciguatoxin isolated from the moray eel in the Pacific has been reported, and appears to be closely related to one of the toxic components isolated from *Gambierdiscus toxicus*[17,18].

Toxic benthic dinoflagellates *Ostreopsis lenticularis* and *Gambierdiscus toxicus* have been isolated from coastal waters of southwest Puerto Rico[19-21]. The abundance and toxicity of the more predominant benthic dinoflagellate, *Ostreopsis lenticularis*, showed seasonal variation[20,22]. Recent reports suggest that bacteria may play a role in toxic dinoflagellate blooms, the toxicity of *O. lenticularis* grown in laboratory culture, and ciguatera fish poisoning[22-25]. The precise origin of the toxins responsible for ciguatera has not been resolved. The chemical and pharmacological relationship between fish "ciguatoxins" and those toxins isolated from presumed benthic microalgal and/or bacterial vectors has not been conclusively resolved. Ciguatoxin isolated from the moray eel in the Pacific and one of the toxins isolated from *Gambierdiscus toxicus* have different histopathological and pharmacological actions[5,26,27].

The diversity and complexity in pharmacological action attributed to ciguatoxic fish extracts ("ciguatoxin(s)") no doubt reflect the variable sources of these materials and the probability that such extracts contain more than one pharmacologically active component[28,29]. The study reported here was conducted to assess the diversity of ciguatoxins found in the Caribbean barracuda and their relationship to the immunological and pharmacological specificity of the standard polyether dinoflagellate toxins, brevetoxin PbTx-3 and okadaic acid.

Materials and Methods

Toxins

Ciguatoxic barracuda tissues (180.4 kg) collected along the southwest coast of Puerto Rico were extracted in aqueous methanol (MeOH), followed by back-extraction of residual water with ethyl acetate (EtAC). 1080 µg toxic extract/g fish tissue extracted were recovered, for a total of 195 gm. Toxic residues were purified initially with Bio-Sil A and subsequently repeatedly fractionated on Sephadex LH20, alternately with MeOH/EtAC (50:50) and ethyl alcohol (95%). Peak toxicity fractions eluted from the LH-20 column at V_e/V_t ratios between 0.56 - 0.58. Pooled fractions within this range were used in the work reported here.

Brevetoxins were purified from laboratory cultures of *Ptychodiscus brevis*, and okadaic acid was obtained from Dr. Robert Dickey at the FDA Dauphin Island Laboratory. Synthetic tritiated PbTx-3 was produced from PbTx-2 by chemical reduction employing cerium chloride and sodium boro-tritiide. Crude PbTx-3 was purified using reverse phase high performance liquid chromatography (HPLC). HPLC-purified toxin had demonstrated specific activities of 10-15 Ci/mM.

High Performance Liquid Chromatography

Samples containing 20-50 mouse units of partially purified fish ciguatoxin were dissolved in 100% methanol and centrifuged (15,000 × g, 10') to remove particulate material. Clear supernatant solutions were subjected to reverse phase (C-18) HPLC using 85% isocratic aqueous methanol as the mobile phase. Fractions were collected at ten minute intervals, regardless of the number of peaks generated within a given interval. This procedure was undertaken to prevent loss of any fractions generated, and allow for the reproduction of separated fractions for further analyses. Following collection of six fractions (60 minutes), the mobile phase was changed to 100% methanol and a single 10 minute fraction was collected.

Toxicity Assays

Methanol was removed from the eluted fractions by flash evaporation and the residues taken up in duplicates of 1.0 ml Wesson oil or 0.9% saline containing 0.1% Emuflor EL-620. Fractions taken up in 0.9% saline-0.1% Emuflor, corresponding to 2 MU equivalents of original extract were injected i.p. into duplicate white Swiss mice (female, 20-25 gm), and corresponding fractions taken up in Wesson oil were administered by gavage to similar sets of animals. Animals were observed for periods of 48 hours. Individual bioassays were terminated when the animals were unable to right themselves when placed on their backs, or when they were in obvious respiratory distress.

Immunoassay

The radioimmunoassay (RIA) was carried out according to the method of Bigazzi *et al.*[30]. This assay utilized specific antibody made against brevetoxin PbTx-3. In the work reported here, keyhole limpet hemocyanin (KLH) was covalently linked to brevetoxin and the KLH-(PbTx-3) conjugate used as the complete antigen. Purified PbTx-3 was dissolved in minimal volumes of redistilled pyridine and succinylated with 10-fold molar excess succinic anhydride. Following separation of unreacted PbTx-3 and succinic anhydride from toxin-succinate using thin layer chromatography (70/30 ethyl acetate/light petroleum), the free carboxyl function on the conjugate was covalently coupled to the ε-amino function of lysyl residues on the KLH using standard procedures[31]. Following coupling, the mixture was dialyzed against PBS (pH 7.4) overnight, and the protein concentration adjusted to yield "toxin equivalents" of 1 mg/ml.

A single goat was immunized with the KLH-PbTx-3 conjugate on alternate weeks, with blood samples taken during the interval weeks for assessment of toxin antibody titers. Blood samples were allowed to clot and the serum separated by centrifugation. Stirred aliquots of antisera were treated with 0.5 ml volumes of saturated ammonium sulfate and allowed to precipitate overnight at 4°C. The mixture was then centrifuged (3000 g), the supernatant solution decanted and saved. The solution was then brought to 50% saturation by the addition of more 0.5 ml volumes of saturated ammonium sulfate, and the precipitate allowed to form overnight once again. The precipitate from this second centrifugation was redissolved in 0.3 ml volumes of original serum and dialyzed against PBS containing 0.01% sodium azide. For long term storage, the antibody solution was dialized against distilled water, and aliquots (approximately 25 ml) lyophilized. These may be reconstituted in PBS (pH 7.4) containing azide as needed.[31]

Synaptosome Binding Assay

Synaptosomes were prepared in multiple runs, generally using material from 4-6 rat brains. Frozen brains were purchased in multiples of 30-50 and stored at -80 °C until used. Synaptosomes prepared according to the method of Dodd *et al.* and stored as a pooled fraction until sufficient synaptosomes (generally from 20 brains) were prepared[32]. Protein was measured on resuspended intact or lysed synaptosomes just prior to binding experiments using the technique of Bradford[33]. Synaptosomes were stable and their activity in binding experiments reproducible for a period of 2-3 months.

Binding of tritiated brevetoxin (PbTx-3) was measured using the rapid centrifugation technique[34]. Binding assays were performed in a binding medium consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium chloride, 5.4 mM potassium chloride, 1 mg/ml BSA, and 0.01% Emuflor EL 620 as an emulsifier for the toxin. Synaptosomes

(40-80 µg total protein), suspended in 0.1 ml binding medium minus BSA, were added to a reaction mixture containing tritiated PbTx-3 and other effectors in 0.9% ml binding medium in 1.5 ml polypropylene microcentrifuge tubes. After mixing and incubating at 4 °C for 1 hour, samples were centrifuged (15,000 g, 2'). Supernatant solutions were aspirated from each tube and the pellets rapidly washed with several drops of a wash medium[35]. Pellets were then transferred to liquid scintillation vials and bound radioactivity measured. Non-specific binding was measured in the presence of 10 µM PbTx-3 and was substracted from total binding to yield a calculated measure of specific binding. Free tritiated probe was measured by counting an aliquot of supernatant solution prior to aspiration.

Results

HPLC Analyses

Figure 1 shows a representative elution pattern of the partially purified barracuda ciguatoxin, monitored at a wavelength of 215 nm. Following an initial peak of material, the most significant quantities detected were in fractions 5 and 7. Fraction 7, the most hydrophobic material found in the barracuda extracts, was recovered from the HPLC column after the 60' elution

Figure 1. HPLC Fractionation of Barracuda Ciguatoxin.
HPLC elution pattern of ciguatoxic barracuda fractions; C-18 reverse phase column, 4.6 mm x 25 cm; flow rate 1.4 ml/min.; mobile phase: isocratic 85% aqueous methanol. Detector at a wave length of 215 nm. Fraction symbols noted at the bottom of each fraction eluted.

period by washing the column with 100% methanol. Ciguatoxic barracuda fractions showed different retention times on C-18 columns and were consistently more hydrophobic than the polyether dinoflagellate toxins, brevetoxin and okadaic acid. The brevetoxins and okadaic acid would elute off the column in fractions 1 and 2[34]. The relative quantities (mg) of material recovered in the fractions reflected those detected by the UV monitor, with the exception that only 22% of the total material recovered in the seven fractions was found in fractions 5 (17%) and 7 (5%), while the second largest quantity (23%) of recovered mass was found in fraction 3, undetected by the UV monitor at 215 nm. The largest fraction of recovered material (36%) was found in fraction 1. Each fraction recovered is identified by an individual symbol, as indicated in Figure 1. These symbols are used in subsequent illustrations of the results obtained in the radioimmuno and synaptosome binding assays.

Toxicity Assays

Results of the toxicity bioassays were numerically ranked as follows; if the animals died within 24 hours the result was scored as a 6, if the animal survived but was clearly sick, it was scored as a 1 , and if the treated animal neither died nor appeared to be sick, as a 0. All animals used in the bioassays either died or appeared to be sick. Thus, all samples were scored either 6 or 1. Peak oral toxicity was found in fractions 1, 2 and 7, while peak i.p. toxicity was seen in fractions 2 and 5. Fraction 2 was distinct in that it was maximally toxic for both oral and i.p. routes of administration, whereas fractions 5 and 7 were distinguished by maximum toxicities in i.p. and oral assays respectively. The pattern of toxicity (oral > i.p. route) of the material recovered in fraction 1 was similar to that seen in fraction 7. Fractions 3, 4 and 6 did not show marked toxicity in either assay, however, test animals were clearly and reproducibly sick after the administration of the materials collected in each of these fractions.

Radioimmunoassay

Activities of the respective HPLC fractions in the RIA and synaptosome binding assays were scored from 6, for those fractions showing the greatest degree of specific displacement, to 1 for those displaying the least effect. For purposes of analysis, scores of 6 and 5 were taken as indicative of significant activity, scores of 4 and 3 of moderate activity and scores of 2 and 1, of minimal or negligible activity.

The results of the RIA displacement assay are illustrated in Figure 2. The % maximum binding (abscissa) refers to the labeled brevetoxin probe, and represents here the % of brevetoxin probe remaining bound in the presence of variable concentrations of the test materials. Increasing amounts of each fraction (ordinate, log scale) resulted in the displacement of the labeled probe as indicated. Thus, in the presence of increasing amounts of pure unlabeled

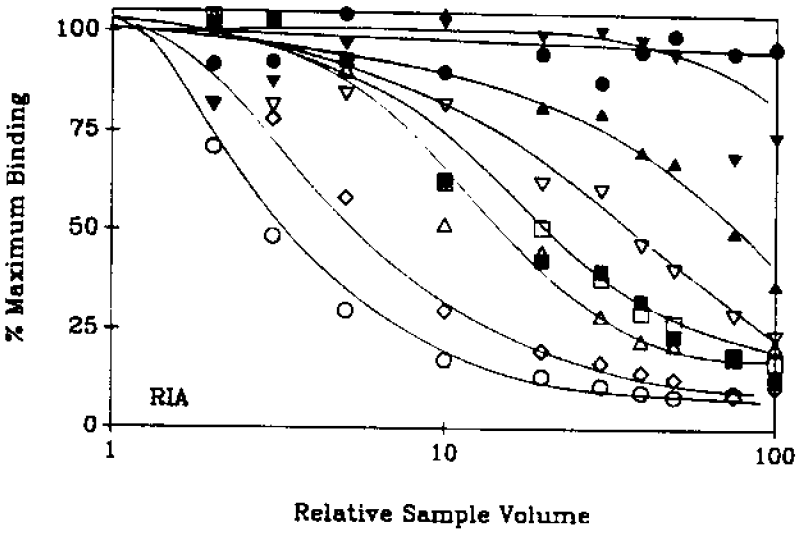

Figure 2. RIA Displacement Assay of Barracuda Ciguatoxin HPLC Fractions. Maximum binding % refers to the displacement of the labeled brevetoxin probe by the test fractions, and represents the % of brevetoxin probe remaining bound in the presence of given amounts of the test materials. Relative Sample Volume indicates the amount of test material added (log scale). Fraction symbols same as noted in Figure 1. Additional materials tested were; crude barracuda ciguatoxin prior to HPLC fractionation (solid circles) and pure PbTx-3 (open circles).

PbTx-3 (O) the labeled probe was most effectively displaced, whereas crude ciguatoxin unfractionated on HPLC (solid circles) and fraction 6 (solid triangles, point down) were least effective in displacing the labeled PbTx-3 probe from its antibody. Among the HPLC fractions, 7 was the most effective competitor of the PbTx-3 probe, followed closely by fractions 1 and 4. Fractions 3, 5, 2 and 6 respectively, showed decreasing abilities to displace the PbTx-3 probe from its antibody. This assay reflects the presence of similar structural components in the antigenic sites (epitopes) of the test molecules and the PbTx-3 probe. Thus, the more similar the structural components of the epitopes, the more effectively will the test molecule compete with the binding of the PbTx-3 probe to its antibody. The epitopes of fractions 7, and to a somewhat lesser degree fractions 1 and 4, contain structures very similar to the epitope of PbTx-3. Okadaic acid on the other hand did not displace tritiated PbTx-3 in these competition studies (Figure 3). Based on the results of this assay, okadaic acid does not appear to be a component of the HPLC fractionated barracuda ciguatoxins.

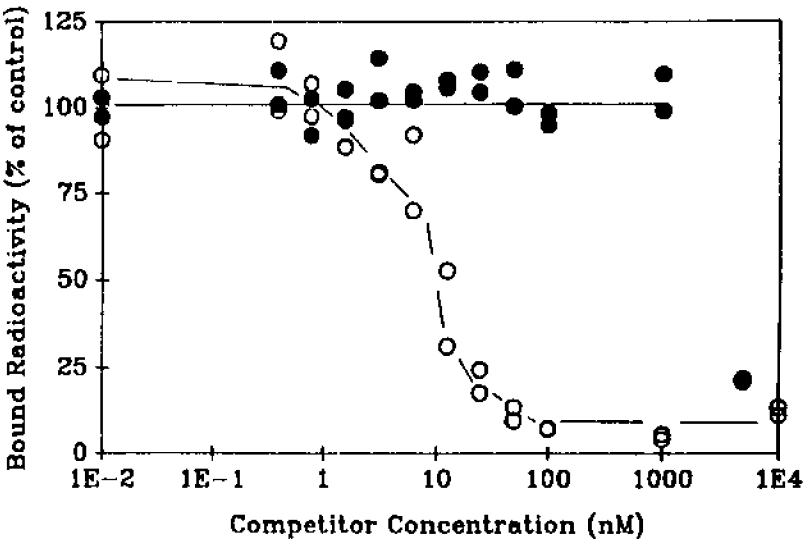

Figure 3. RIA Displacement Assay of Okadaic Acid.
Maximum binding % refers to the displacement of the labeled brevetoxin probe, and represents the % of brevetoxin probe remaining bound in the presence of given amounts of okadaic acid (solid points) and unlabeled pure PbTx-3 (open circles). Relative Sample Volume indicates the amount of material added (log scale).

Synaptosome Assay

The results of the synaptosome assay are illustrated in Figure 4. In this assay test fractions compete with tritiated PbTx-3 for binding sites on rat brain synaptosomes. These assays reflect the similarity in molecular topography of toxins for binding sites that are not necessarily the same as those recognized by antibodies. The degree to which the test fractions displace the binding of the PbTx-3 probe to the synaptosomes (sodium channel receptors) is a measure of their composite potency relative to PbTx-3.

The % maximum binding (abscissa) once again refers to the PbTx-3 probe as noted above, and the ordinate indicates the amount of each fraction added to compete with the binding of the PbTx-3 probe with synaptosome sodium channel components. Pure PbTx-3 (O) displaced the probe most effectively, while fraction 7 was the most effective competitor of the probe among the HPLC fractions of the barracuda ciguatoxin. The maximum specific displacement activity of fraction 7 (open diamond) was closely followed by fractions 4 and 2, with fractions 1 and 3 showing somewhat less activity respectively. Thus, fraction 7, the most hydrophobic of the HPLC fractions of the barracuda ciguatoxin had the highest composite potency compared to PbTx-3 itself. Hydrophobicity has been correlated with increased potency among the brevetoxins[36].

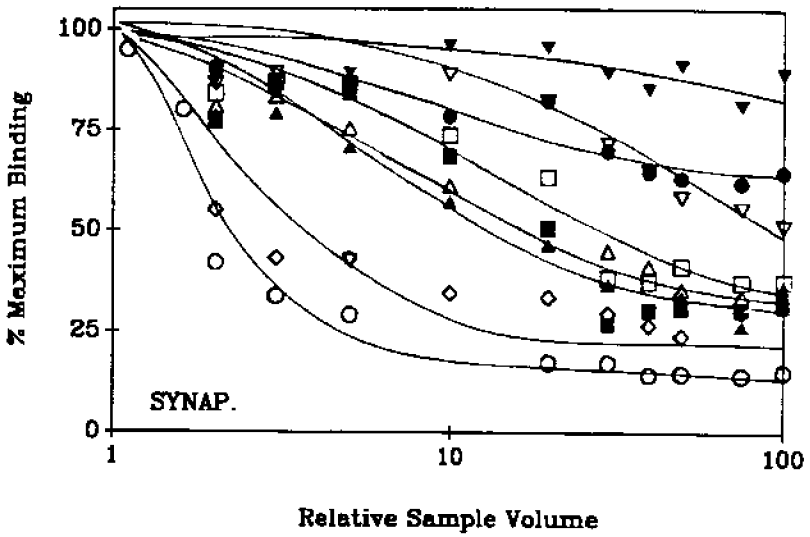

Figure 4. Synaptosome Displacement Assay of Barracuda Ciguatoxin HPLC Fractions.
Maximum binding % refers to the displacement of the labeled brevetoxin probe by the test fractions, and represents the % of brevetoxin probe remaining bound in the presence of given amounts of the test materials. Relative Sample Volume indicates the amount of test material added (log scale). Fraction symbols same as noted in Figure 1. Additional materials tested were; crude barracuda ciguatoxin prior to HPLC fractionation (solid circles) and pure PbTx-3 (open circles).

Discussion

The results of toxicity, radioimmuno and synaptosome binding assays are summarized in Figure 5. In this figure the Relative Maximal Response (abscissa), numerically scored from 6 (maximum activity) to O (no activity) is shown for each HPLC fraction of the partially purified barracuda ciguatoxin (ordinate). HPLC fractions of the barracuda ciguatoxin differed widely in their toxicity, depending on whether they were administered orally or by i.p. inoculation. Oral toxicity was found in fractions 1, 2, and 7, while only fractions 2 and 5 showed i.p. toxicity. Only fraction 2 was toxic when administered by both routes. Oral toxicity may indicate materials that are truly "ciguatoxic."

Results of the binding competition studies showed that fractions 1 ,4 and 7 had significant activity (scored 6 and 5) in both the radioimmuno and synaptosome binding assays. Fraction 2 is particularly interesting because of its high oral and i.p. toxicity and the fact that it showed significant activity in the synaptosome assay, coupled with low RIA activity. This highly toxic material did not contain the antibody binding portion (epitope) of the PbTx-3 antigen

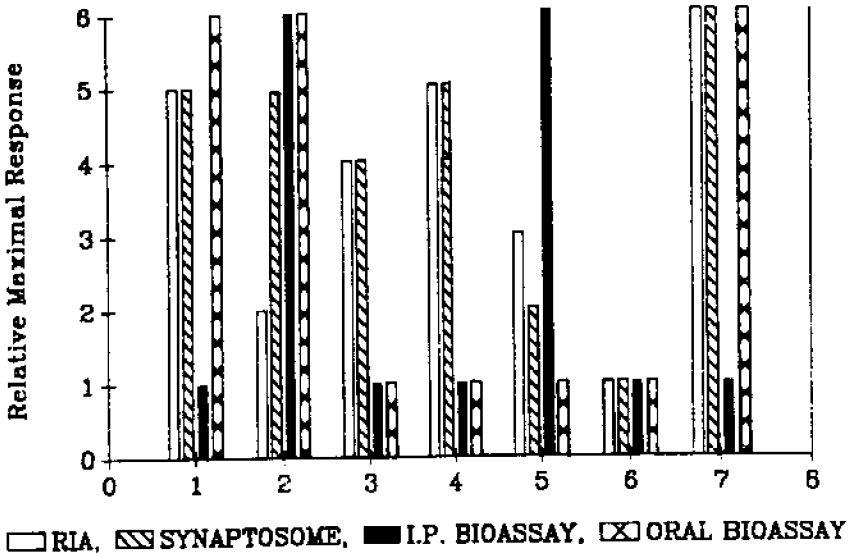

Figure 5. Assay Summary: Barracuda Ciguatoxin HPLC Fractions.
Relative Maximal Response of the barracuda ciguatoxin HPLC fractions in the RIA (clear bar), Synaptosome Assay (hatched bar), I.P. Toxicity Assay (solid bar) and Oral Toxicity Assay (crossed bar). See text for explanation.

which was used to elicit antibody production. Thus, the toxic material in this fraction of the barracuda ciguatoxin may either be a non-polyether type toxin or a polyether toxin that is not epitopic with the brevetoxin employed in these studies. The resolution of this question will require further study.

The RIA and synaptosome assays showed roughly the same degree of activity in each of fractions 3, 4 and 6. These fractions, which were not toxic to the test animals, showed moderate, significant and negligible activities respectively in both binding assays. Fraction 4 is interesting in that it contained material that was epitopic with PbTx-3, with a similar composite potency in the synaptosome assay, and yet was not toxic to the test animals. Fractions 4 and 7 have well correlated, significant activities in both binding assays, yet differ markedly in their toxicities. The relationship between this presumed polyether, PbTx-3 like material and fraction 7 remains to be determined.

All assays considered together suggest that peak oral toxicity was associated with fractions 1, 2 and 7. Fractions 2 and 7 showed significant activity (score 5 or 6) in the synaptosome assays, closely followed by fraction 1 with a moderate level of activity in this assay. Significant activities were seen in the RIA assay in fractions 1 and 7. Thus, the overall pattern of relative activity was nearly identical in these fractions despite marked differences in hydrophobicity (elution times). Fraction 2 was distinct from fractions 1 and 7 in that it

showed high i.p. toxicity, and negligible immunoassay activity, as noted above. Fractions 3 and 4 displayed negligible toxicities, however, they showed moderate and significant activity in both the RIA and synaptosome assays. Fraction 4 may be a polyether, PbTx-3 like material that is not toxic to mice. Peak i.p. toxicities were found in fractions 2 and 5, however, these fractions displayed widely different activities in oral toxicity and synaptosome binding. Fraction 5 was distinct from all other fractions in that it showed high i.p. toxicity, only moderate to negligible activities in the binding assays, and no oral toxicity. Fraction 6 showed little activity in any of the four assays, however, this material reproducibly made animals sick.

On the basis of present results, there appear to be at least five chromatographically different components in the crude ciguatoxic barracuda extracts, fractions 1, 2, 3-4, 5 and 7. Four of these components, fractions 1, 2, 5, and 7 were toxic. Whether these represent different toxins or metabolites of the same toxin remains to be resolved. The crude barracuda ciguatoxin had multiple toxins, the more hydrophobic of which (fractions 5 and 7) appeared to be distinguished on the one hand (fraction 5) by components of high i.p. toxicity with only negligible similarity to brevetoxin PbTx-3, and on the other (fraction 7), components of high oral toxicity, similar in activity to PbTx-3. Further work is in progress to resolve the nature and relationship between these components.

Acknowledgements

This work was supported in part by research grants from the Sea Grant Program of the University of Puerto Rico, the NIH-MBRS Program, University of Puerto Rico at Mayagüez (TRT), and the U.S. Army Med. Res. and Dev. Command, contract #DAMD 17-88-C-8148 at the University of Miami (DGB). Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

LITERATURE CITED

1. Craig, C.P. 1980. Editorial: It's always the big ones that should get away. JAMA 244: 272-273.
2. Lawrence, D.N., M.B. Enríquez, R.M. Lumish and A. Maceo. 1980. Editorial: Ciguatera fish poisoning. JAMA 244: 254-258.
3. Olsen, D.A., D.W. Nellis and R.S. Wood. 1984. Ciguatera in the eastern Caribbean. Mar. Fish.Rev. 46: 13-18.
4. Ragelis, E. P. 1984. Ciguatera seafood poisoning: Overview. In: Seafood Toxins, (E.P.Ragelis, ed.). ACS Symposium Series #262, American Chemical Society, Washington, D.C. pp. 25-36
5. Anderson, Donald M. and Phillip S. Lobel. 1987. The continuing enigma of ciguatera. Biol. Bull., 172:89-107.

6. McMillan, J.P., H.R. Granade and P. Hoffman. 1980. Ciguatera fish poisoning in the United States: Preliminary studies. J. Coll. Virgin Is. 6: 84-107.
7. Tacket, C. 1981. Studies of epidemiological and clinical aspects of ciguatera. Paper read at First Conference on Ciguatera, Caribbean Fisheries Management Council, San Juan, Puerto Rico.
8. Escalona de Motta, G.,J. F. Feliú and A. Izquierdo. 1986. Identification and epidemiological analysis of ciguatera cases in Puerto Rico. Nat. Mar. Fish. Rev. 48: 14-18.
9. Todd, E.C.D. 1992. How ciguatera affects Canadians. In: Proceedings of the Third International Conference on Ciguatera Fish Poisoning (T.R. Tosteson ed.). Polyscience Publishers, Quebec, Canada. See pp. 199-214.
10. de Sylva, Donald P. 1963. Systematics and Life History of the Great Barracuda. University of Miami Press, Coral Gables, Florida.
11. Tosteson, T.R., D.L. Ballantine and H.D. Durst. 1988. Ciguatoxic barracuda frequency in southwest Puerto Rico. Toxicon 46:795-801.
12. Randall, J.E. 1958. A Review of ciguatera tropical fish poisoning, with a tentative explanation of its cause. Bull. Mar. Sci. Gulf Carib. 8: 236-267.
13. Helfrich, P. and A.H. Banner. 1963. Experimental induction of ciguatera toxicity in fish through diet. Nature 197: 1025-1026.
14. Yasumoto, T., I. Nakajima, R. Bagnis and R. Adachi. 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. Bull. Jpn. Soc. Sci. Fish., 43: 1021-1026.
15. Yasumoto, T., A. Inoue, R. Bagnis and M. Garson. 1979. Ecological survey on a dinoflagellate possibly responsible for the induction of ciguatera. Bull. Jap. Soc. Sci. Fish. 45: 395-399.
16. Adachi, R. and Y. Fukuyo. 1979. The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov. collected in a ciguatera endemic area. Bull. Jpn. Soc. Sci. Fish. 45:67-71.
17. Murata, M., A.M. Legrand, Y. Ishibashi and T. Yasumoto. 1989. Structures of ciguatoxin and its congener. J. Am. Chem. Soc. 111:8929-8931.
18. Yasumoto, T., M. Murata, Y. Ishibashi, M. Fukui and A. M. Legrand. 1992. Structure determination of ciguatoxin of moray eels and *Gambierdiscus toxicus* . In: Proceedings of the Third International Conference on Ciguatera Fish Poisoning (T.R. Tosteson ed.). Polyscience Publishers, Quebec, Canada. See pp. 3-12.
19. Ballantine, D.L., A.T. Bardales, T.R. Tosteson and H.D. Durst. 1985. Seasonal abundance of *Gambierdiscus toxicus* and *Ostreopsis sp.* in coastal waters of southwest Puerto Rico. Proc. Fifth Inter. Coral Reef Cong. 4:417-422.
20. Ballantine, D.L., T.R. Tosteson, and A.T. Bardales. 1988. Population dy-

namics and toxicity of natural populations of benthic dinoflagellates in southwest Puerto Rico. J. Exp. Mar. Biol. Ecol. 119:201-212.

21. Tosteson, T.R., D.L. Ballantine, C.G. Tosteson, A.T. Bardales, H.D. Durst, and T.B. Higerd. 1986. Comparative toxicity of *Gambierdiscus toxicus, Ostreopsis* cf. *lenticularis* and associated microbial flora. Mar. Fish. Rev. 48:57-59.
22. Tosteson, T.R., D.L. Ballantine, C.G. Tosteson, V. Hensley and A. Bardales, 1989. Associated bacterial flora, growth and toxicity of cultured benthic dinoflagellates *Ostreopsis lenticularis* and *Gambierdiscus toxicus*. Appl. Environ. Microbiol. 55:137-141.
23. Buck, J.D. and R.H. Pierce. 1989. Bacteriological aspects of Florida red tides: A revisit and newer observations. Estuarine, Coastal and Shelf Science 29: 317-326.
24. González, I., C.G. Tosteson, V. Hensley and T.R. Tosteson. 1991. Role of Associated Bacteria in Growth and Toxicity of Benthic Dinoflagellates. ASM Annual Meeting. Dallas, Texas, May 5-9. Abstr. #Q 123.
25. Kodama, Arthur M., Y. Hokama, T. Yasumoto, M. Fukui, S.J. Manea and N. Sutherland. 1989. Clinical and laboratory findings implicating palytoxin as cause of ciguatera poisoning due to *Decapterus macrosoma* (Mackerel). Toxicon 27:1051-1053.
26. Terao, K. and T. Yasumoto. 1992. Pathomorphological studies on experimental maitotoxicosis and ciguatoxicosis in mice. In: Proceedings of the Third International Conference on Ciguatera Fish Poisoning (T.R. Tosteson ed.). Polyscience Publishers, Quebec, Canada. See pp. 61-78.
27. Ohizumi,Y. 1992. Mechanisms of pharmacological and toxicological action of ciguatoxin and maitotoxin. In: Proceedings of the Third International Conference on Ciguatera Fish Poisoning (T.R. Tosteson ed.). Polyscience Publishers, Quebec, Canada. See pp. 79-88.
28. Vernoux, J.P. and A. El. Andaloussi. 1986. Hétérogénéité des ciguatoxines extraites de poissons peches aux Antilles francaises. Biochimie 68: 287-291.
29. Vernoux, J.P. and F. Talha. 1989. Fractionation and purification of some muscular and visceral ciguatoxins extracted from Caribbean fish. Comp. Biochem. Physiol. 94B: 499-504.
30. Bigazzi, P., K. Wicker, E- Goryski, R. Zeschke, J. Puleo, G. Andres and S. Gutcho. 1973. Procedures using labeled antibodies or antigens. In: Methods in Immunodiagnosis, (N-R. Rose and P.E. Bigazzi eds.). John Wiley and Sons, New York. p. 107.
31. Abraham, G.E. and P.K. Grover. 1971. Covalent linkage of steroid hormones to protein carriers for use in radioimmunoassay. In: Competitive Protein Binding Assays, (W. Odell and W. Daughaday, eds.). J. P. Lippincott, Philadelphia. p.140.

32. Dodd, P.R. J.A. Hardy, A.E. Oakley, J.A. Richardson, E.K. Perry and J.P. Delaunoy. 1981. A rapid method for preparing synaptosomes: Comparison with alternative procedures. Brain Res. 226:107.
33. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein. Analyt. Biochem. 72:248.
34. Trainer, V.L., R.A. Edwards, A.M. Szmant, A.M. Stuart, T.J. Mende and D.G. Baden. 1990. Brevetoxins: unique activators of voltage-sensitive sodium channels. In: Marine Toxins: Origin, Structure and Molecular Pharmacology, (S. Hall and G. Strichartz, eds.). ACS Symposium Series 418, Washington D.C. pp 165-177.
35. Baden, D.G., T.J. Mende, J. Walling and D.R. Schultz. 1984. Specific antibodies directed against toxins of *Ptychodiscus brevis* (Florida's redtide dinoflagellate). Toxicon 22:783.
36. Baden, D.G., Kathleen S. Rein, Masao Kinoshita and Robert E. Gawley. 1991. Computational modeling of the polyether ladder toxins brevetoxin and ciguatoxin. In: Proceedings of the Third International Conference on Ciguatera Fish Poisoning (T.R. Tosteson ed.). Polyscience Publishers, Quebec, Canada. See pp. 117-130.

Computational Modeling of the Polyether Ladder Toxins Brevetoxin and Ciguatoxin

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Abstract

Monte Carlo and standard computational methods were used to generate conformational models of the brevetoxins and ciguatoxin. These were used to construct a model of polyether ladder toxin interaction with the voltage-sensitive sodium channel which explains on a molecular level how brevetoxins and ciguatoxin might maintain voltage-sensitive channels in an open configuration.

Introduction

Sodium channel isolated from rat brain consists of three separate and distinct protein subunits: the α-subunit, and the ß1 and ß2-subunits, which together comprise the channel in a 1:1:1 stoichiometry[1]. The α-subunit is a glycoprotein of approximately 260 kilodaltons (kD), and is a transmembrane protein which binds neurotoxins at specific loci or topographic sites. The two ß-subunits are smaller molecular weight peptides (each of about 30 kD) and are integral membrane subunit glycoproteins. Schematically, the channel has been illustrated as shown in Figure 1.

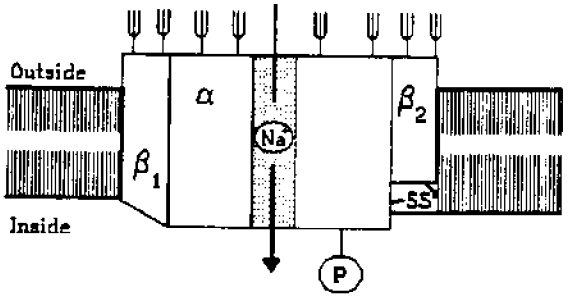

Figure 1. Subunit Structure of the Voltage Sensitive Sodium Channel.
Schematic subunit structure of the voltage-sensitive sodium channel showing disulfide bonds (S-S), phosphorylation sites (P), and glycosidation sites (pitchforks)[1].

Using primary structure data, Catterall developed a model for how the α-subunit inserts itself into the membrane of excitable cell types[2]. Each α-subunit consists of four homologous domains; each homologous domain being composed of six transmembrane peptide sequences S1-S6, with the highly positively charged S4 regions (shaded in the figure) being most highly conserved[3]. These S4 domains have been postulated to completely transverse the membrane, and all four S4 regions in concert contribute to the ion shuttling ability of the α-subunit (Figure 2)[4].

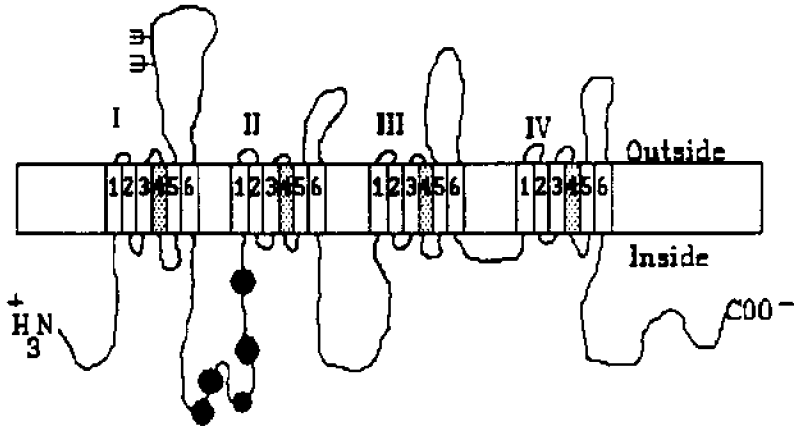

Figure 2. Functional Map of the Sodium Channel α-Subunit.
Catterall's functional map of the α-subunit of the voltage-sensitive sodium channel. The S4 regions (shown shaded) are thought to be responsible for ion transport character of the transmembrane-spanning protein.

The polyether ladder toxins brevetoxin and ciguatoxin represent a novel class of marine toxins first characterized in 1981 (Figure 3)[5,7]. Ciguatoxin is the most recent toxin in the group[7]. These three classes of compounds have in common a number of structural features, including: trans/syn stereochemistry along the entire backbone, with oxygen atoms alternating between the top and bottom; each ether oxygen constitutes a one atom bridge between adjacent rings; each toxin has regions of rigid, semirigid, and flexible character; the two brevetoxin backbones have lactone functions on one end, with an enone function on the other; the structure of ciguatoxin has not been fully elucidated, but the vicinal allylic diol may be oxidized *in situ* by an inducible cytochrome P450 oxidase or constitutive alcohol dehydrogenase to an enone (*vide infre*), and the spirocyclic acetal on the other end may be opened to a dihydroxyketone or a hemiacetal. Thus, there appears to be electrophilic functional groups on either end of each polyether ladder.

These common structural features are consistent with the observation that all three toxin types bind to the same unique site of the voltage-sensitive

sodium channel[8]. Additionally, the fact that the reactive functional groups are at either end, and the finding that all three have (or in the case of ciguatoxin, might be enzymatically activated to have) an enone function as the most reactive functionality, is highly suggestive of a common mode of binding and activity. Pharmacologically, the least toxic of the three types is PbTx-2 (brevetoxin B), followed by PbTx-1 (brevetoxin A), and the most potent is ciguatoxin. The present study seeks to examine the computer-generated population of structural conformers which fall between the energy constraints one would expect under normal biological conditions in living systems. Based on the apparent correlation between flexibility and potency, we sought to develop a mechanistic model for polyether ladder toxin interaction with the voltage sensitive sodium channel (VSSC).

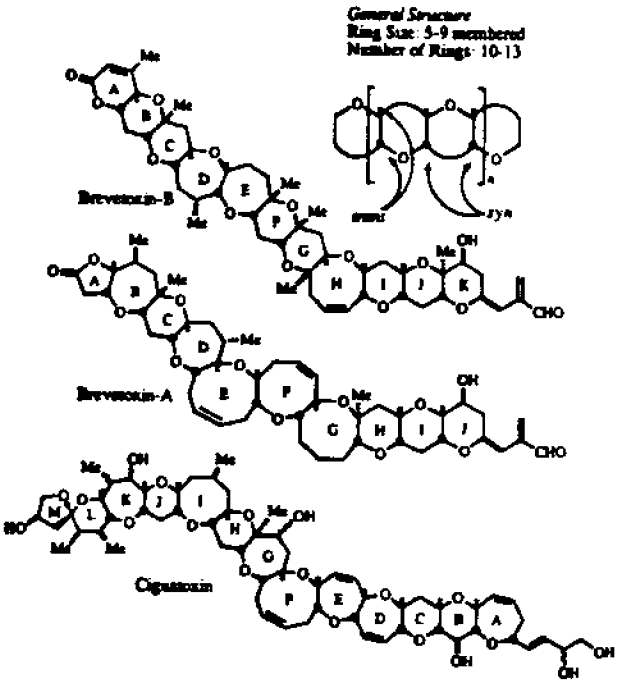

Figure 3. The Polyether Ladder Toxins PbTx-2 (Brevetoxin-B), PbTx-1 (Brevetoxin A), and CgTx (Ciguatoxin).

Materials and Methods

The most efficient method for randomly sampling conformational space makes use of internal coordinate Monte-Carlo subroutine techniques[9]. This approach has recently been compared to other computational algorithms to locate the 256 low energy minima of cycloheptadecane (16 rotatable bonds),

and was found to be far superior to its competitors[10]. The Monte Carlo method is incorporated into the molecular modeling software Macromodel, developed by Prof. Clark Still at Columbia University. Because the Monte Carlo method has not been explicitly tested on asymmetric structures like these ladder toxins, we have approached the problem of multiple ring flexibility with caution.

For PbTx-2 type toxins, there are only two areas where the molecule might flex: the two 7-membered rings D and E, and the 8-membered ring H. All other rings in the molecule are held rigid by virtue of their trans-fused decalin-like geometries. Thus, these hinge points were modeled independently of one another using Monte Carlo methods on the smaller models. The starting structures were randomly varied using the standard variables of Batchmin version 2.7 (the Macromodel batch-mode program). All bonds not specifically locked by their inclusion in a rigid ring were varied. Thus, for example, the ring fusion between rings C and D of PbTx-2 is rigid whereas the ring fusion between rings D and E is not rigid.

Synaptosome binding protocols and inhibition constant calculations, and HPLC analyses for toxin hydrophobicity were carried out as described previously[8].

Results

Much of the pharmacological data available on the brevetoxins surrounds PbTx-3, the reduced aldehyde-reduced primary alcohol derivative of PbTx-2. Some correlations in the literature show that within a structural class of brevetoxin, i.e. PbTx-2 type or PbTx-1 type, minor substituents play an insignificant role in affecting toxicity or inhibition constant binding character[8]. However, there was shown to be a statistically significant difference between the two brevetoxin classes. By using radioactive derivatives of

Table 1. Correlation of Potency with Binding Constants and Hydrophobicity.

Toxin	Mouse LD_{50}	Synaptosome Binding			HPLC Relative Hydrophobicity
		K_D	B_{max}	K_i	
PbTx-2 Type	200	2.6	6.80	2-3	least
PbTx-1 Type	100	0.8-1.0	6.30	0.5-0.7	middle
Ciguatoxin	0.45	(0.10)	(6.80)	0.14	most

either PbTx-1 or PbTx-2, specific binding parameters such as binding affinity (expressed as K_D, the dissociation constant) and number of sites (B_{max}, binding maximum expressed as pmol/mg synaptosomal protein), it is possible to compare directly the relative binding affinities and maxima. Moreover, derived values for ciguatoxin dissociation constants and binding maxima can be calculated, there being no radioactively labeled material for direct binding studies.

Purified natural toxins within either the PbTx-2 or PbTx-1 toxin class used as competitive inhibitors of radioactive toxin of the same class failed to show any statistical correlation, i.e. all class PbTx-2 toxins exhibited inhibition constants equivalent to one another. PbTx-1 toxins were uniformly and statistically more efficient at displacing PbTx-2 type toxin from site 5 of the VSSC than were PbTx-2 type toxins. Inhibition constant studies indicate to us that PbTx-1 brevetoxins bind with a higher affinity to the specific binding site than do PbTx-2 type toxins. Ciguatoxin, by our studies and by literature accounts, competitively inhibits brevetoxin binding at much lower concentrations than either of the brevetoxin classes. Inhibition constants for ciguatoxin displacing PbTx-2 type brevetoxin from its specific site are in the 0.1-0.2 nM concentration range.

Thus, the dissociation constants calculated for each of the three toxin types progress from the least potent PbTx-2 class at 2.6 nM, to the PbTx-1 class at 0.8-1.0 nM, and culminate with ciguatoxin, displaying a calculated dissociation constant of 0.1 nM. Mouse intraperitoneal bioassay progresses in exactly the same manner, although the magnitude of the derived values exceeds the differences in specific binding data. As expected, the inhibition constant data parallels the dissociation constant and mouse bioassay data. High performance liquid chromatography retention on C-18 reverse phase columns indicates that hydrophobicity increases from PbTx-2 type to PbTx-1 type to ciguatoxin. These data correlations seem to indicate that toxicity is related to binding affinity, which is in turn related to relative hydrophobicity. That binding maximum does not vary from toxin type to toxin type is reassuring, for it indicates to us that specific site 5 behaves like a homogeneous binding domain when exposed to these toxins, and further, that we cannot distinguish sub-populations of high affinity binding sites (Table 1). It is the pharmacological and bioassay data that we seek to correlate with the structural characteristics of each toxin type as determined by computer-aided molecular mechanics. As described below, we believe we have developed a working model which explains the variable onset of symptoms and curious alcohol-related recurrence of ciguatera symptoms in humans, and which also lends credibility to the observations of reduced reversibility and rapid onset times in toxin derivatives which possess conjugated aldehyde systems versus α, β unsaturated primary alcohol systems.

The molecular topography involved in binding and expression of activity by the polyether ladder toxins can be modeled as activity (A, triangles) and binding (B, squares) loci on the toxins, as shown in Figure 4. The inherent flexibility of each toxin permits either more (c) or less (b, then a) effective simultaneous interaction of the A and B domains with specific sites on the VSSC. A cursory examination of the structures in Figure 3 reveals that PbTx-2 has 16 rotatable bonds, PbTx-1 has 31, and ciguatoxin has over 40.

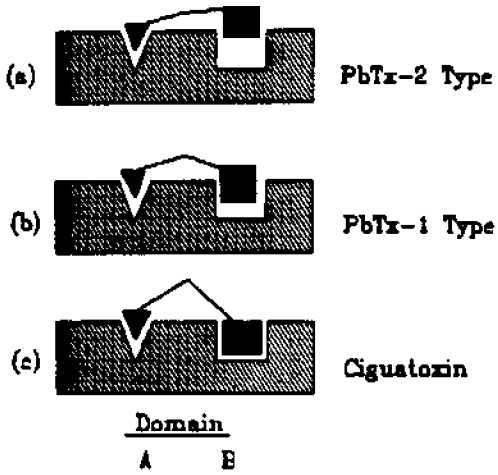

Figure 4. Schematic Model of Polyether Latter Toxin Interaction with VSSC. Toxin flexibility increases from (a) to (b) to (c), and correlates with increasing potency. It is proposed that increased flexibility allows for more favorable interaction of binding (B) and activity (A) centers located on distal ends of each polyether ladder toxin.

While our long-term goal is to evaluate the conformational space of all three polyether ladder toxin types, we have begun our efforts with the most rigid of the three types. Examination of the structures suggested that PbTx-2 would be most rigid. Indeed, Lin *et al.* originally described the structure as a "rigid ladder-like structure"[5]. The 8-membered ring of PbTx-2 diol-dibenzoate was calculated to prefer the crown conformation by several kcal/mol[5].

Internal coordinate Monte Carlo modeling of the G-H-I rings, in which all torsions of the 8-membered ring are varied except for the double bond in the two ring fusion bonds, reveals only two conformers <6 kcal/mol above global minimum. These conformations differ by 4.06 kcal/mol which corresponds to greater than 99.9% of the lower energy conformer. By contrast, modeling of the C-D-E-F rings shows a number of conformers and suggests that the toxin is not rigid at all. Stereo views of the seven lowest energy isomers and their

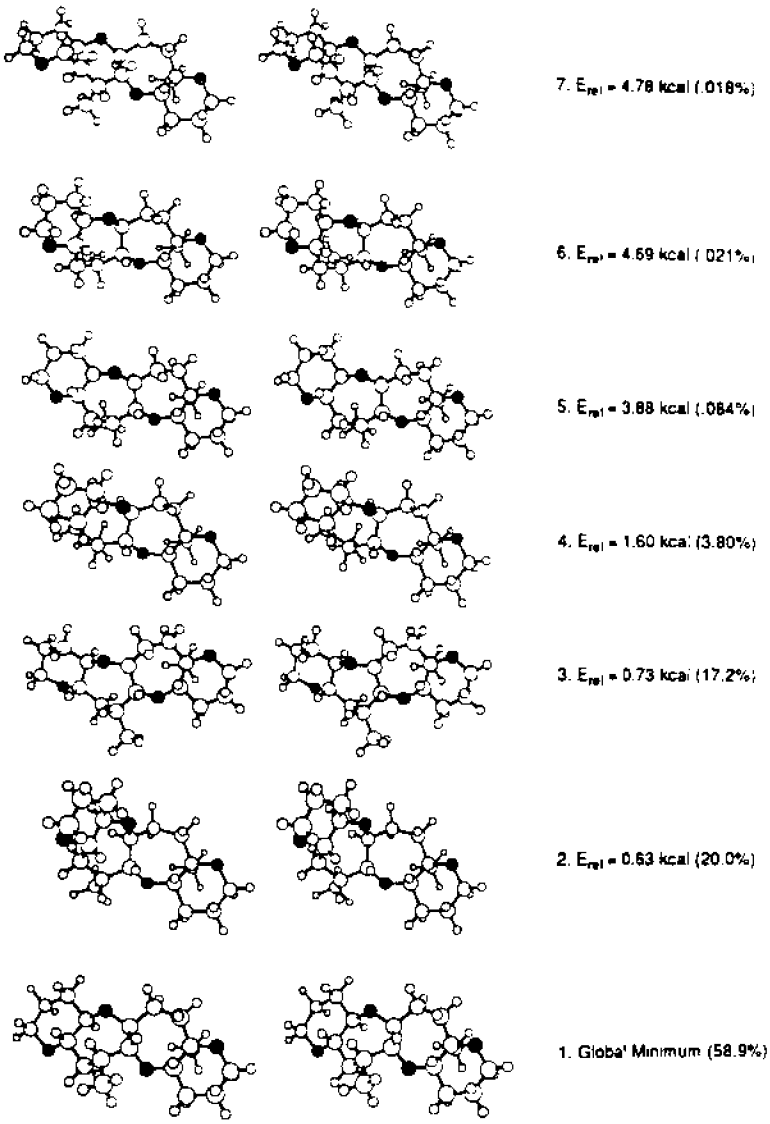

Figure 5. Conformational Analysis of the 6-7-7-6- Ring Region (Rings C-F) of Brevetoxin B. Stereoviews of the 7 lowest energy conformers of the model compounds, with relative energies and the Boltzmann distribution of each at 298°K.

Boltzmann populations are shown in Figure 5. Note that the four lowest energy structures, which differ by only 1.6 kcal/mol comprise greater than 99% of the total population. Note also that structures 2 and 4 possess an approximate 90° bend at the D- ring.

With these preliminary results in hand, we used the internal coordinate Monte Carlo technique to probe the conformations of PbTx-2. Although the complete details of these calculations will be reported elsewhere, the method did locate a number of conformations, including all four of the low energy C-D-E-F conformations corresponding to structures 1-4 of Figure 5. Interestingly, the relative positions of the three lowest energy conformations were scrambled. For example, the lowest energy conformation of the toxin contains the D-E configuration corresponding to conformation 3 in Figure 5. Conformation 1 of figure 5 appears next, at 0.26 kcal/mol above the global minimum. Then comes conformer 2, at 0.57 kcal/mol above global minimum and conformer 4 at 1.82 kcal/mol above the global minimum. Figure 6 illustrates the superimposition of the global minimum and the 0.57 kcal/mol

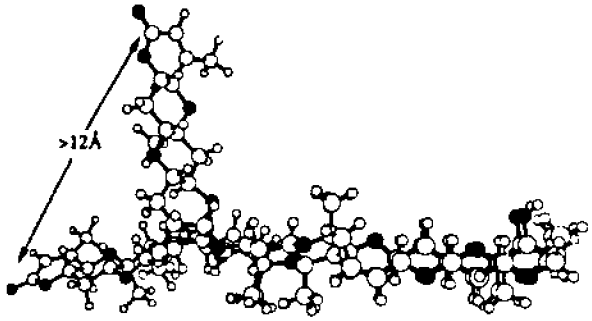

Figure 6. Superimposed Conformers of PbTx-2. The two structures correspond to the global instant minimum (extended) and the +0.57 kcal/mmol (bent) structure. The K ring is located on the right side.

structures, and illustrates the dramatic effect of D-ring flexibility on the position of the A-ring relative to the stationary K-ring. The A-ring sweeps over 12 angstroms by this single ring flip. These 4 structures comprise greater than 98% of the total population and we would expect that within this composite population lies a conformation or conformations that are "active" with respect to VSSC interaction.

For ciguatoxin, the computational chemistry was more difficult, there being more than two hinge regions. Manual manipulation of the 9-membered F ring has identified several structures within 5 kcal/mol of instant global minimum. By performing a similar superimposition of two of the generated structures, (ΔE = 0.8 kcal/mol) we see that the M ring in ciguatoxin may sweep a distance greater than 35 angstroms (Figure 7).

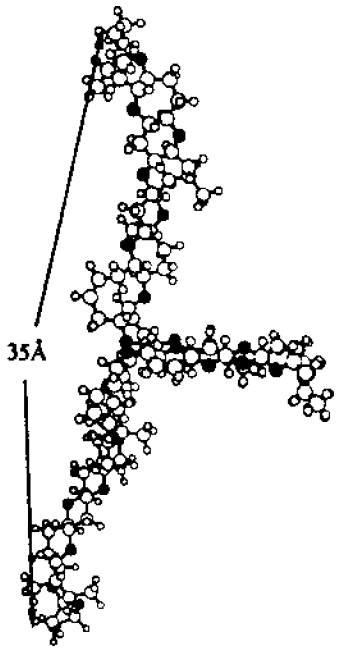

Figure 7. Superimposed Conformers of ciguatoxin. By flexing only the E-F-G region, and differing in energy by 0.8 kcal/mole, a 35 angstrom spread between the location of the M ring. The mostly rigid A-E ring is located on the right of the figure.

As neurotoxins, the polyether ladder toxins interact with the VSSC based primarily on their binding to a single specific site called site 5, located on the α-subunit of the channel. Unpublished photoaffinity probe results indicate that brevetoxin binds in a 1:1 stoichiometry to Domain IV of the channel. In all likelihood, site 5 is located on the extracellular loop between polypeptide segments S5 and S6 (Ph.D. dissertation work of V.L. Trainer, University of Miami).

We propose that brevetoxins interact with the α-subunit of the voltage-sensitive sodium channel as illustrated in Figure 8. We suggest that free movement of the sliding helix (S4 region) is hampered by the polyether ladder toxins so that the channel remains in a conducting state as follows: (1) the ε-amino group of a lysine on or near the S4 helix adds to the toxin enal (in either Schiff base or Michael fashion) to form a reversible S4-toxin conjugate; (2) the toxin flexes to wrap around or against the extracellular loop between S5 and S6, and is held in place by a combination of hydrophobic interactions, hydrogen bonding, or reaction with a second electrophilic site on the other end of the toxin (such as the lactone of the brevetoxins, or the masked carbonyl at the L-M ring fusion of ciguatoxin).

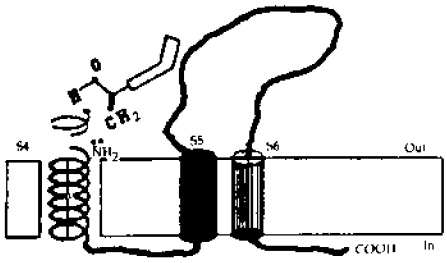

Figure 8. Model of Toxin Interaction With VSSC.
The transmembrane S4 helix is proposed to be held in place due to ion pairing by a spiral path of "+" charged arginine residues wrapped around the helix. Ion pairing is perturbed by membrane depolarization, which results in a screw-like motion through a 60° rotation and a 5 angstrom outward movement[2]. Toxin could be attacked by an exposed amino group forming a Schiff's base or Michael addition, thereby perturbing normal kinetics.

These two steps correspond to activity and binding, illustrated schematically in Figure 4, although available data do not distinguish which is which. We believe both steps are required for the full expression of polyether ladder toxin action. This model is consistent with several intriguing facts; squid or crayfish axons exposed to toxin exhibit depolarization which can be reversed by washing, but the reversibility is dependent on the length of exposure; upon binding to voltage clamped neurons, closed channels were opened and channel kinetics were modulated to exbibit slow inactivation kinetics[11]; toxins administered to VSSC in rat brain synaptosomes can be reversibly bound if washout occurs within 20 min to 1 hr of application; and all three toxin types exhibit competitive displacement equilibrium binding constants in radioactive brevetoxin Rosenthal Analyses[8]. Interestingly, the reversibility of toxin action is lessened for the more flexible toxins.

Our hypothesis is that the increased flexibility is reflected in the second "wrap-around" step described above. Note also that the calculated differences in energy for the toxin conformers allow significant populations of the bent isomers even in the absence of external influences. Moreover, differences of 2-3 kcal/mol could be easily overcome by hydrophobic interactions or hydrogen bonding, which might significantly stabilize a higher energy conformer relative to the calculated global minimum.

The α, ß unsaturated enal system required for the proposed action is present in the brevetoxins and may be produced enzymatically in ciguatoxin (see Figure 9) by the constituitive enzyme alcohol dehydrogenase, but more likely by an inducible cytochrome P450 oxidase. It is interesting to note that persons afflicted with ciguatera often relate recurrent ciguatera symptoms with the consumption of alcohol. It has always been considered merely

Figure 9. Activation of Ciguatoxin by Cytochrome P450.
Proposed mechanism by which ciguatoxin could be activated via a cytochrome P450 oxidase to produce an α, ß unsaturated enal system like the brevetoxins. Such an enal may be required for the activity expressed by any of the polyether ladder toxins.

anecdotal, but perhaps there is a biochemical basis for ciguaintoxication, since alcohol detoxification induces cytochrome P450 oxidase activity.

In summary, this working model describes the computational, pharmacological and binding results thus far generated, and consolidates our thoughts on the specific interaction between these toxins and the VSSC. Additional work in progress includes chemical stabilization of Schiff's base by reduction following incubation with radioactive toxin, and isolation and characterization of the adduct. Synthetic polyether toxins are also being developed, utilizing the information gathered concerning hinge regions and activity and binding centers. We anticipate fully developing quantitative structure-activity relationships (QSAR) for polyether ladder toxins which will aid in the complete description of voltage-sensitive sodium channel topography and toxinology.

Acknowledgements

This work was supported in part by the US Army Medical Research and Development Command, DAMD17-88-C-8148. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army.

Literature Cited

1. Catteral, WA. 1989. Molecular properties of voltage-sensitive sodium channels. ICSU Short Reports. 9:14.
2. Catterall, WA. 1986. Molecular properties of voltage-sensitive sodium channels. Ann. Rev. Biochem. 55:953.
3. Noda, M., T. Ikeda, T. Kayano, H. Suzuki, H. Takashima, M. Kurasaki, H. Takahashi and S. Numa. 1986. Existence of distinct sodium channel messenger RNA's in rat brain. Nature. 320:188.
4. Tanabe, T., H. Takashima, A. Mikami, V. Flodkerzi, H. Takahashi, K.

Kangawa, M. Kojina, J. Matsuo, T. Hirose and S. Numa. 1987. Primary structure of the receptor for calcium channel blockers from skeletal muscle. Nature. 328:313.

5. Lin, Y., M. Risk, S. Ray, D. VanEngen, J. Clardy, J. Golik, J. James and K. Nakanishi. 1981. Isolation and structure of brevetoxin B from the "red tide" dinoflagellate *Ptychodiscus brevis* (*Gymnodinium breve*) J. Am. Chem. Soc. 103:6773.
6. Shimizu, Y., H. Chou, H. Bando, G. VanDuyne and J. Clardy. 1986. Structure of brevetoxin-A (GB-1), the most potent toxin in the Florida red tide organism *Gymnodinium breve* (*Ptychodiscus brevis*) J. Am. Chem. Soc. 108: 514.
7. Murata, M., A-M. Legrand, Y. Ishibashi and T. Yasumoto. 1989. Structure of ciguatoxin and its cogeners. J. Am. Chem. Soc. 111:8929.
8. Trainer, V.L., R.A. Edwards, A.M. Szmant, A.M. Stuart, T.J. Mende and D.G. Baden. 1990. Brevetoxins: Unique activators of voltage-sensitive sodium channels. ACS Symposium Series 1990 418: 166.
9. Chang, G., W. Guida and W.C. Still. 1989. An internal coordinate Monte Carlo Method for searching conformational space. J. Am. Chem. Soc. 111:4379.
10. Saunders, M., K. Houk, Y. Wu, W.C. Still, W, M. Lipton, G. Chang and W. Guida. 1990. Conformations of cycloheptadecane: A comparison of methods for conformational searching J. Am. Chem. Soc. 112:1419.
11. Huang, J.M.C., C.H. Wu and D.G. Baden. 1984. Depolarizing action of a red tide brevetoxin in axonal membranes. J. Pharm. Exp. Ther. 229:615.

Antibody Production and Development of a Radioimmunoassay for the PbTx-2-Type Brevetoxins.

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Abstract

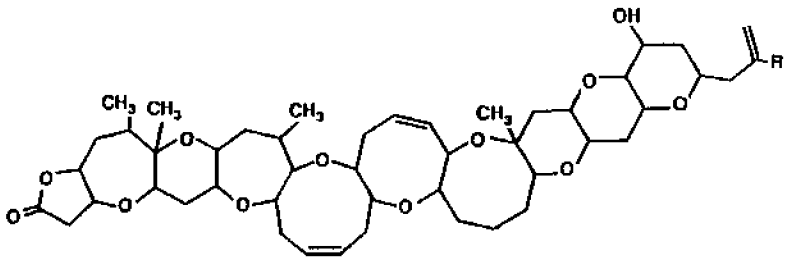
A goat polyclonal antiserum specific for brevetoxins containing the PbTx-2 backbone structure was raised against PbTx-3 conjugated to bovine serum albumin. This antiserum was used to develop a sensitive, specific radioimmunoassay. The minimum detection limits for PbTx-2, PbTx-3, and PbTx-9 were approximately 300 pg. Cross-reactivity with PbTx-1 occurred at 100-fold higher concentrations. No cross-reactivity was detected against saxitoxin, palytoxin, or the diarrhoeic shellfish toxins okadaic acid, pectenotoxin-6, or yessotoxin.

The same detection limits were achieved when measuring brevetoxins in human urine and in extracts of clams (*Mercenaria mercenaria*). The successful development of a radioimmunoassay for brevetoxin detection in shellfish extracts is an important advance in the monitoring of seafood stocks. The ability to detect toxins in human urine suggests potential for the diagnosis of neurotoxic shellfish poisoning and for monitoring or confirming human exposure.

Introduction

The brevetoxins are a group of polyether neurotoxins produced by the dinoflagellate *Ptychodiscus brevis* . These toxins are extremely potent to both mammals and fish[1]. During blooms of *P. brevis*, known as Florida red tides, brevetoxins can be concentrated to levels dangerous to man by filter-feeding molluscs[2]. Human intoxications from neurotoxic shellfish poison of contaminated shellfish and massive fish kills can result from the presence of *P. brevis* in the nearshore marine environment[3,4].

Nine structurally distinct brevetoxins have been described to date[5]. Each is a derivative of one of the two polyether backbone structures illustrated in Figure 1. In this work, we use the PbTx nomenclature system and the terms PbTx-1-type and PbTx-2-type to differentiate between the backbone structures[6].



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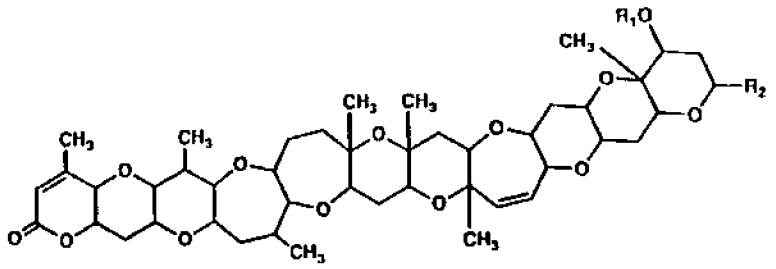

B

Figure 1. Structures of the Brevetoxins

(A) PbTx-l-type brevetoxins:
PbTx- 1: R=$CH_2C(CH_2)CHO$
PbTx-7: R=$CH_2C(CH_2)CH_2OH$
PbTx-10: R=$CH_2CH(CH_3)CH_2OH$

(B) PbTx-2-type brevetoxins:

PbTx-2:	R_1=H,	R_2=$CH_2C(=CH_2)CHO$
PbTx-3:	R_1=H,	R_2=$CH_2C(=CH_2)CH_2OH$
PbTx-5:	R_1=Ac	R_2=$CH_2C(=CH_2)CHO$
PbTx-6 :	R_1=H	R_2=$CH_2C(=CH_2)CHO$ (27, 28 epoxide)
PbTx-8:	R_1=H	R_2=CH_2COCH_2Cl
PbTx-9:	R_1=H	R_2=$CH_2CH(CH_3)CH_2OH$

For the most part, detection of the brevetoxins has been by means of *in vivo* bioassays based upon the mouse bioassay of McFarren or the fish bioassay of Baden *et al.*[7,8]. While useful in determining the general toxicity of a sample, these assays possess neither the specificity nor the sensitivity desired in a research setting. Further, today's climate of reduced acceptance by both the

general public and the scientific community of the use of animals in research, coupled with increasingly stringent regulation of such research, has made the development of sensitive, specific *in vitro* assays even more desirable.

In an initial attempt to produce a radioimmunoassay, Baden *et al.* described a polyclonal antiserum against the brevetoxins[9]. This serum bound PbTx-2 and PbTx-3 with equal affinity, and showed about 50% cross-reactivity with brevetoxins containing the PbTx-1 backbone structure[10]. Competition curves demonstrated a significant decrease in antibody binding to [^{3}H]PbTx-3 in the range of 2-5 ng unlabeled PbTx-3 in phosphate-buffered saline. However, no data were presented regarding detection limits in the presence of biological fluids or tissue extracts, and cross-reactivity with other marine toxins was not addressed. This group is now developing an enzyme-linked immunosorbent assay (ELISA) using this antiserum, with encouraging results (Baden, personal communication)[11].

We report here the production of specific antibodies and development of a radioimmunoassay for the brevetoxins. The assay is specific for those toxins containing the PbTx-2-type polyether backbone structure and little cross-reactivity occurred with PbTx-1. No cross-reactivity was observed with other marine toxins known to accumulate in shellfish. We further demonstrate that the assay is equally effective in assaying crude shellfish extracts and human urine.

The successful development of a radioimmunoassay for the brevetoxins in biological samples is an important advance in dinoflagellate toxinology. We feel it also holds great potential for monitoring shellfish stocks and the diagnosis and epidemiology of neurotoxic shellfish poisoning.

Materials and Methods

Toxins

Unlabeled brevetoxins were supplied by Dr. D.G. Baden (University of Miami, FL). [^{3}H]PbTx-3 (9-14 Ci/mmol) was prepared and purified by Dr. Baden as previously described[6]. Each was in excess of 99% pure and exhibited a single peak of UV absorbance at 208 nm. Radiolabeled toxin was kept as a stock solution of 100 µg/ml in ethanol at -20°C. Unlabeled brevetoxins were stored in methanol (100 µg/ml) at -20°C. Potency was confirmed by the *Gambusia* bioassay[8].

Okadaic acid, pectenotoxin-6, and yessotoxin were a generous gift from Dr. Takeshi Yasumoto (Tohoku University, Sendai, Japan). Saxitoxin was supplied by Dr. Sherwood Hall (U.S. Food and Drug Administration, Washington, D.C.), and palytoxin was received from the Hawaii Biotechnology Institute (Aeia, HI).

Antigen Preparation and Immunization

PbTx-3 was conjugated to bovine serum albumin (BSA) according to a modified version of the procedure of Baden *et al.*[9]. PbTx-3 (2.5 mg plus approximately 0.01 μCi [^{3}H]PbTx-3 as a tracer for the reaction) was dissolved in 0.1 ml freshly distilled pyridine containing a 10-fold molar excess of succinic anhydride. The reaction vessel was sealed and the solution stirred in a water bath at 85°C for 2 hr. The reaction mixture was then dried under a stream of nitrogen and streaked onto a 10 × 20 cm silica gel high performance thin-layer chromatography plate (Whatman High Performance Diamond System, HP-KF, 200 μm layer, Whatman Chemical Separations, Inc.). Development in a solvent system of chloroform/methanol/trifluoroacetic acid (100:10:1) resolved two major bands. The predominant broad band at Rf=0.27-0.35 contained 85-90% of the tracer radioactivity and corresponded to the PbTx-3 hemisuccinate derivative. This fraction was scraped, eluted with methanol, and dried in a reaction vial.

The free carboxyl group on the succinylated PbTx-3 derivative was covalently coupled to the e-amino groups on the lysine residues of BSA by a standard carbodiimide condensation[12]. Dry succinate derivative was dissolved in 50% aqueous pyridine at 50 mg/ml. Carbodiimide reagent (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide, 100 mg/ml in 50% aqueous pyridine was added in 10-fold molar excess and the mixture was stirred gently at room temperature for 1 hr. Bovine serum albumin (20 mg/ml in distilled water) was then added dropwise in an equimolar ratio of toxin/lysine residues and the reaction mixture stirred gently overnight at room temperature. The resulting cloudy solution was dialyzed for 24 hours against several changes of 0.9% NaCl and the antigen concentration adjusted with normal saline to 0.5-1 mg PbTx-3/ml.

An adult female goat was administered toxin/BSA conjugate containing 0.25-0.5 mg conjugated PbTx-3 via intramuscular injection in Freund's complete adjuvant. Injections were given in 1 ml total volume, half administered into each hind leg. After the initial dose, subsequent boosts were given biweekly in Freund's incomplete adjuvant. Blood samples were taken beginning 8 weeks post-immunization and analyzed for PbTx-3 binding activity.

Characterization of Anti-PbTx-3 Binding Activity

Serum was diluted 1:20,000 with phosphate-buffered saline (PBS), pH 7.4, containing 0.01% emulsifier (Emulphor EL-620, GAF Corp.). Triplicate tubes containing antiserum and increasing concentrations of [^{3}H]PbTx-3 were incubated 2-4 hours at 4°C. Nonspecific binding of toxin to serum constituents was measured by using parallel tubes containing pre-immune serum matched for total protein concentration. After incubation, 0.5 ml of a suspension of 2 mg/ml dextran-coated charcoal was added. The tubes were mixed, incubated 15 min at 4°C, and centrifuged 15 min at 1500 × g. Aliquots of 0.75 ml of the clear

supernatant were transferred to scintillation vials, acidified with 50 µL 1N HCl, and counted in a Beckman liquid scintillation counter with a counting efficiency of 45%. At each concentration of radiolabeled PbTx-3, specific binding was calculated as the difference between total and nonspecific binding. Rosenthal plots were constructed by plotting bound/free vs bound DPM at each label concentration, and binding affinity (KD) and binding capacity (B_{max}) were estimated from these data.

Competition curves, including standard curves for PbTx-l, PbTx-2, PbTx-3 and PbTx-9, were constructed by incubating antiserum with increasing amounts of unlabeled competitor in the presence of a constant [^{3}H]PbTx-3 concentration. The amount of radiolabeled toxin bound in the supernatant was quantified as above and plotted versus competitor concentration. Each point was plotted as the mean of triplicate determinations. Variation from the mean was typically less than 3%. Toxin concentrations in unknown samples were determined by comparison with standard curves.

Solid-phase Extraction of Urine

Urine samples were processed over a solid-phase extraction (SPE) column prior to analysis. Up to 5 ml of urine was loaded onto a 6 ml SPE column (C18 BondElut, Analytichem International, Harbor City, CA) and pushed through with a syringe at a rate of approximately 3 ml/min. The column was washed once with 3 ml of water, again with 3 ml of 70% methanol, and the sample eluted with 3 ml of 100% methanol. The methanol fraction was then evaporated to dryness and redissolved in assay buffer to the original sample volume.

Preparation of Shellfish Extracts

Clams (*Mercenaria mercenaria*) were purchased fresh at a local grocery store. The clam was removed whole from the shell, weighed and homogenized to a thick slurry with a Brinkmann tissue homogenizer in a minimum volume of distilled water. This slurry was extracted three times with two volumes of acetone by stirring for 15-20 min at room temperature followed by filtration through Whatman #1 filter paper. The filtrates were pooled and evaporated to dryness under vacuum. The residue was dissolved in 90% methanol (2 ml/g original tissue weight) and liquid-liquid partitioned three times with equal volumes of light petroleum ether (BP 30-60°C). The ether fraction was discarded and the 90% methanol fraction again evaporated to dryness. The residue was taken up in 100% methanol at 1 ml/g original tissue weight, sealed in serum vials and stored at -20°C.

Data Analysis

All radioimmunoassay data, including the generation of best-fit standard curves, were analyzed with the IBM PC Radioimmunoassay Data Reduction System, version 4.11[3].

Results

The conjugation of PbTx-3 to BSA as described here resulted in a highly immunogenic product. The overall conjugation efficiency of the two-step process ranged from 37-49%, and the molar ratio of toxin/BSA in the final product ranged from 7.6-13.9 (n=4). Higher conjugation efficiencies were achieved when we used freshly recrystallized succinic anhydride in the succinylation step.

Anti-PbTx-3 antibodies appeared rapidly in the goat serum after the initial immunization. By the first bleed at week 8, the binding capacity had reached 0.5 nmoles PbTx-3/ml serum, where it remained constant for several weeks. After cessation of boosts at week 10, the binding capacity remained constant for 6 weeks before rapidly diminishing to very low levels. Resuming boosts at this time resulted in a rapid increase to approximately twice the

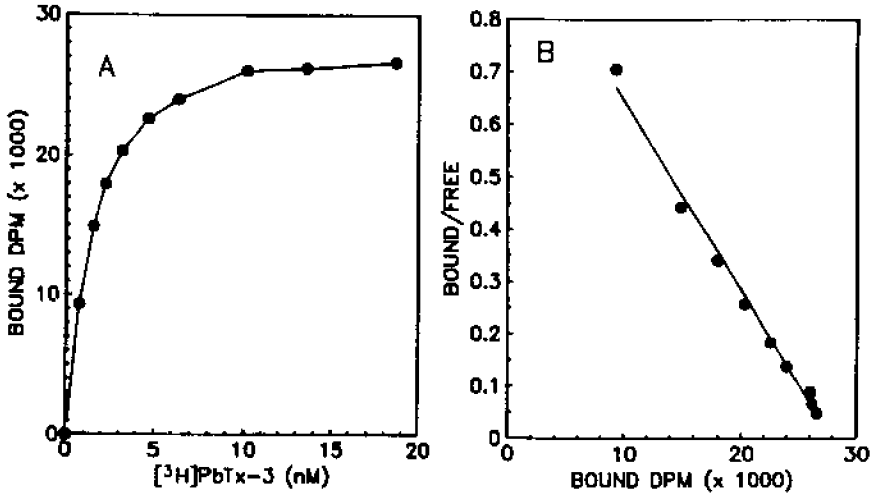

Figure 2. Characterization of PbTx-3 Binding Activity in Goat Antiserum. Serum was diluted 1:20,000 with PBS and binding at increasing [^{3}H]PbTx-3 concentrations determined as described in Materials and Methods. Plotted points are means of triplicate determinations. Variation from the mean was typically 3% or less. (A) Saturability of [^{3}H]PbTx-3 binding. (B) Rosenthal analysis of binding data. In this representative experiment, K_D= 0.8 nM, B_{max}= 8 µg/ml.

previous levels. A second "resting" of the animal for several months resulted in an even greater rebound effect. Estimates of the binding capacity of the later bleeds by Rosenthal analysis of binding data were 6.7-8.9 nmoles/ml serum (Figure 2B).

Antiserum Affinity and Specificity

Binding curves (Figure 2A) demonstrated the saturability of [^{3}H]PbTx-3 binding. Rosenthal analysis (Figure 2B) indicated a single class of binding

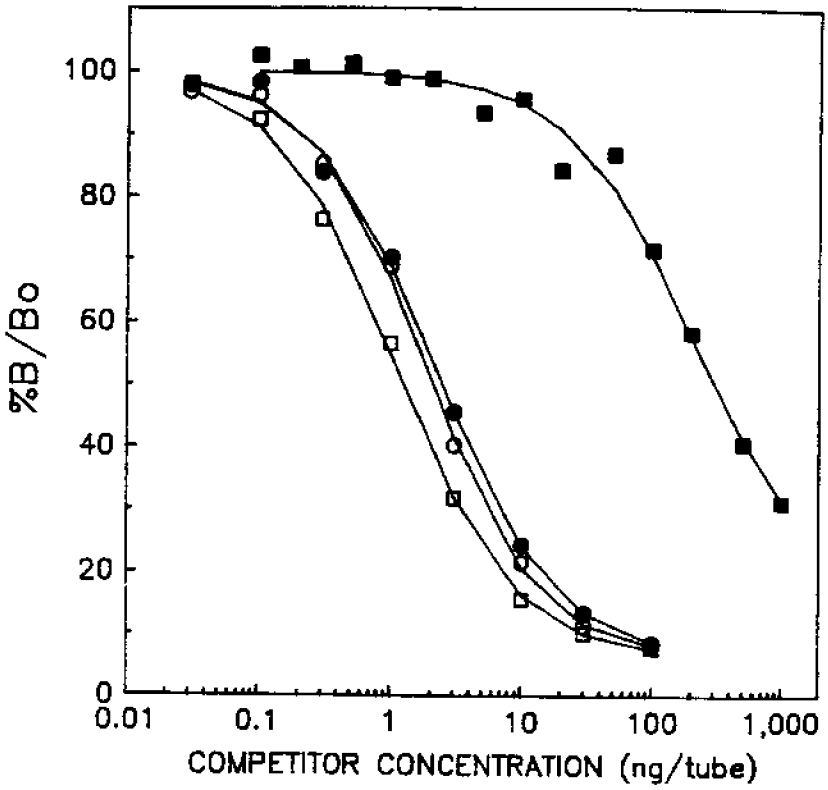

Figure 3. Standard Competition Curves for PbTx-1 and PbTx-2-Type Brevetoxins. Binding of [^{3}H]PbTx-3 was determined as described in Materials and Methods in the presence of increasing amounts of competitor toxins. Plotted points are means of triplicate determinations. Variation of the mean was typically 3% or less; error bars have been omitted for clarity. Lines are computer generated best-fit curves. PbTx-2-type toxins: (○) PbTx-2 (slope = 1.01, ED_{50}= 1.81 ng), (●) PbTx-3 (slope = 0.93, ED_{50}= 2.13 ng), (□) PbTx-9 (slope = 0.95, ED_{50}= 1.08 ng). Toxin containing the PbTx-1-type backbone: (■) PbTx-1 (slope =0.91, ED_{50}= 200 ng).

sites with an apparent affinity constant (K_D) of 1 nM (0.5-2.1 nM, n=11). Standard competition curves for PbTx-2, PbTx-3, and PbTx-9 (Figure 3) showed these toxins to be nearly equipotent in their ability to inhibit the binding of labeled toxin by the antiserum. The linear portion of the standard curves lay between 0.3-10 ng unlabeled toxin/assay tube.

The competitor concentrations necessary to inhibit 50% of [^{3}H]PbTx-3 binding (IC_{50}) were calculated from the computer-generated best-fit curves (Figure 3). The IC_{50} values calculated for PbTx-2, PbTx-3, and PbTx-9 were 1.81 (±.08), 2.13 (±.11), and 1.08 (±.04) ng/tube, respectively. In contrast, the IC_{50} value calculated for competition by PbTx-1 was 200 (±13) ng/tube. The linear portion of all curves had a slope of 1 (±.09).

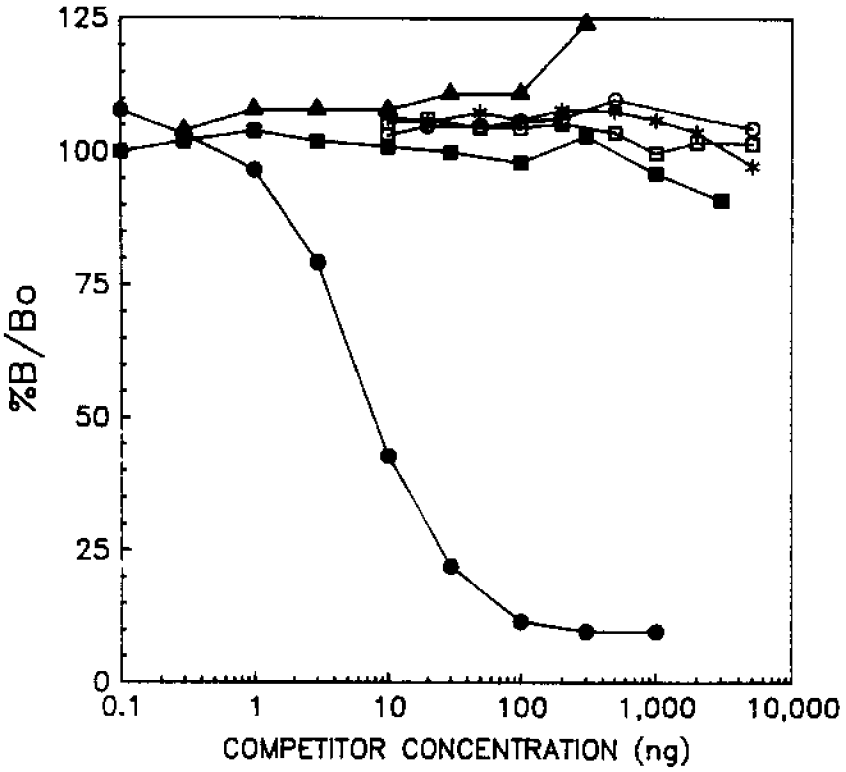

Figure 4. Lack of Cross-Reactivity of Anti-PbTx Serum with Other Marine Toxins. Binding of [^{3}H]PbTx-3 was determined as described in Materials and Methods in the presence of increasing amounts of other marine toxins. Plotted points are means of triplicate determinations. Variation from the mean was typically 3% or less; error bars were omitted for clarity. (*) okadaic acid, (□) pectenotoxin-6, (○) yessotoxin, (■) saxitoxin, (▲) palytoxin, (●) PbTx-3 control.

Competition experiments with the diarrhoeic shellfish toxins, okadaic acid, pectenotoxin-6, and yessotoxin, the paralytic shellfish toxin saxitoxin, and the zoanthid toxin palytoxin (Figure 4) demonstrated no inhibition of [^{3}H]PbTx-3 binding by any of these toxins over the concentration ranges tested.

Radioimmunoassay of Urine

Greater than 5% urine in the assay tubes interfered significantly with the assay (data not shown). Processing the urine over a C_{18} SPE column was necessary to eliminate this interference. Control experiments with urine spiked with trace amounts of radiolabeled PbTx-3 demonstrated negligible loss of radiolabel in the water and 70% methanol wash steps, and >99% of the administered radiolabel eluted with 100% methanol. Interfering contaminants

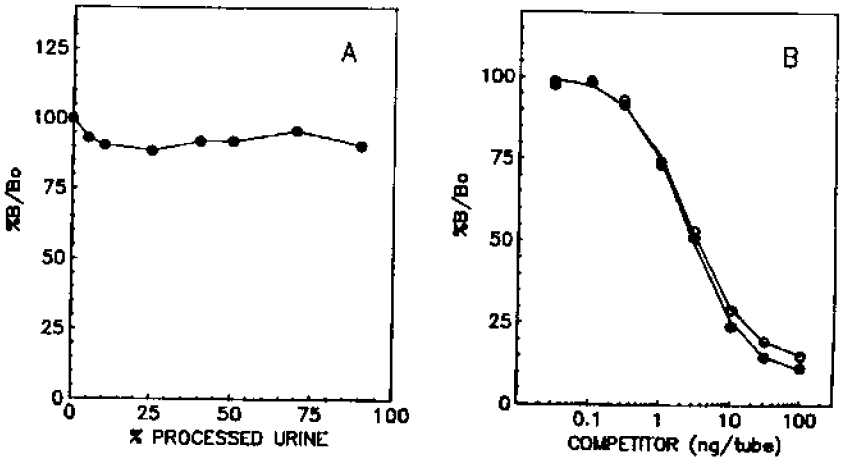

Figure 5. Effect of Processed Urine on Brevetoxin Radioimmunoassay Sensitivity. Human urine was processed through a C_{18} solid-phase extraction column as described in Materials and Methods to remove interfering substances. (A) The effect of increasing amounts of processed urine on [^{3}H]PbTx-3 binding by the antiserum. (B) Standard competition curves for unlabeled PbTx-3 in the presence of (●) PBS (slope = 1.08, ED_{50} = 2.4 ng) or (○) 50% processed urine (slope = 1.07, ED_{50}= 2.4 ng). Plotted points are means of triplicate determinations. Variation from the mean was typically 3% or less; error bars were omitted for clarity. Standard curves are computer-fitted.

were effectively removed by this process. No significant binding interference was detected in assays containing up to 90% processed urine (Figure 5A). A standard competition curve generated in the presence of 50% processed urine (Figure 5B) demonstrated no change in the calculated IC_{50} when compared a control curve generated in PBS (2.44 ng vs. 2.41 ng, respectively).

The urine of a normal human volunteer (M.P.) was used to assess the overall accuracy of the procedure for urine analysis. Urine was spiked with PbTx-2 at 5, 2.5, and 1.25 ng/ml prior to SPE processing. The values determined by radioimmunoassay were 4.3 (±0.2), 2.3 (±0.2), and 1.4 (±0.1) ng/ml, respectively (Table 1).

Table 1. Brevetoxin Immunoassay: Analyses of Spiked Samples in Urine and Shellfish Extracts.

Matrix	Toxin	Concentration	RIA Result
Urine	PbTx-2	5 ng.ml	4.3 (±0.2)
		2.5 ng/ml	2.3 (±0.2)
		1.25 ng/ml	1.4 (±0.1)
Clam Extract	PbTx-3	10 µg/g	10.8 (±0.6)
		5 µg/g	5.6 (±0.5)

Radioimmunoassay of Shellfish Extracts

Crude shellfish extract was used directly in the assay without SPE treatment. No significant interference occurred at dilutions greater than 1:30 (33 mg tissue equivalents/assay tube) (Figure 6A). Standard curves generated in PBS, SPE-processed, and crude extract (10 mg tissue equivalents/assay tube) had identical slopes and IC_{50} values (Figure 6B). Analysis of crude clam extract spiked with 5 and 10 µg PbTx-3/g tissue yielded toxin values of 5.6 (±0.5) and 10.8 (± 0.6) ng/ml, respectively (Table 1).

Discussion

Saturation curves and Rosenthal analysis of binding data indicated a single class of antibodies with an apparent affinity constant (K_D) of approximately 1 nM. As the antiserum is polyclonal, this suggests the predominance

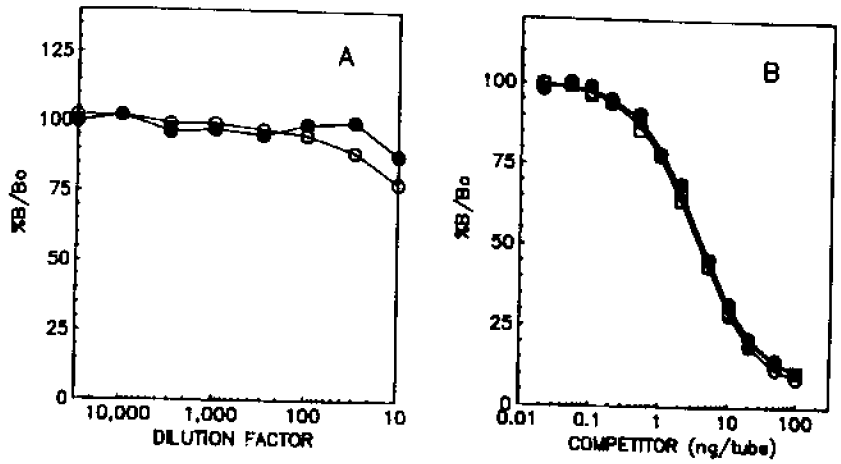

Figure 6. Effect of Shellfish Estract on Brevetoxin Radioimmunoassay Sensitivity. Crude clam (*Mercenaria mercenaria*) extracts were prepared as described in Materials and Methods and dissolved in methanol (1 g original tissue/ml). (A) The effect of crude clam extract (●) or SPE-processed clam extract (○) on [^{3}H]PbTx-3 binding by the antiserum. (B) Standard curves for unlabeled PbTx-3 in the presence of (○) PBS (slope = 1.05, EC_{50} = 3.33 ng), (●) crude clam extract (slope = 1.02, EC_{50}= 3.55 ng), or (□) SPE-processed extract (slope = 0.99, EC_{50}= 3.10 ng). Final extract dilutions were 1:100 (10 mg original tissue equivalents/tube). Plotted points are means of triplicate determinations. Variation from the mean was typically 3% or less; error bars were omitted for clarity. Curves are computer-fitted.

of a single class or the simultaneous occurrence of multiple classes with affinity constants similar enough that they cannot be resolved by Rosenthal analysis. The binding capacity (B_{max}) derived from the Rosenthal analysis reached 6.7-8.9 nmoles/ml serum (7-8 µg/ml serum) in the latest bleeds. This allowed the assay to be run at antiserum dilutions of up to 1:25,000. Varying the dilution from 1:10,000 to 1:25,000 did not affect assay sensitivity (data not shown). Typically, assays were run at 1:10,000 dilution to minimize sample counting times.

The minimum detection limits, as determined by the linear portions of the standard competition curves for PbTx-2, PbTx-3 and PbTx-9, were approximately 300 pg/assay tube. While other PbTx-2-type brevetoxins have yet to

be evaluated in this system, significant cross-reactivity with PbTx-5, PbTx-6, and PbTx-8 is expected, as the differences between all of these toxins lie at or near the site of conjugation to the protein carrier[9]. Conversely, PbTx-1 was bound to a much lesser degree (minimum detection limit approximately 30 ng/ml), probably due to the different backbone structure. We hypothesize similar results with PbTx-7 and PbTx-10.

The assay described here appears to be specific for the brevetoxins. No other marine toxin tested cross-reacted with this serum. Especially notable in this regard were the diarrhoeic shellfish toxins pectenotoxin-6, yessotoxin, and okadaic acid, each of which possesses some cyclic polyether character, and are known to accumulate in shellfish. Lack of cross-reactivity by saxitoxin and palytoxin was expected because neither toxin shares structural similarity to the brevetoxins. Still to be tested are the ciguatera toxins, including the ciguatoxins and maitotoxins, as well as the new and as yet only partially characterized toxins of *Ostreopsis lenticularis*[14]. These compounds will be investigated as they become available.

We believe this assay holds great promise for the detection of brevetoxins in shellfish stocks. Experiments with clams artificially exposed to *P. brevis* cultures in the water column under laboratory conditions are underway. Further, the ability to detect these toxins in human urine suggests potential for the diagnosis of neurotoxic shellfish poisoning and for monitoring or confirming human exposure to the brevetoxins.

Literature Cited

1. Poli, M.A. 1988. Laboratory procedures for detoxification of equipment and waste contaminated with brevetoxins PbTx-2 and PbTx-3. J. Assoc. Off. Anal. Chem. 71:1000-1002.
2. Tester, P.A., R.P. Stumfp and P.K. Fowler. 1988. Red Tide, the first occurrence in North Carolina waters: an overview. In: Proc. Oceans '88 Conf. (Institute of Electrical and Electronics Engineers, and Marine Technological Society, Sponsors), IEEE, New York. pp. 808-811.
3. Music, S.I., J.T. Howell and C.L. Brumback. 1973. Red tide: its public health implications. J. Fla. Med. Assoc. 60:27-29.
4. Steidinger, K.A. and E.A. Joyce, Jr. 1973. Florida red tides. Florida Dept. Nat. Resources Ed. Ser. No. 17, Fla. Dept. Nat. Res., St. Petersburg.
5. Baden, Daniel G. 1989. Brevetoxins: unique polyether dinoflagellate toxins. FASEB J. 3:1807-1817.
6. Poli, M.A., T.J. Mende and D.G. Baden. 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. Mol. Pharmacol. 30:129-135.
7. McFarren, E.F., H. Tanabe, F.J. Silva, W.B. Wilson, J.E. Campbell and K.H. Lewis. 1965. The occurrence of a ciguatera-like poison in oysters,

clams, and *Gymnodinium breve* cultures. Toxicon 3:123.

8. Baden, D.G., T.J. Mende and R.E. Block. 1979. Two similar toxins isolated from *Gymnodinium breve.* In: Toxic Dinoflagellate Blooms (D.L. Taylor, and H.H. Seliger, eds.). Elsevier New York. pp. 327-334.
9. Baden, D.G., T.J. Mende, J. Walling and D.R. Schultz. 1984. Specific antibodies directed against toxins of *Ptychodiscus brevis* (Florida's red tide dinoflagellate). Toxicon 22:783-789.
10. Baden, D.G., T.J Mende, A.M. Szmant, V.L. Trainer, R.A. Edwards and L.E. Roszell. 1988. Brevetoxin binding: molecular pharmacology versus immunoassay. Toxicon 26:97-103.
11. Trainer, V.L. and D.G. Baden. 1989. Enzyme immunoassays of brevetoxins. In: Toxic Marine Phytoplankton, (E. Granelli, B. Sundstrom, L. Elder and D.M. Anderson, eds.). Elsevier, New York. pp 430-435.
12. Abraham, G.E. and P.K. Grover. 1971. Covalent linkage of steroid hormones to protein carriers for use in radioimmunoassay. In: Competitive Protein Bindinq Assays ,(W.O.. Odell and W.H. Daughaday, eds.). J.B. Lippincott, Philadelphia. pp. 140-148.
13. Rodbard, D., P.J. Munson and A. De Lean. 1978. Improved curve-fitting, parallelism testing, characterization of sensitivity and specificity, validation, and optimization of radioligand assays. In: Radioimmunoassay and Related Procedures in Medicine, Vol.1. International Atomic Energy Agency, Vienna. pp. 469-504.
14. Tindall, D.R., D.M. Miller and P.M. Tindall. 1990. Toxicity of *Ostreopsis lenticularis* from the British and United States Virgin Islands. In: Toxic Marine Phytoplankton, (E. Granelli, B.G.G. Sundstrom. L. Elder and D.M. Anderson, eds.). Elsevier, New York. pp- 424-429.

Ecology and Fisheries

Excretion of Ciguatoxin from Moray Eels (Muraenidae) of the Central Pacific

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Abstract

Ciguatera is a disease in humans caused by consumption of otherwise edible fishes that have become contaminated with ciguatoxin through the marine food chain. A long-standing dogma in ciguatera research is that once contaminated, fishes retain the original level of toxicity for many years. In this study we analyzed the pattern of change in toxicity of viscera of 218 moray eels, *Lycodontis javanicus* (Bleeker) collected from Teaoraereke, Tarawa, in the Republic of Kiribati over a 500 day period. Our results indicated that the concentration of ciguatoxin (per g of viscera) declined exponentially over the 500 day period of this study ($p<0.001$). All 47 samples from Teaoraereke tested toxic to mice, indicating that recruitment of non-toxic eels could not explain this decrease in toxicity. The half-life for ciguatoxin efflux from the viscera of moray eels was determined to be 264 days. Over the 500-day period, the size of the viscera did not vary significantly. Interestingly, no relationship could be established between the toxicity of viscera and viscera weight. The half-life determined in our study assumes that ciguatoxin input into eels stopped prior to the start of our collections. The calculated efflux rate will underestimate the actual efflux rate if the input of ciguatoxin did not stop at this time but was merely reduced. The efflux rate of ciguatoxin is likely to vary between fish species (and perhaps even between individuals of a species) and may in part determine the ciguatera risk of a species. The decline in viscera toxicity paralleled a reduction in the number of ciguatera cases in Tarawa. Ciguatoxin was separated from a minor, less polar form of ciguatoxin by high performance liquid chromatography. The implications of these results are discussed and a model describing the ciguatera risk of a fish species is proposed.

Introduction

Humans in tropical and sub-tropical areas that eat fish are at risk from ciguatera. This risk stems from the ability of otherwise edible fishes to become contaminated with ciguatoxin through the marine food chain[1-3]. The

structure of ciguatoxin in moray eels has been elucidated[3]. Ciguatoxin apparently arises from a less-polar form of ciguatoxin produced by wild *Gambierdiscus toxicus* which is oxidized (three times) to ciguatoxin as it passes through the marine food chain[3].

Many species of herbivorous, detritivorous, omnivorous and carnivorous fishes have been implicated in ciguatera[4-9]. However, certain fishes, i.e. *Lutjanus bohar* Forsskål, *Lycodontis javanicus* Bleeker, *Scomberomorus commerson* Lacepede, *Sphyraena spp.* and *Epinephelus spp.*, are most likely to cause ciguatera in particular areas. Outbreaks of ciguatera are often biphasic in nature with an initial upsurge followed by a gradual decline in the incidence of ciguatera in an area[9,10]. In this paper we report the potential for the excretion (decay) of ciguatoxin in fishes and propose a model that can account for increases or decreases in ciguatera risk within and between fish species. This conclusion stems from an analysis of viscera toxicity in a demersal fish, the moray eel (*Lycodontis javanicus*) collected from Tarawa, Republic of Kiribati, an area where ciguatera has been endemic for several decades (Tebano and McCarthy, unpublished results).

Materials and Methods

Eel collection

Moray eels (*Lycodontis javanicus*) were captured in fish-baited cage traps set at various locations on ocean reefs adjacent to the island of Tarawa (Figure 1). The viscera (including liver) of each eel was removed and stored frozen prior to air dispatch to Brisbane, Australia. On arrival in Brisbane viscera were still in either a chilled or frozen state. Viscera were pooled (n=1-18) to a convenient sample weight for extraction (0.3 to 1.0 kg). Each sample of viscera contained only viscera of similar size to allow the relationship between size and toxicity to be determined. The date of arrival of viscera in Brisbane was recorded as the collection date and represented the collection of eels up to one month prior to that date. During this study, eels captured from the ocean reef adjacent to the villages of Teaoraereke, Bikenibeu, Bariki and Betio (Figure 1) were found to be similarly toxic. However, a pooled sample of viscera from five eels from the ocean reef adjacent to Tanea did not contain detectable ciguatoxin. The flesh of one eel from Teaoraereke tested nontoxic. This study reports the analysis of viscera toxicity of 217 eels (47 pooled samples) collected from the ocean reef adjacent to Teaoraereke in the center of the toxic zone on Tarawa. Eels were obtained from nine collection dates over a 500-day period.

Extraction of ciguatoxin

Viscera samples (n=47) were thawed and cooked in a plastic cooking bag and then refrozen prior to extraction. Cooking denatured proteins that oth-

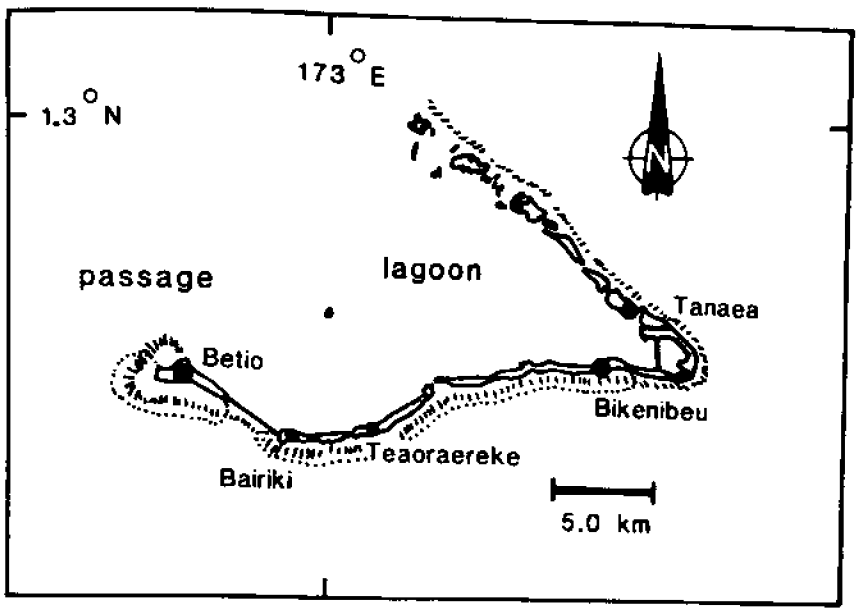

Figure 1. Eel Collection Site.
Map of the southern half of Tarawa, Republic of Kiribati. The dashing indicates the outer barrier reef. The areas reported toxic in 1983 are indicated by the dotted line (adapted from Tebano and McCarthy, unpublished). Eels were collected initially from outer reefs adjacent to the five villages indicated. Only results of toxicity testing of eels collected on outer reefs adjacent to Teaoraereke from September 1987 to January 1989 were used in this study.

erwise hampered homogenization in acetone. Frozen samples were chopped into small pieces and minced in a hand-operated meat mincer to yield a fine slurry. Ciguatoxin was extracted from the viscera with acetone for 15 minutes using an air powered homogenizer (Ystral) fitted with 20 T shaft. Each viscera sample was extracted twice with acetone at room temperature. A third extraction with acetone yielded <0.15% of total toxicity[11].

Partial purification of ciguatoxin

Two liquid-liquid partitioning schemes were compared to determine the most efficient method for isolating ciguatoxin. Both schemes first extracted a 90% aqueous methanol phase with hexane (Figure 2). Method I then used the standard diethyl ether-water partitioning, while method II (Figure 2) used a modification of this method (25% ethanol added to the aqueous phase) suggested by Vernoux *et al.*[12]. For this purpose a minced sample of viscera was divided into two equal portions for extraction. After four diethyl ether extractions using method I, a total of 0.9 g of lipid and 1,160 mouse units (m.u.) of ciguatoxin were recovered. Four additional diethyl ether extractions yielded

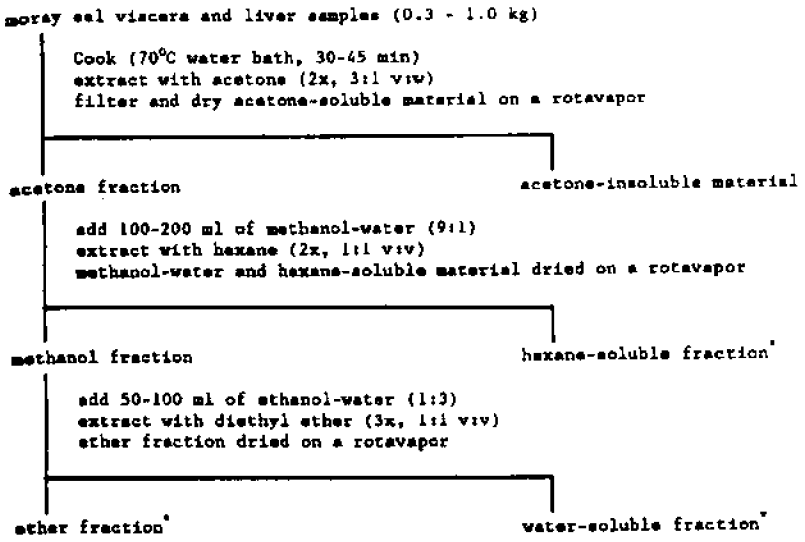

Figure 2. Extraction of Eel Ciguatoxin.
Procedure used for the extraction and partial purification of ciguatoxin. Fractions marked by an asterisk were tested for toxicity in mice.

0.3 g and 930 m.u. of ciguatoxin and an additional extraction of the aqueous phase using method II yielded 2.1 g and 145 m.u. of ciguatoxin. Extraction of the other portion by method II alone (three diethyl ether extractions) yielded 7.0 g and 2,750 m.u. of ciguatoxin. Consequently, method II (Figure 2) was used for all assays reported here. This method has the additional advantage of a rapid separation of the diethyl ether-25% aqueous ethanol phases compared with an often difficult separation of the diethyl ether-water phases (method I). Much of the high yield of lipid impurities obtained using Method II could be removed with -20°C acetone precipitation (acetone precipitate contained ~ 5% of lethality). The hexane and water phases contained no detectable ciguatoxin (at 1 g/kg dose) although the high yield of impurities in these fractions precluded a sensitive assay.

Characterization of toxin content of viscera

A portion of the diethyl ether fraction was purified to homogeneity by low pressure and high pressure liquid chromatography (HPLC). Fast atom bombardment (FAB) mass spectral measurements were performed on two toxic fractions separated by HPLC.

Bioassay

A portion (1-5 mg) of each diethyl ether fraction was suspended in 0.5 ml 5% Tween 60 saline and assayed in duplicate by i.p. injection into 20 ± 2 g Quackenbush mice of either sex. For each mouse the signs and the time to

death were recorded. The relationship between dose and time to death was used to quantify each fraction and is approximated by:

$$\log \text{m.u.} = 2.3 \log (1 + T^{-1})$$

where, m.u. = number of mouse units of ciguatoxin injected and T = time to death in hours[11].

One m.u. is the LD50 dose of ciguatoxin for a 20 g mouse. Based on the data of Tachibana one m.u. = 9 ng ciguatoxin[20]. Hexane and 25% aqueous ethanol soluble fractions were intermittently assayed in mice at doses up to 1 g/kg.

Statistical analysis

Data are expressed as the mean ±1 population standard deviation. Linear regression analyses were performed on unweighted data or on data weighted with the number of fish per sample.

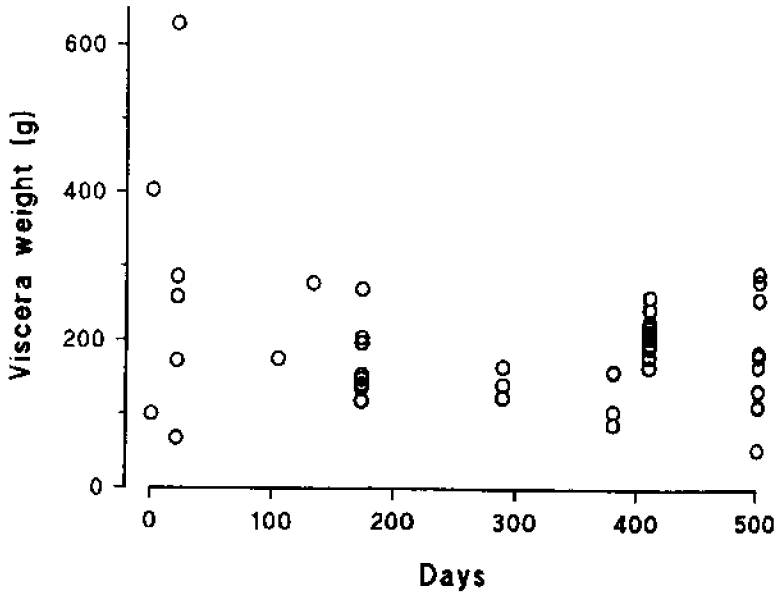

Figure 3. Eel Viscera Weights vs. Time.
Average weight of moray eel viscera collected over a 500 day period. Data represent the average viscera weight (sample weight/number of fish per sample) for each of 47 samples obtained during nine collections. Viscera weight did not change significantly over time. Eel collections commenced in September, 1987.

Results

Moray eel collection

Whole viscera from 217 moray eels, collected over a 500-day period from one ciguatera-prone site on Tarawa were pooled into 47 samples. The average weight of whole individual eels from a subsample of 38 eels was 3.6 ±2.3 kg with individual weights ranging from 0.6 kg to 10 kg. A total of 35.9 kg of pooled eel viscera were collected from the 217 eels. Figure 3 shows the average viscera weight for each of the 47 pooled samples. No significant change occurred in the average weight of viscera collected over the period of this study.

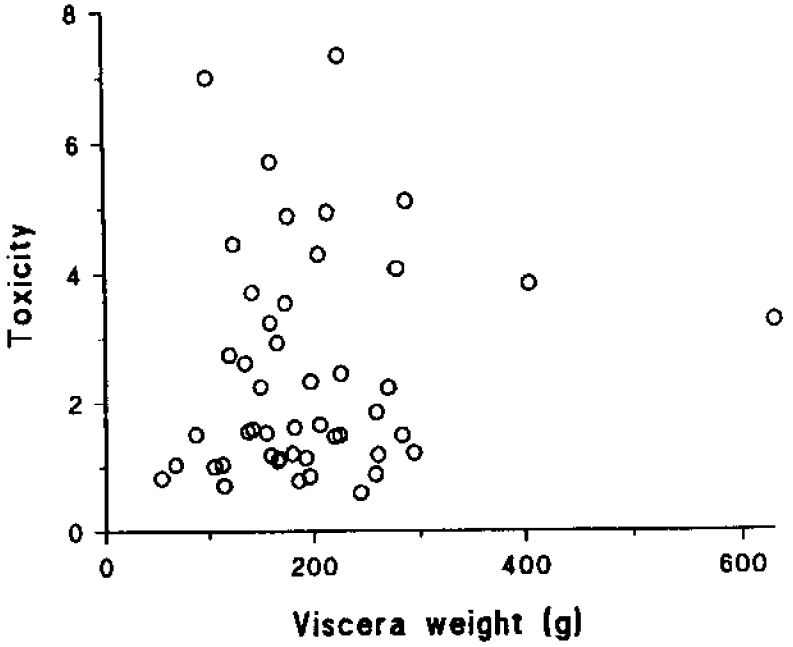

Figure 4. Eel Viscera Toxicity vs. Viscera Weight.
Sample toxicity (mouse units per g viscera) vs. average viscera weight. Data represent the toxicity of the 47 samples collected during the study period.

Viscera toxicity

A total of 99,200 m.u. of ciguatoxin was extracted from 35.9 kg of eel viscera. The average toxicity was 2.43 ± 1.69 m.u. per g viscera (n=47) and ranged from 0.59 to 7.3 m.u. per g. No significant relation was found between toxicity and average viscera weight (Figure 4). The toxicity of viscera (n=47) was found to decline significantly over the 500-day period of the collections (Figure 5). This decline was significant for both weighted (using number of fish per sample) or unweighted data. The weighted negative

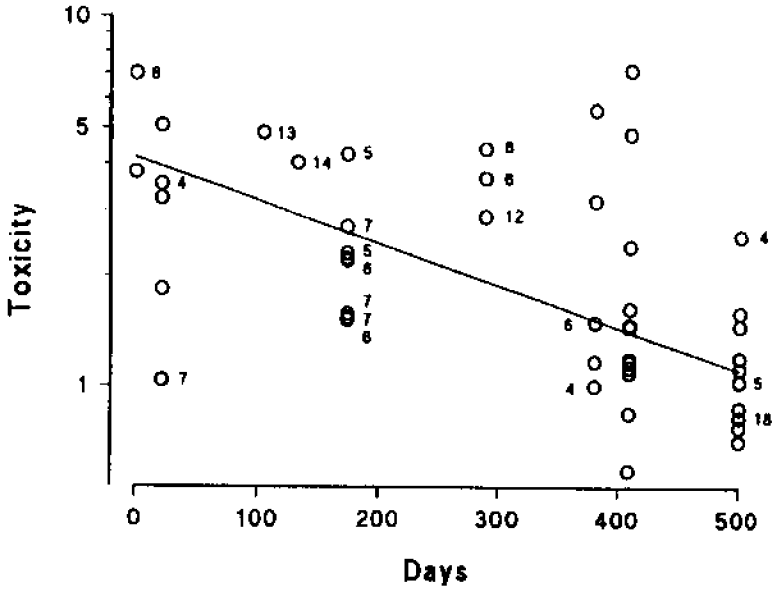

Figure 5. Eel Viscera Toxicity vs. Time.
Toxicity (mouse units per g viscera) of eel viscera over a 500 day period. Note log scale for y axis. Numbers adjacent to each data point indicate the number of fish pooled for that sample, Samples of 3 or less are unmarked for clarity. Toxicity declined significantly over the 500 day period (n=47, $p<0.001$). Eel collections commenced in September 1987.

linear regression of log toxicity vs time is approximated by:

$$\log y = 0.62 - 0.00114x \quad (p<0.001)$$

where y = toxicity (mouse units/g viscera) and x = days from start of collection.

The equation for the weighted data was chosen as the most appropriate as it takes into account the high variability in viscera toxicity. The exponential relationship for efflux (decay) was chosen as the simplest model to explain the observed decrease in toxicity. The slope of this regression estimated that the half-life for the efflux (decay) of ciguatoxin stored in the viscera was 264 days. Averaging the toxicity for each collection date (n=9) estimated the half-life for the decay of ciguatoxin was 316 days. All 47 samples contained detectable levels of ciguatoxin and no seasonal fluctuations in toxicity were evident.

Characterization of the toxin content of viscera

Two toxins were isolated by reverse phase HPLC (Figure 6). The more-

polar toxin was the major component representing approximately 90% of total toxicity. This toxin chromatographed similar to ciguatoxin from the Spanish mackerel, *Scomberomorus commerson* and was regarded as ciguatoxin[13]. The approximate MH^+ m/z of this toxin was determined to be 1111.3, similar to 1111.584 reported for ciguatoxin by Murata *et al.*[3]. The less-polar toxin induced signs in mice similar to ciguatoxin and had a MH^+ m/z of 1095.5, indicating it differed from ciguatoxin by the absence of one oxygen atom. Presumably, one hydroxyl group on ciguatoxin was missing in this

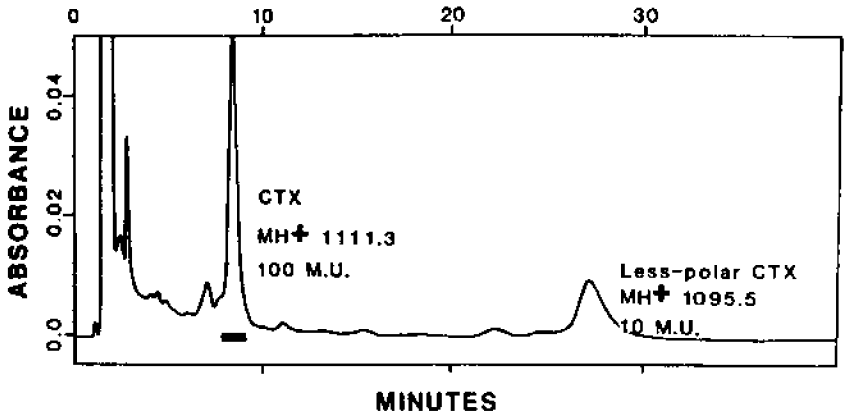

Figure 6. HPLC Analysis of Eel Ciguatoxin.

HPLC elution profile of a semi-purified fraction containing 120 mouse units (m.u.) of ciguatoxin (CTX). Chromatography was performed on a Hamilton PRP-1 reverse phase column (5µm, 150 x 4.1mm) eluted with acetonitrile-water (1:1) at 0.5 ml/min and monitored at 206 nm. Ciguatoxin was eluted at 8.4 minutes as a homogeneous peak indicated by the dark bar. A less-polar toxin eluted at 27 minutes as a broad homogeneous peak. The MH^+ m/z for ciguatoxin and the less-polar toxin was 1111.3 and 1095.5, respectively. The approximate number of mouse units (mu) is indicated for both toxins.

less polar toxin.

Discussion

The concentration of ciguatoxin in the viscera of a population of moray eels collected from one site within the toxic area of Tarawa was monitored over a 500-day period (1987-1989), using a modified extraction procedure and the mouse bioassay. In this period there was a significant exponential decline in the concentration of ciguatoxin in the eel viscera. The half-life for this decline in toxicity was estimated to be 264 days. We propose that this loss stems from excretion and/or decay of ciguatoxin. Excretion comprises the loss from eels of stored native ciguatoxin and decay comprises conversion of ciguatoxin to a less-toxic moeity within eels. The calculated half-life assumes

that the concentration of ciguatoxin in the diet of eels reduced to zero before the commencement of eel collections. The actual half-life will be shorter if the concentration of ciguatoxin in the diet of eels reduced only partially prior to these collections. Parrotfishes and surgeonfishes taken from reefs near Teaoraereke eight months after the last eel collection contained ciguatoxin. As these fishes can be part of the diet of moray eels it is likely the actual half-life for the excretion (decay) of ciguatoxin from eels is considerably shorter than 264 days[7]. Case history data on fish poisoning (including ciguatera) collected by the South Pacific Epidemiological and Health Information Service from 1973 to 1989 (Figure 7) indicate a general increase in the

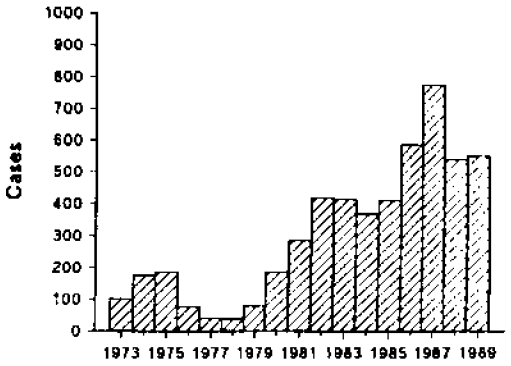

Figure 7. Annual Incidence of Fish Poisoning in the Republic of Kiribati. Data (1973 to 1989) provided by the South Pacific Commission Epidemiological and Health Information Services, and includes mostly cases of ciguatera as well as other forms of fish poisoning including histamine poisoning.

number of ciguatera cases over this period. The upsurge in ciguatera in 1986/87 may reflect a transient increase in ciguatoxin production that could explain the high levels of ciguatoxin found in eels at the start of the collection period reported here.

At least two other mechanisms could contribute to the observed reduction in eel toxicity. First, immigration of non-toxic eels may have ocurred as a result of our collections depleting eel stocks. This is considered unlikely for; (a) all 47 samples contained significant levels of ciguatoxin, (b) the collection site is in the center of a large toxic zone and, (c) our collections did not influence eel size over the collection period (assuming viscera weight is proportional to whole weight as found for other fish species)[14]. Eel growth would contribute to a reduction in the concentration of ciguatoxin in their tissues of eels. Quantifying the effect of growth was not possible, as data on the growth rate of moray eels were not available. To explain the reduction in concentration of ciguatoxin in eel viscera by growth alone, these eels would

have had to double in size in 264 days.

This study revealed that the concentration of ciguatoxin in the viscera of eels from Tarawa did not correlate with eel viscera weight. Lack of correlation presumably extends to whole-eel weight, as typically viscera weight correlates with whole weight for fishes[14]. Vernoux found a correlation between size and toxicity for *Caranx latus* Agassiz but not for the closely related *Caranx bartholomaei* Curier captured in the same area[15]. *L. bohar* was found to

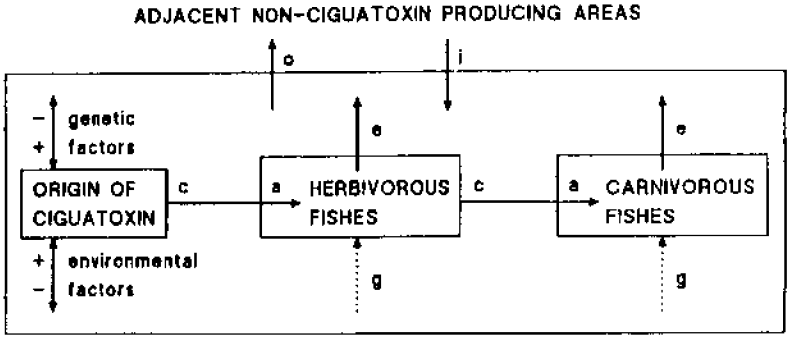

Figure 8. Ciguatera Risk: A Model

A model describing the ciguatera risk of herbivorous (including detritivorous and omnivorous fishes) and carnivorous fishes in an area producing ciguatoxin. The risk from ciguateric fishes is driven by the rate of ciguatoxin (CTX) production. This rate is increased (+) or decreased (-) by the influence of both genetic and environmental factors. The horizontal arrows indicate the transfer of ciguatoxin through the food chain from the benthos to herbivorous and carnivorous species. Factors influencing the concentration of ciguatoxin within and between fish species are indicated; c = concentration of CTX in the total diet of a particular species of fish; a = first pass efficiency of assimilation of CTX from the diet into the tissue of a particular fish species; o,i = rate of emmigration (including mortality) of particular toxic fish species and immigration of non-toxic fish species, respectively; e = rate of excretion (decay) of stored ciguatoxin from a tissue of a particular fish species (leading to a reduced CTX concentration); g = growth of individual fish species (leading to a dilution of CTX in fishes).

have a correlation between size and toxicity irrespective of location[10,16].

A model incorporating the influence of key variables (excretion, growth, first-pass assimilation efficiency, concentration of ciguatoxin in diet, immigration and emigration) is proposed to explain the ciguatera risk posed by fish species in a ciguatera-endemic area (Figure 8). The rate of production of ciguatoxin (and related compounds) by benthic dinoflagellate species including *Gambierdiscus toxicus* determines the overall level of ciguatoxin in the system and consequently the overall level of ciguatera risk[3]. Genetic and environmental factors are proposed as the key factors influencing ciguatoxin

production[9,17,18]. This model assumes that fishes assimilate ciguatoxin from their diet, as indicated by feeding studies with *Acanthurus xanthopterus* Valenciennes[18]. This is a basic requirement of the food chain hypothesis originally proposed by Randall[1]. However, no assessment of the efficiency of assimilation of ciguatoxin from the diet has been undertaken for any fish species. The potential to excrete ciguatoxin has been investigated for a few species. *L. bohar* may not excrete ciguatoxin, while *Sphyraena barracuda* (Walbaum) may excrete ciguatoxin more rapidly than eels[20,21]. Between-species variability in excretion, assimilation and the dietary concentration of ciguatoxin are likely to explain most of the variability in ciguatera risk between species. For each species the magnitudes of the variables presented in the model may specifically determine the relationship between fish size and toxicity. Within-species variability in these factors may explain why some individuals are more toxic than others.

This model can explain the different general patterns of ciguatera that are observed[10]. Both genetic and environmental factors influencing the periodicity of ciguatera risk and the import of new genetic material (perhaps a ciguatoxin-producing strain of *G. toxicus*) may explain the first appearance of ciguatera in an area. The rate of decline of ciguatera risk will be determined by factors including excretion, growth and mortality. This proposed model may be useful in the development of management strategies that may be implemented to reduce the ciguatera risk in an area.

Until procedures to detect toxic fishes become available that are suitable for large scale screening, the use of a bio-indicator species is desirable to measure the level of ciguatera risk in an area. Moray eels are a sensitive indicator of ciguatera and appear useful for the long-term monitoring of ciguatera. Herbivorous species are likely to be more useful bio-indicators if details of short-term changes in ciguatoxin production are required.

The eels from Tarawa contained at least two toxins. The major toxin is similar in molecular weight and chromatography to the major ciguatoxin from eels from French Polynesia and is similar in chromatography to ciguatoxin from Spanish mackerel[22,13]. A less polar form of ciguatoxin has also been isolated from Tarawa eels. This toxin differs in molecular weight from ciguatoxin by the loss of one oxygen (presumably by the loss of one hydroxyl group). This toxin may be the same as the less polar toxins previously reported to occur in eel viscera[23]. The relationship between "ciguatoxins" from eels and the "ciguatoxin" found in the flesh and viscera of other fish species remains to be established[11,24-27]. Species specific differences in assimilation and conversion of the less polar ciguatoxin produced by *G. toxicus* may explain the different composition of "ciguatoxins" present in different fish species[3]. The presence in fishes of a class of ciguatoxins which have no detectable toxicity should not be discounted.

Acknowledgements

This project was supported by the Fishing Industry Research and Development Council, Australia. We thank Mr. T. Tebano for assistance with moray eel collection, Dr. J. Mcleod for mass spectral measurements, Dr. D. Die for statistical advice and Mr. K. Jones (Waters Associates) for the use of a photo diode array detector. We thank also Miss S. Brice and Mrs. H. Smyth for general assistance, Mr. G. Smith for photography and Mrs. E. Donovan for typing the manuscript.

Literature Cited

1. Randall, J.E. 1958. A review of ciguatera, tropical fish poisoning, with a tentative explanation of its cause. Bull. Mar. Sci. Gulf Carribean 8: 236-267.
2. Scheuer, P.J., W. Takahasi, J. Tsutsumi and T. Yoshida. 1967. Ciguatoxin: Isolation and chemical nature. Science 155: 1267-1268.
3. Murata, M., A.M. Legrand, Y. Ishibashi and T. Yasumoto. 1989. Structures of ciguatoxin and its congener. J. Am. Chem. Soc. 111: 8929-8931.
4. Cooper, M.J. 1964. Ciguatera and other marine poisonings in the Gilbert Islands. Pac. Sci. 18: 411-440.
5. Halstead, B.W. 1967. Poisonous and Venomous Marine Animals of the World. Vol. 2. Vertebrates. U.S. Goverment Printing Office, Washington, D.C., 330 pp
6. Helfrich, P., T. Piyakarnchana and P.S. Miles. 1968. Ciguatera fish poisoning: 1. The ecology of ciguateric reef fishes in the Line Islands. Bernice P. Bishop Museum, Occasional Papers. 23: 305-369.
7. Randall, J.E. 1979. A survey of ciguatera at Enewetak and Bikini, Marshall Islands, with notes on the systematics and food habits of ciguatoxic fishes. Fish. Bull. 78: 201-249.
8. Gillespie, N.C., R.J. Lewis, J.H. Pearn, A.T.C. Bourke, M.J. Holmes, J.B. Bourke and W.J. Shields. 1986. Ciguatera in Australia. Occurence, clinical features, pathophysiology and management. Med. J. Australia 145: 584-590.
9. Bagnis, R., J. Bennett, M. Barsinas, J.H. Drollet, G. Jacquet, A.M. Legrand, P.H. Cruchet and H. Pascal. 1988. Correlation between ciguateric fish and damage to reefs in the Gambier Islands (French Polynesia). Proc. 6th Inter. Coral Reef Symp. 2: 195-200.
10. Helfrich, P. and A.H. Banner. 1968. Ciguatera fish poisoning: II. General patterns of development in the Pacific. Bernice P. Bishop Museum, Occasional Papers 23: 371-382.
11. Lewis, R.J. and R. Endean. 1984a. Ciguatoxin from the flesh and viscera of the barracuda, *Sphyraena jello*. Toxicon 22: 805-810.

12. Vernoux, J.P., N. Lahlou, S.A. El Andaloussi, N. Riyeche and L. Ph. Magras. 1985. A study of the distribution of ciguatoxin in individual Caribbean fish. Acta Tropica 42: 225-233.
13. Lewis, R.J. and R. Endean. 1984b. Mode of action of ciguatoxin from the Spanish mackerel, *Scomberomorus commersoni*, on the guinea-pig ileum and vas deferens. J. Pharmacol. Exp. Ther. 228: 756-760.
14. Weatherley, A.H. and H.S. Gill. 1987. The Biology of Fish Growth. Academic Press, London, 443p.
15. Vernoux, J.-P. 1988. La ciguatera dans l'île de Saint-Barthélémy: Aspects épidémiologiques, toxicologiques et préventifs. Oceanol. Acta 11: 37-46.
16. Banner, A.H. 1974. The biological origin and transmission of ciguatoxin. In: Bioactive Compounds from the Sea, (H.J. Humm and C.E. Lane, eds.). Marcel Dekker, New York. pp.15-36,
17. Gillespie, N., R. Lewis, J. Burke and M. Holmes. 1985. The significance of the absence of ciguatoxin in a wild population of *G. toxicus*. Proc. 5th Inter Coral Reef Cong., 4: 437-441.
18. Lewis, R.J., N.C. Gillespie, M.J. Holmes, J.B. Burke, A.B. Keys, A.F. Fifoot and R. Street. 1988. Toxicity of lipid-soluble extracts from demersal fishes at Flinders Reef, southern Queensland. Proc. 6th Inter Coral Reef Symp., 3: 61-65.
19. Helfrich, P. and A.H. Banner. 1963. Experimental induction of ciguatera toxicity in fish through diet. Nature 197: 1025-1026.
20. Banner, A.H., P. Helfrich and T. Piyakarnchana. 1966. Retention of ciguatera toxin by the red snapper, *Lutjanus bohar*. Copeia, 2: 297-301.
21. Tosteson, T.R., D.L. Ballantine and H.D. Hurst. 1988. Seasonal frequency of ciguatoxic barracuda in southwest Puerto Rico. Toxicon 26: 795-801.
22. Legrand, A.M., M. Litaudon, J.M. Genthon, R. Bagnis and T. Yasumoto. 1989. Isolation and some properties of ciguatoxin. J. Appl. Phycol.1: 183-188.
23. Tachibana, K. 1980. Structural studies on marine toxins. Ph.D. Dissertation, Univ. of Hawaii. 142 p.
24. Nukina, M., L.M. Koyanagi and P.J. Scheuer. 1971. Two interchangeable forms of ciguatoxin. Toxicon. 22: 169-176.
25. Chungue, E., R. Bagnis, N. Fusetani and Y. Hashimoto. 1977. Isolation of two toxins from a parrot fish *Scarus gibbus*. Toxicon 15: 89-93.
26. Vernoux, J.-P. and A. El. Andaloussi. 1986. Hétérogénéité des ciguatoxines extraites de poissons péchés aux Antilles françaises. Biochimie 68: 287-291.
27. Vernoux J.-P. and F. Talha. 1989. Fractionation and purification of some muscular and visceral ciguatoxins extracted from Caribbean fish. Comp. Biochem. Physiol. 94B: 499-504.

Seasonal Abundance and Toxicity of *Gambierdiscus toxicus* Adachi et Fukuyo from O'ahu, Hawai'i

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Abstract

Inshore reef environments of the island of O'ahu were monitored for the presence of the benthic dinoflagellate *Gambierdiscus toxicus* from September, 1988 to December, 1989. Low population levels ranging from 15 cells/g alga to 225 cells/g alga were found in association with the macroalgae *Spyridia filamentosa, Acanthophera spicifera,* and *Dictyota sandvicensis. S. filamentosa* was the preferred substrate.

Several clonal and batch cultures of *G. toxicus* were isolated and grown in the laboratory. Lethality of crude acetone and methanol extracts were assayed by intraperitoneal (i.p.) injection into mice. The nature of the toxins was assessed by their effect on the isolated guinea-pig atrium.

Introduction

With the successful elucidation of the molecular structure of ciguatoxin by Yasumoto and co-workers, the focus of ciguatera research has once again turned toward ecological aspects[1]. Historically, ciguatera has primarily presented problems in ecology and therapy. The ecological concerns centered on the identification of the primary toxin producer, the factors governing its geographical and temporal fluctuations, details of the toxin transmission from dinoflagellate via microalgae, herbivorous fish, and carnivorous fish to man. The discovery of *Gambierdiscus toxicus* Adachi et Fukuyo as a principal toxin producer laid the foundation for meaningful research into the factors which influence the genesis of a critical concentration of toxic dinoflagellates that is likely to cause detectable fish toxicity and hence human intoxication[2,3].

Ciguatera intoxication has not been a major public health hazard in Hawaii, but it exists, occasionally makes the headlines in the newspapers and represents a latent threat to commercial and sport fisheries[4]. Although *G. toxicus* populations had been quantitatively related to macroalgal substrates at a single site, no systematic evaluation of *G. toxicus* seasonal distribution along

the shoreline of the island of O'ahu had been carried out[5]. We now report such a survey, which includes toxicity assays.

METHODS

Collection of Algae

Macroalgae were collected at eight sites (Figure 1) on the island of O'ahu by wading or snorkeling. Algal samples were removed from the substratum

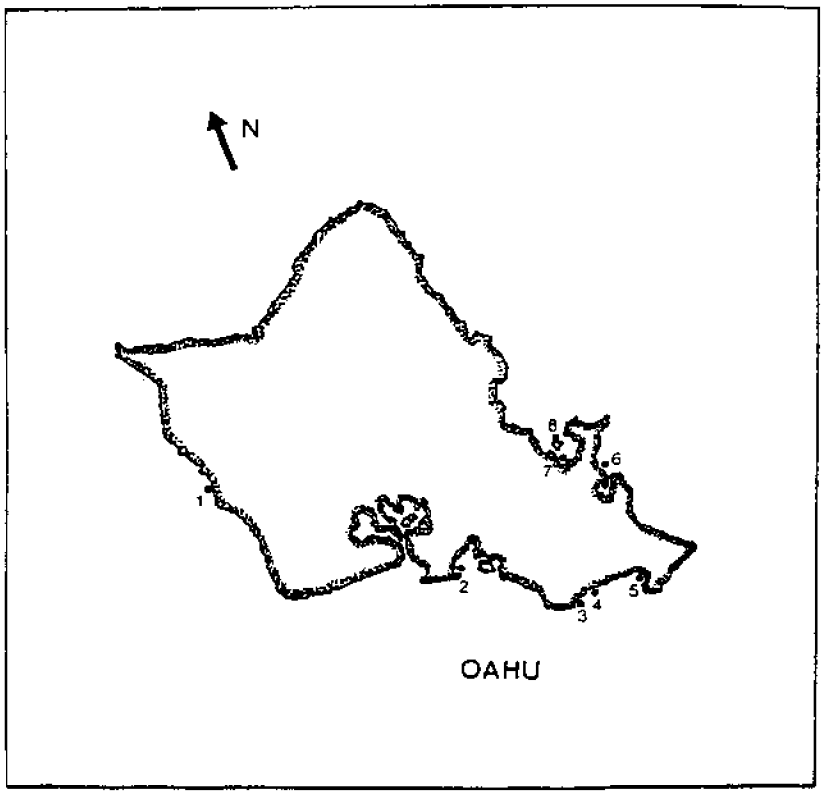

Figure 1. Dinoflagellate Collection Sites.
A map of the island of O'ahu showing the location of collection sites: (1) Lualualei Beach Park, (2) The reef runway, Keehi Lagoon side, (3) Black Point, (4) Kahala Beach, (5) Hawaii Kai, (6) Kailua Beach Park, (7) Kaneohe Bay, (8) Coconut Island.

and sealed within plastic bags with a minimum of disturbance. Additional specimens of each alga collected were preserved in 5% formalin in seawater for later identification and for an assessment of the substrate specificity of the dinoflagellate species. At the time of collection, note was made of the ambient water temperature, depth and substratum type for each algal species sampled.

After being returned to the laboratory, the bags containing the algae were shaken vigorously and the resulting detrital suspension filtered successively through 250 μm and 35 μm mesh filters. This filtrate was examined microscopically for the presence of dinoflagellates. Tentative identification of any dinoflagellates observed was made according to Steidinger and Carlson[6,7]. The quantification of the dinoflagellate-algal substrate relationship (expressed as the mean number of dinoflagellate cells/g alga) was achieved using a calibrated counting cell.

Algal Culture

Cells of *Gambierdiscus* were removed from the filtrate using sterile drawn Pasteur pipettes and washed six times in sterile media using glass spotting plates. Single cells were subsequently dispensed into 10 ml of medium in 16 mm borosilicate glass tubes. "Bulk" cultures comprising 20-50 washed cells were similarly prepared. The tubes were maintained at 25°C± 1.0°C under a light cycle of 14:10 light-dark. Initially, continuous illumination as prescribed by Carlson was used[7]. The light source was provided by 34 watt, cool white fluorescent tubes (General Electric; total light flux 0.64 × 10 quanta, cm^{-2} sec^{-1}). The tubes were left undisturbed for approximately one month and then examined for the presence of growth. Fifty (50) ml cultures of certain clonal and "bulk" cultures were prepared by inoculating cells (20 cells/ml) into 50 ml of medium in 125 ml Erlenmeyer flasks. These flasks were maintained under the conditions described above. The medium in these flasks was replaced every 3-4 weeks. Cell concentrations of these cultures were assessed by removing 1 ml aliquots from the cultures and counting the number of cells present using a calibrated counting chamber.

Dinoflagellate cultures were periodically assessed for the presence of bacterial contamination using the following method. Aliquots (0.1 ml) of the dinoflagellate cultures were inoculated into 5 ml of culture media enriched with peptone (10 g/l) and yeast extracts (5 g/l). Broth cultures remaining clear three weeks post-inoculation were deemed to be axenic. Any contaminants were isolated from the broth cultures and purified by repeated streaking on nutrient seawater agar. Stock cultures of these contaminants were maintained on nutrient seawater agar.

Axenic cultures of each clonal and batch stock culture were obtained by the following method. Samples of each stock culture (100 - 200 cells) were washed in sterile medium using glass spotting dishes and transferred to ES medium supplemented with antibiotics (Penicillin:Streptomycin; 2:1). After 48 hours the cells were placed in a 50 ml flask containing 20 ml of normal ES medium. This process is being repeated at 3-4 week intervals.

Evaluation of toxicity

For toxicity studies, batch and clonal cultures of *Gambierdiscus toxicus* were prepared as follows. Fernbach flasks each containing 1.5 l of ES medium were

inoculated with seed cultures of the dinoflagellate (approx. 200 ml; 200 cells/ml). The cells were maintained for 3 - 4 weeks as described previously, at which time the cell density had reached 1 - 1.5x10^6 cells/ml.

The cells were harvested by filtration onto glass fiber filters (Whatman GF/A), washed with deionized distilled water to remove salts and lyophilized. The washed, dried cells were sonicated in acetone and extracted in acetone over 48 hours. The extract suspension was filtered and the acetone-insoluble material extracted in methanol over 48 hours. Solvent was removed from both the acetone and methanol extracts under vacuum (Buchi, Rotavapor) and the residues taken to dryness under a stream of nitrogen. The crude extracts were stored at -20°C.

Male and female mice (Swiss white; 18-23g) were injected i.p. with doses of the dinoflagellate extracts prepared as fine suspensions in 1% Tween 80 in physiological saline. Signs expressed in the mice following administration of the extracts were identified according to the criteria detailed in Hoffman *et al.* and recorded at 1 hr and then at intervals up to 48 hours[8]. The mice were used and sacrificed in accordance with ethical standards.

The cardiotoxic effects of selected extracts prepared from cultured *G. toxicus* were evaluated as follows. Male guinea pigs (300-500g) were sacrified, the hearts excised, and placed in oxygenated Krebs-bicarbonate solution. The left and right atria were dissected free and used separately to study the effects of the *G. toxicus* extracts upon the electrically-stimulated and spontaneously elicited contraction, respectively. The left atrium was stimulated by rectangular pulses (1.5 threshold voltage, 4 msec duration and at 1.5 Hz) delivered through a Grass SD9 stimulator. The isometric responses of both the left and right atria were measured using force transducers and recorded on a Grass polygraph. The crude extracts were dissolved in 10% methanol and 100 µl aliquots of the solutions added to the organ bath to give a final concentration of 10 µl/ml.

Results

The results of the geographical survey are summarized in Table 1. The Kahala beach site (No. 4) appeared to provide a suitable location for seasonal monitoring. Two species of dinoflagellates, *G. toxicus* and *Ostreopsis siamensis* were assessed monthly for a period of 16 months. As seen in Figure 2, the cell density showed a marked steady increase during that time.

Lethality of batch and clonal cultures was determined by i.p. injection into mice. Those extracts (A for acetone, M for methanol) from batch and clonal cultures which elicited toxic symptoms in mice are indicated by (+) in Table 2. Those extracts which caused death are denoted by (L). No acetone extract proved to be lethal. Cultures of *Prorocentrum lima* also yielded lethal methanol extracts.

Table 1. Species Macroalgae from Sites on the Island of O'ahu.

Algal Species	Site	Substrate	G. toxicus	O. siamensis
Chlorophyta				
Halimeda opuntia	4	sand	—	—
H. discoidea	4	sand	—	—
Enteromorpha sp	7	mud	—	—
Phaeophyta				
Turbinaria ornata	4	rock	—	—
Sargassum polyphyllum	4	rock	—	—
S. echinocarpum	3	rock	—	—
Padina sp. 1	4	rock	+	—
Padina sp. 2	5	rock	—	—
Dictyota dichotoma	4	sand	—	—
D. sadvicensia	4	sand	—	—
D. acutiloba	4	sand	—	—
Rhodophyta				
Spyridia filamentosa	1	rock	—	—
"	3	sand/rubble	+	+
"	4	sand/rubble	+	+
"	5	sand/mud	+	+
"	6	sand	—	—
"	7	mud	—	—
"	8	sand	+	+
Acanthophora spicifera	2	mud	—	+
Plocamium sadvicense	3	rock	—	—
Laurencia obtusa	3	rock	—	—
L. succisa	4	rock	—	—
Grateloupia filicina	4	sand	—	—
Unidentified Algae				
Brown filamentous	8	sand	—	—
Brown filamentous	7	mud	—	—
Green Filamentous	7	mud	—	—

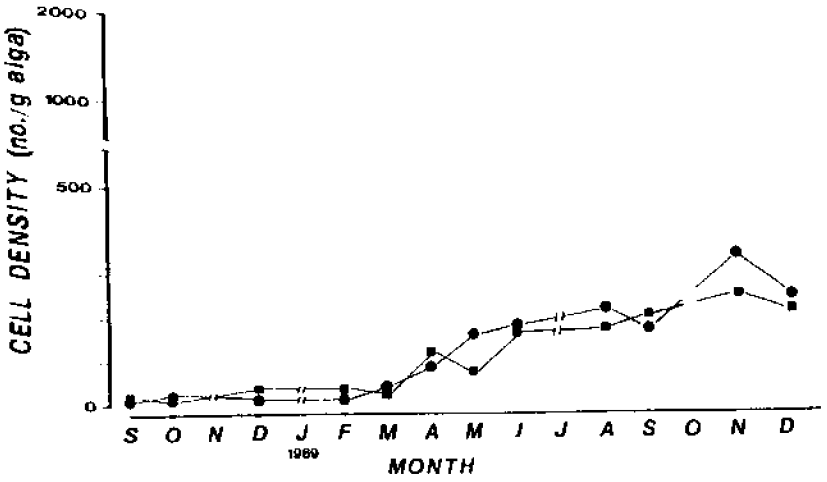

Figure 2. Dinoflagellate Densities vs. Time.
Seasonal variation of *G. Toxicus* (●) and *Ostreopsis siamensis* (■).

Both acetone and methanol extracts of Hawaiian *Gambierdiscus toxicus* (Batch g. tox. KB 18/1) have been found to be cardiotoxic when tested upon the isolated guinea pig atrium. Representative traces obtained in the course of these experiments are presented in Figure 3. The acetone extract (A, 10 µg/ml) of *G. toxicus* exerted a positive inotropic effect upon both the electrically stimulated (Figure 3, panel 1) and spontaneously beating atria (Figure 3, panel 3). This extract also elicited a positive chronotropic effect upon the spontaneously beating atrium (Figure 3, panel 3). The positive inotropic effects of the acetone extracts of *G. toxicus* were blocked in the presence of the adrenergic blockers propanolol (Pro) and phentolamine (Phe) and the sodium ion channel blocking agent tetrodotoxin (TTX). The effects of the *G. toxicus* acetone extract upon the isolated guinea-pig atria closely resembled those described for ciguatoxin and for extracts of certain species of toxic fish[9]. The effects of the methanol extracts (M; 10 µg/ml) of *G. toxicus* upon the electrically stimulated guinea-pig atrium are shown in Figure 3, panel 2. The extract induced a positive inotropic response similar to that recorded previously for maitotoxin.

Discussion

In view of the widely quoted but as yet unproven hypothesis that ciguatera outbreaks follow the creation of new surfaces from natural or man-made causes, we had expected a high *G. toxicus* concentration at Station 2, where the reef had been disturbed for the construction of a runway for the Honolulu airport[10]. This was not the case. Kahala beach on the SE shore of O'ahu proved to be the collection area of choice. Again as in an earlier survey the red

Table 2. Cell Extract Yields, Toxicity to Mice of Crude Extracts from Cultured Dinoflagellates.

Clonal Cultures	Yield of Cells (mg)	Yield of Extract (mg)		Toxicity to Mice	
		A	M	A	M
Batch Culture					
GT; KB 1/18					
(A)	23.4	13.0	24.0	+	+(L)
(B)	35.6	1.6	5.0	+	+(L)
TG. tox.; Stn 4	22.2	13.0	13.4	+	+(L)
Clonal Cultures					
GT 174	242.2	22.0	14.0		
GT 177	269.9	12.0	16.0	+	+(L)
PL 4 (A)	90.3	41.3	7.9	-	+(L)
PL 4 (B)	108.3	12.1	6.4	-	+(L)

GT - *Gambierdiscus toxicus*
PL - *Prorocentrum lima*
KB - Kahala Beach

alga *Spyridia filamentosa* (Coulfen) Harvey (*Ceramiaceae, Gigartinales, Florideae, Rhodophyta*) was the preferred host of *G. toxicus*[5].

Cell density throughout the 16 months of the survey was very modest, no doubt a reflection of the fact that normally ciguatera poisonings on O'ahu are infrequent and often are caused by fish caught outside Hawai'i.

The cultures, as elsewhere yielded two distinct toxic fractions, which by solubility, symptomatology in mice, and their effect on the isolated guinea-pig atrium were related to, or identical with, maitotoxin and ciguatoxin[11]. The relationship and biogenesis of these two toxins continue to be unresolved.

Acknowledgments

This research was supported by the U.S. Army Medical Research Acquisition Activity and by an M.B.R.S. (NIH) scholarship to M.M.K.S.

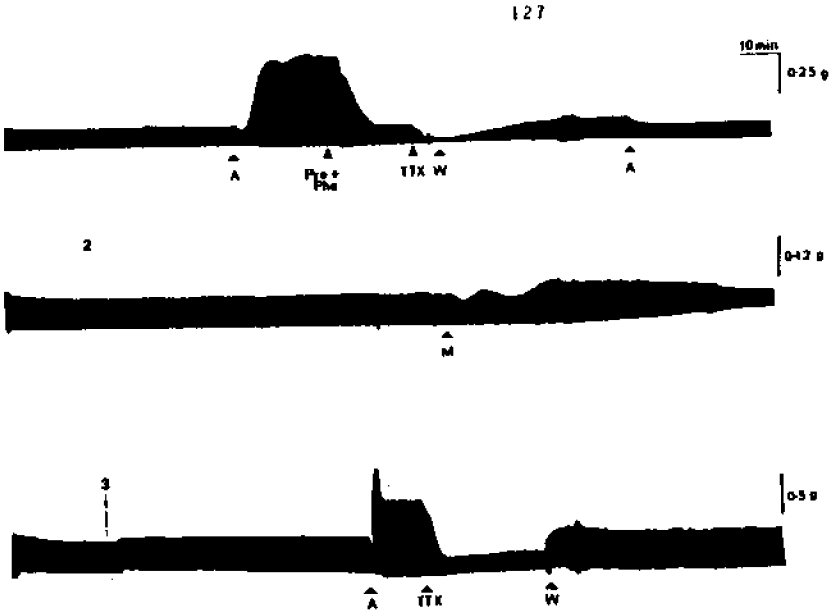

Figure 3. Effect of *G. toxicus* Extracts on Isolated Guinea Pig Atrium.
The inotropic and chronotropic effects of the acetone and methanol extracts of *Gambierdiscus toxicus*. Panels 1 and 3 show the effects of the acetone (A, 10 µg/ml) extract on the electrically-stimulated atrium (panel 1) and spontaneously-beating atrium (panel 3) of the guinea-pig. The effects of the addition of tetrodotoxin (TTX, $5x10^{-7}$ M) and propranolol and phentolamine (Pro, $1x10^{-6}$ M; and Phe, $5x10^{-7}$ M) on the extract-induced responses of the atria are shown. Panel 2 shows the effects of the methanol extract of *G. toxicus* (M, 10 µg/ml) on the electrically-stimulated guinea-pig atrium.

Literature Cited

1. Murata, M., A.M. Legrand, Y. Ishibashi and T. Yasumoto. 1989. Structures of ciguatoxin and its congener. J. Am. Chem. Soc. 111:8929-8931.
2. Yasumoto, T., I. Nakajima, R. Bagnis and R. Adachi. 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. Bull. Jpn. Soc. Sci. Fish. 43:1021-1026.
3. Adachi, R. and Y. Fukuyo. 1979. The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov. collected in a ciguatera-endemic area. Bull. Jpn. Soc. Sci. Fish. 45:67-71.
4. Anderson, B.S., J.K. Sims, N. Wiebenga and M. Sugi. 1983. The epidemiology of ciguatera fish poisoning in Hawaii, 1975-1981. Haw. Med. J. 42:326-334.

5. Shimizu, Y., H. Shimizu, P. J. Scheuer, Y. Hokama, M. Oyama and J.T. Miyahara. 1982. *Gambierdiscus toxicus*, a ciguatera-causing dinoflagellate from Hawaii. Bull. Jpn. Soc. Sci. Fish. 48:811-813.
6. Steidinger, R.A. 1983. A re-evaluation of toxic dinoflagellate biology and ecology. In: Progress in Phycological Research, Vol. 2, (F.E. Round and V.J. Chapman, eds.). Elsevier, New York. pp 147-188
7. Carlson, R.K. 1984. Distribution, periodicity and culture of benthic/epiphytic dinoflagellates in a ciguatera-endemic region of the Caribbean. Ph.D. dissertation, Southern Illinois University, 305 p.
8. Hoffman, P.A., H.R. Granade and J.P. McMillan. 1983. The mouse ciguatoxin bioassay: A dose-response curve and symptomatology analysis. Toxicon 21:363-369.
9. Miyahara, J.T., C.K. Kambayashi and Y.Hokama. 1989. Pharmacological characterization of the toxins in ciguateric fishes. In: Mycotoxins and Phycotoxins 88 (S. Natori, K. Hashimoto and Y. Ueno, eds.). Elsevier, New York. pp. 399-406.
10. Randall, J.E. 1958. A review of ciguatera, tropical fish poisoning, with a tentative explanation of its cause. Bull. Mar. Sci. Gulf Carib. 8:236-267.
11. Durand-Clement, M. 1987. Study of production and toxicity of cultured *Gambierdiscus toxicus*. Biol Bull. 172:108-121.

Societal Impact

Public Health, Epidemiological and Socioeconomic Patterns of Ciguatera in Tahiti

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Abstract

The study deals with 30 year follow-up of the number of human cases, clinical features and evolution of the disease, the names of the ciguateric fishes, fishing areas and the social and economic influence of ciguatera on Tahitian life.

The evaluation of ciguateric morbidity was carried out by the census of officially recorded cases since 1960, by the analysis of standard clinical and epidemiological questionnaires for each patient, filled out by medical and paramedical staff, and by direct inquiries in the population. A significant increase of the recorded cases occurred in 1965, the yearly number growing suddenly from 64 to 305. Then the incidence rate per 1,000 inhabitants ranged from 4 to 7, with an average of about 480 cases each year, from 1965 to 1989. During the period of the study, nearly 80 species, from 27 fish families, involving various trophic levels were incriminated. They include mainly groupers, snappers, emperor-fish, jacks, surgeonfish, parrotfish, mullets, wrasses and trigger-fish. A great number of the toxic fishes came from other islands than Tahiti, mainly from the Tuamotu archipelago and they were sold on the market place of Papeete. It has been estimated that each year on an average, ciguatera results in the loss of about 4,000 days of work and 3,000 tons of reef fish, that are banned from sale on the market-place in the Tahitian community.

Introduction

The first report on toxic fish from Tahiti appeared in the journal of a boatswain's mate of the Bounty from 1788 to 1791[1]. For the last two centuries the presently most populated island of French Polynesia (135,000 inhabitants) has been concerned with ciguatera fish poisoning. Studies of overall epidemiological and clinical features have already been published, however, no long-term survey of the specific human and economic patterns of ciguatera in the big volcanic island has ever been carried out[2,3].

The aim of the present work is to report the follow-up of various param-

eters such as reported cases of ciguatera (from 1960), poisonous fishes, toxic fishing areas, clinical features (from 1965), monthly cases (from 1974) socioeconomic influence and the relationship between symptomatology and ingested fish caught around the island of Tahiti (from 1987).

Materials and Methods

The evaluation of patients took place as part of an epidemiological research program in progress since 1965. A standardized one page questionnaire was used to obtain some information about the clinical features, the names of the poisonous fishes and the area of capture. The information was gathered from patients whose statements and clinical findings were highly suggestive of ciguatera. Most of the questionnaires were completed by an examining physician, and in a few cases nurses filled in the requested data. A rough estimation of lost working days and of the cost of treatment associated with attacks of ciguatera was made based on the length of the confinement either at home or in hospital, without any normal professional activity, for all the patients examined and treated at the Luis Malardé Institute Clinic over the past three years.

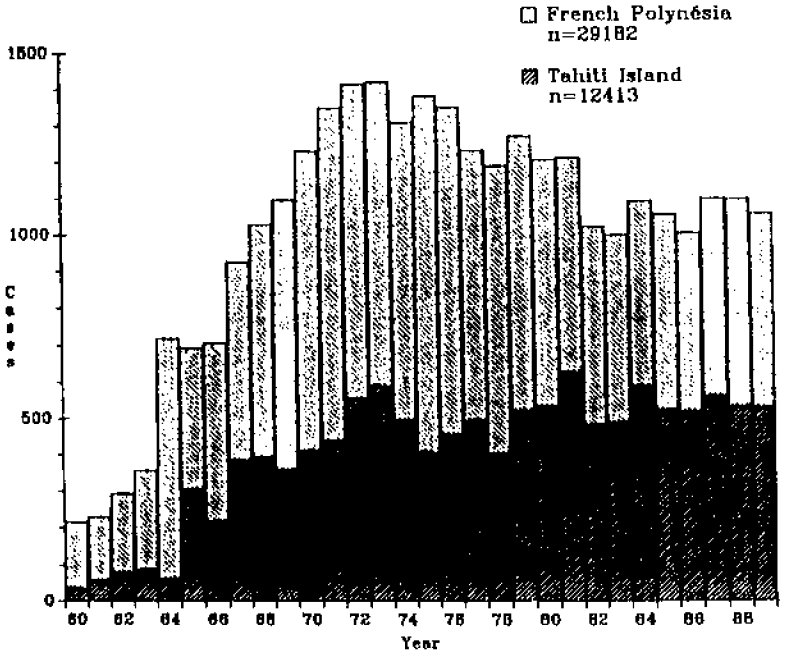

Figure 1. Ciguatera in Tahiti and French Polynesia.
Ciguatera cases in Tahiti and in French Polynesia (1960 - 1989).

A comparison of the frequencies of symptoms associated with the toxic fish and their food habits (herbivores or carnivores) was established by the Pearson's chi square test.

Results

Incidence of ciguatera

From January 1, 1960 to December 31, 1989, 12,413 cases of ciguatera fish poisoning were recorded in Tahiti, out of a total of 29,182 in French Polynesia. The yearly distribution is indicated in Figure 1. It must be acknowledged that reporting procedures have changed during this period. From 1960 through 1964, only the severe cases of ciguatera were reported by physicians to the Health Department. The 431 cases treated dur-

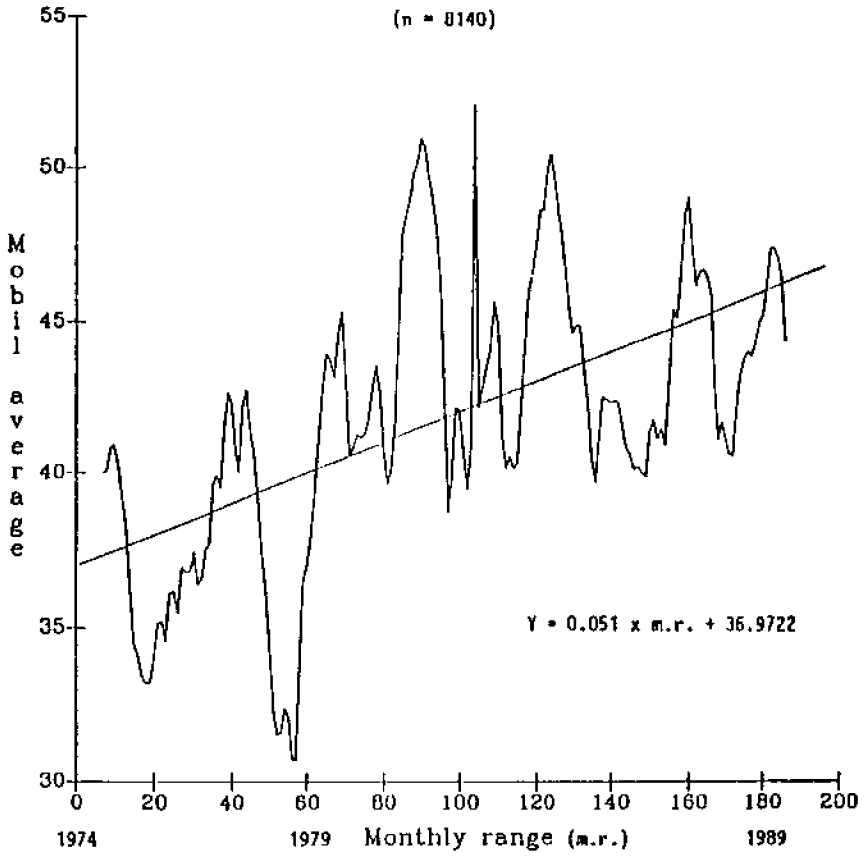

Figure 2. Ciguatera in Tahiti: General Trend.
Secular trend of ciguatera cases in Tahiti (1974 - 1988).

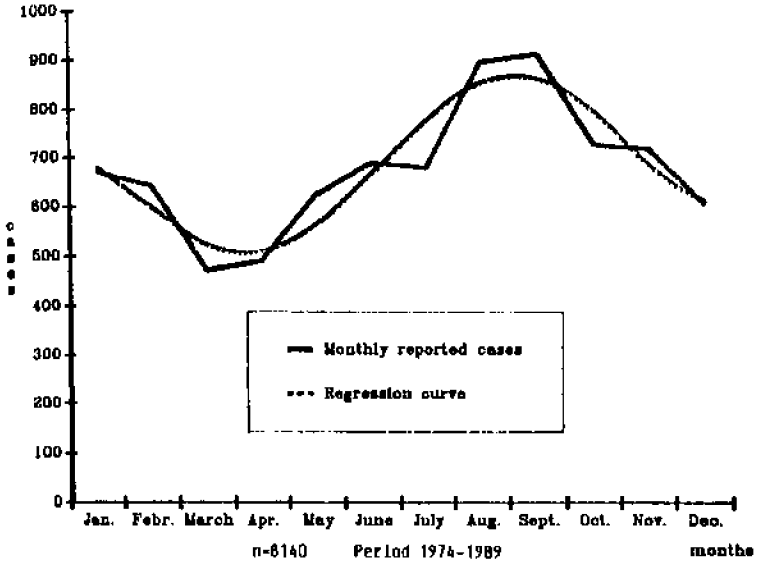

Figure 3. Monthly Frequency of Ciguatera in Tahiti.
Monthly ciguatera cases in Tahiti (1974 - 1989).

ing these five years were anamnestic and were taken from the various consulting books of the Tahiti hospital. The yearly average of less than 100 cases is very low. From 1965 to 1973, more cases were reported, thanks to a new program of epidemiological monitoring of ciguatera and the 3,842 cases for the nine year period gives an average of 428 cases per year. From 1974 to 1989, the reporting was even better after ichtyosarcotoxemia had been classified as a monthly reported disease by the South Pacific Commission. During this third period, 8,140 patients complained of ciguatera attacks in Tahiti, with an average of 508 cases per year. A very light increase in the number cases may be observed during this period. Taking the month of January 1974 as a reference, the general trend of the disease may be described by the formula (Figure 2):

$$Y=0.051 \times \text{monthly range} + 36.9722$$

Seasonal patterns were also apparent, with periodic significant changes in incidence, decreasing in March-April and increasing in August-September (Figure 3).

Geographical distribution of toxic fish

This study covers the period 1965-1989. For the 11,984 cases in which the toxic fish were identified, less than 50% came from Tahitian waters, more than 30% were caught in peripheral islands and nearly 20% had an unknown

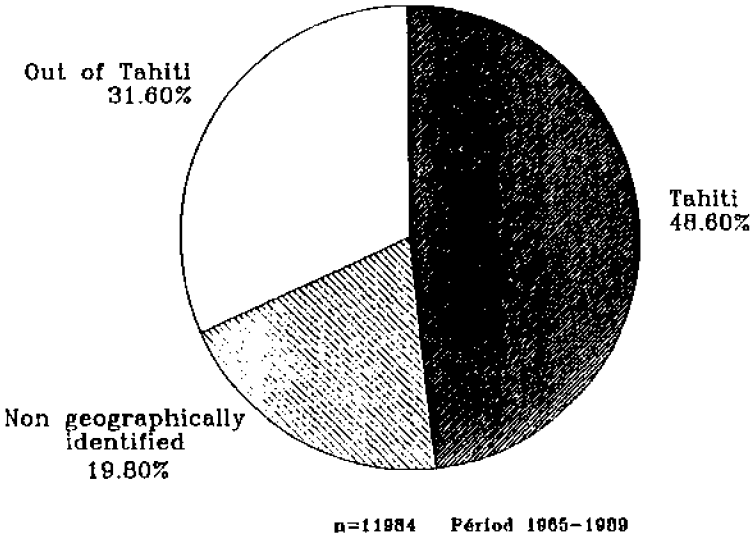

Figure 4. Ciguatera in Tahiti: General Origin of Toxic Fish.
Ciguatera cases in Tahiti and rough origin of toxic fish.

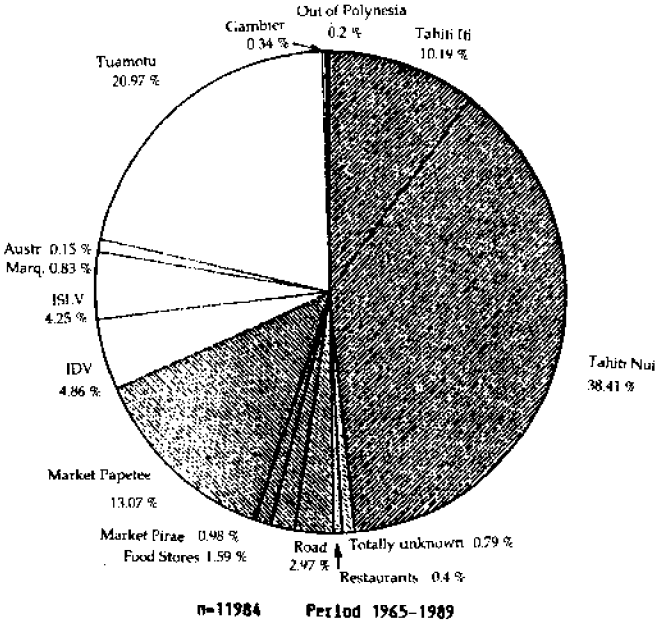

Figure 5. Ciguatera in Tahiti: Detailed Origin of Toxic Fish.
Ciguatera cases in Tahiti and detailed origin of toxic fish.

geographical origin (Figures 4 and 5). In Tahiti, no district was safe from endemic ciguatera. More than 2/3 of the cases (68.01%) were due to fish caught on the west coast. Nearly half the toxic fishes (49%) came from the urban area consisting of Mahina, Arue, Pirae, Papeete, Faaa, Punaauia, Paea districts (Figures 6 and 7). The Taiarapu Peninsula (Tahiti iti) provided less than 27% of the local toxic fishes.

Toxic fishes caught in the peripheral island waters responsible for 31.6% of the total attacks of ciguatera in Tahiti (Figure 5), came from all the archipelagoes. However, in Tahiti the most important supplier of poisonous fishes was the Tuamotu archipelago (2/3 of the cases) leaving less than 30% coming from other Society Island archipelagoes. Toxic fishes of unkown geographical origin were mainly (67%) distributed through the Papeete market place, 15% purchased from roadside vendors, 8% in food stores and only 2% were supplied by restaurants. Close to 2/3 of the poisonous fishes bought on the market place, based on available data, came from the Tuamoto atolls.

Distribution of the toxic fishes according to ichthyological family

Ciguatera fishes were identified in 11,669 cases during the 1965-1989 period (Figure 8). Considering all geographical origins, nearly 80 species of

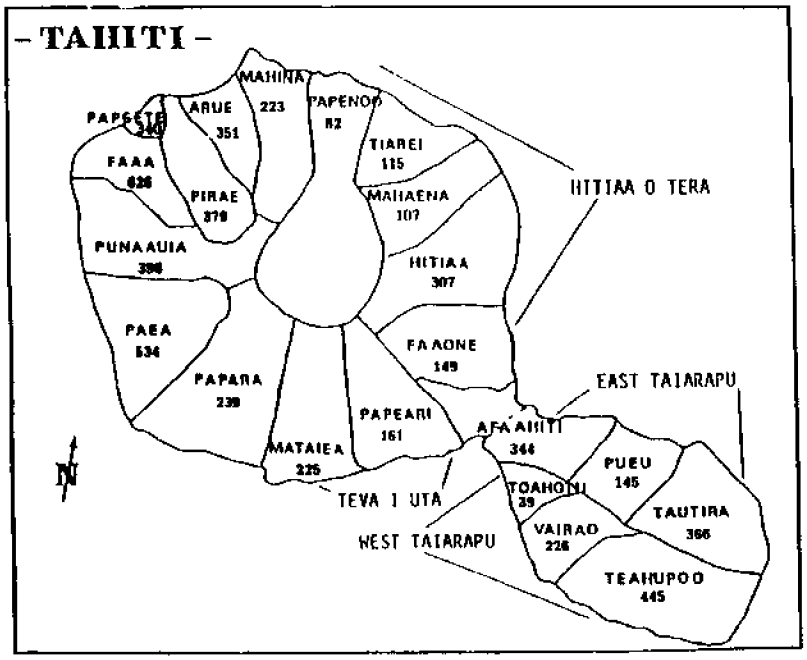

Figure 6. Ciguatera in Tahiti: Verified Cases and Distribution of Toxic Fish. Distribution of official cases of ciguatera induced by fishes caught in the various districts of Tahiti from 1965 to 1989.

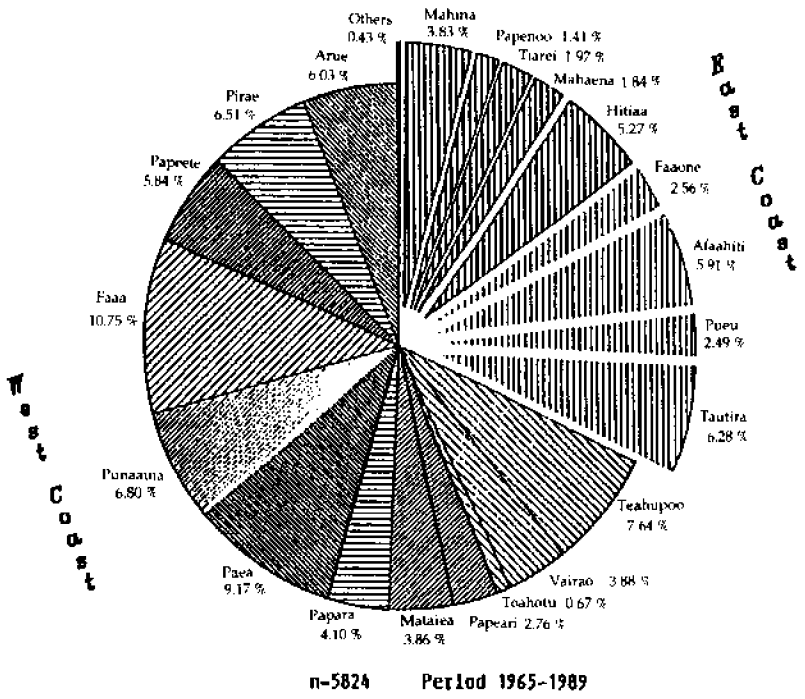

Figure 7. Ciguatera in Tahiti: Cases Caused by Tahitian Fish.
Detailed district origin of the toxic fish caught in Tahiti waters.

fish belonging to 27 families from the various trophic levels were implicated. The most often poisonous fishes for the patients of Tahiti were surgeon-fish (Acanthurids) and groupers (Serranids), each family involved in nearly 20% of the cases. Jacks (Carangids) and snappers (Lutjanids, Sparids, Etelids) were each poisonous in nearly 12% of the cases, while emperor-fish (Lethrinids), parrot-fish (Scarids), wrasses (Labrids) were each pathogenic in 10 to 7.5% of the cases, and Mugilids in less than 5% of the cases. Triggerfish (Balistids), moray eels (Murenids) and barracudas (Sphyraenids) accounted for 2.5 to 1% of the cases. Less than 1/3 of all these toxic fishes are herbivores.

This pattern is not valid if we consider the specific distribution of only the Tahitian toxic fish, which was estimated accurately for the last three years (Figure 9). The herbivorous fishes, Acanthurids and Mugilids were both responsible for more than half the cases, with only two species implicated, the surgeonfish, *Ctenochaetus striatus* (35%) and the mullet, *Crenimugil crenilabis* (15%). The chief toxic carnivorous fishes were jacks (especially *Caranx melampygus*) involved in nearly 12% of the cases, groupers (mainly *Cephalopholis argus* and *Epinephelus tauvina*) and snappers (mainly *Lutjanus gibbus* and *L. monostigmus*) with 10% of the cases for each family. The emperor-fish (*Lethrinus miniatus*) was implicated in close to 7% of the cases.

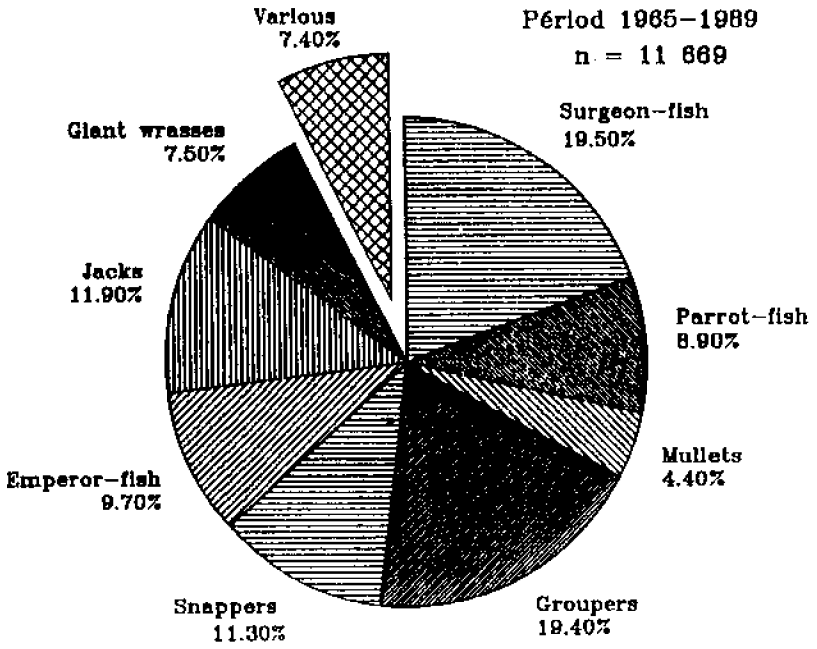

Figure 8. Ciguatera in Tahiti: Distribution of Causitive Fish.
Cases of ciguatera in Tahiti and toxic fish (all geographical origins included).

Relationship between clinical features and toxic species

A study was carried out on 629 patients examined at the Louis Malardé Institute Clinic, to establish a correlation between significant differences in some clinical features of ciguatera and the food habits of the Tahitian toxic fish. Four syndromes were characterized: digestive (taking mainly into account vomiting and diarrhea), neurosensory (mainly tingling and numbness, sensory reversal to cold, muscle and joint pains, chilliness, dizziness, itching), neuromotor (mainly asthenia, and lack of coordination), and cardiovascular (cardiac arrhythmia and decreased blood pressure) (Figure 10).

Digestive signs (diarrhea) were significantly more frequent after ingestion of carnivorous fish (83.55%) than after ingestion of herbivorous fish (71.07%, $p<0.001$). No differences were observed in the frequency of occurrence of neurosensory symptoms. Neuromotor signs (general weakness and lack of coordination) were more often present ($p<0.02$) in consumers of carnivorous fishes (62.80% and 16.11%, respectively) than in consumers of herbivorous fishes (52% and 9.53%).

The most significant differences were observed for the cardiovascular signs. Bradycardia and the fall of the blood pressure were more frequent ($p <$

10^{-6}) in the consumers of carnivores (respectively 21.7% and 19.07%) than in the consumers of herbivores (4.92% and 4.61%). In the four families more especially studied, groupers, jacks, snappers and emperor-fishes, the lowest and the highest frequencies of occurrence of cardiovascular disturbances were observed in jacks and snappers, respectively.

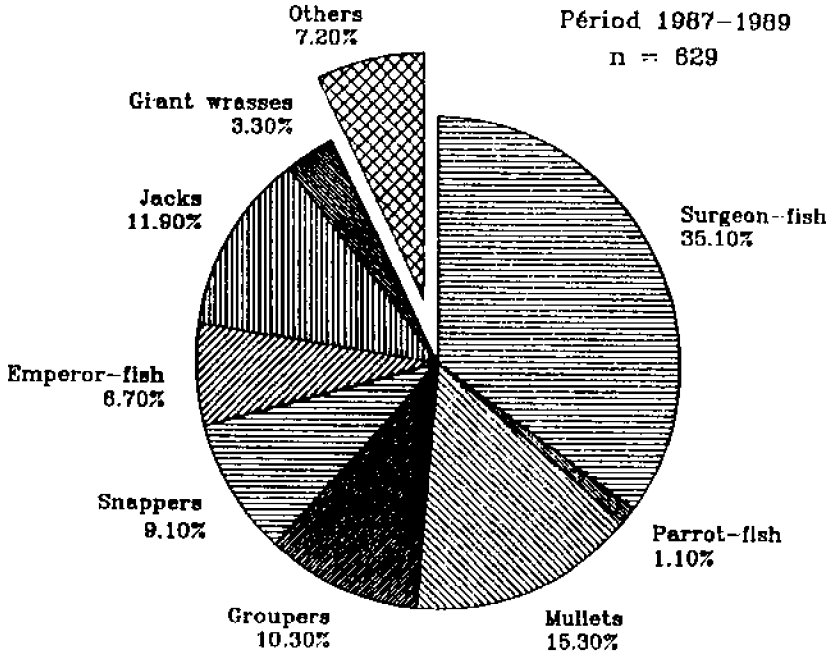

Figure 9. Ciguatera in Tahiti: Distribution of Causative Tahitian Fish. Ciguatera cases involving toxic fish from Tahiti only.

Socioeconomic features

A rough evaluation of the social impact of ciguatera was attempted through a survey carried out from 1987 to 1989 among the patients at the Louis Malardé Institute Clinic. The main criteria of evaluation were the length of the rest at home (generally) or in the hospital (in a few cases), together with the inability to resume normal activity. Results indicated that about 1/3 of the patients who experienced a ciguatera attack had to be confined to bed. The incapacity to work usually ranged from two to seven days, occasionally lasting three or four weeks. For the 629 patients surveyed during the period, ciguatera resulted in the loss of about 4,000 working days. The average cost of the nonproductive days, on the basis of the minimum monthly wage, was close to $100,000 (US).

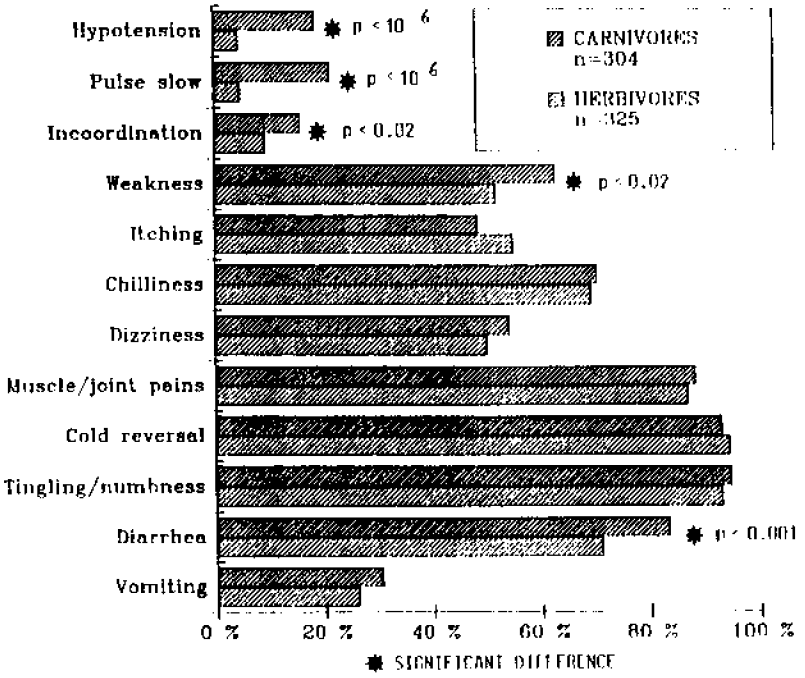

Figure 10. Ciguatera Symptoms Correlated with Consumption of Carnivorous as Compared with Herbivorous Tahitian Fish.
Frequency of some common or typical ciguatera symptoms by main groups of fish caught in Tahitian waters (1987 - 1989).

Due to the lack of a simple, fast, reliable and cheap assay for the detection of ciguatoxicity, several species of reef fishes are banned from sale on the market place of Papeete. These are the groupers *Plectropomus leopardus* (tonu), *Epinephelus microdon* (hapuu), the snappers *Lutjanus bohar* (haamea), *L. monostigmus* (taivaiva), *L. rivulatus* (haputu), the giant wrasse *Cheilinus undulatus* (mara), the great barracuda *Sphyraena barracuda* (ono), the surgeonfish *Ctenochaetus striatus* (mito), the moray-eels and the trigger-fish generally. This law protecting the consumer results in a loss of earning in the order of about 1 million dollars (US) for the native fishermen.

Discussion

The light increase in the number of cases in Tahiti during the 1974-1989 period may be associated with the demographic growth. The ciguatera incidence rate remained the same at about 4 per 11,000 inhabitants for the period.

Seasonal variations were observed in the incidence of ciguatera. The period of lowest incidence corresponded with the end of the hot and rainy season, while the period of highest incidence was found in the cold and dry seasons. The only significant correlation with a biological parameter that appeared concerned the macroalgal population. In high islands especially, the algal covering is the lowest in March-April and the highest in August-September (Payri, personal communication). A large algal covering is necessary for *Gambierdiscus toxicus* to develop in abundance.

Concerning the geographical origin of toxic fish, the relatively minor role of Tahitian fish in the ciguatera morbidity may be associated with the decrease of reef-fish stock by overfishing. The higher occurrence of toxic fishes along the west coast of the main island might be associated with the larger number of both fishermen and fish. Over the last three years, the urban reef area of Tahiti supplied half the toxic fishes. This pattern might be related to the greater reef damage caused by human activity. On the other hand, in 1987, 2/3 of the patients of the urban area, where 100,000 people lived (75% of the total island population), were poisoned by fish from the Tuamotu Islands.

Available data on the relationship between the clinical features and toxic fishes consumed confirm the higher richness and severity of the clinical picture induced by carnivorous fish, as previously suggested[5]. This study also revealed a significantly higher frequency of diarrhea after poisoning by carnivorous fish. It had previously been reported that digestive and neurosensitive disorders predominated in patients poisoned by herbivorous fish[2,5]. However, the samples of surveyed patients in the previous study were smaller and dealt more with systemic syndromes than isolated symptoms or signs. Regarding the strong cardiovascular toxicity of the snappers, it must be noted that in Tahiti, most of the people treated in the hospital for a ciguatera attack were poisoned by snappers. Such a pattern could be associated either with a higher amount of ciguatoxin in the flesh or the occurrence, apart from the ciguatoxin, of one or several other toxins which are very active on the cardiac muscle and blood pressure. Present studies on the various ciguatoxin-like substances or other toxins present in ciguatoxic fish are very likely to provide an answer to these questions in the future.

Acknowledgements

This study was jointly supported by the Government and the Territory Assembly of French Polynesia, and by the French Ministry of Research, to whom we express our gratitude. We also thank the officials of the local Public Health Department and the staff of the various medical centers of Tahiti for their cooperation in the collection of information, as well as Mr. J. Lagardère for revising our English.

Literature Cited

1. Morrison, J. 1972. The journal of a boatswain's mate of the Bounty. London Editions, 1935. pp. 129-130. .
2. Bagnis, R. 1968. Clinical aspects of ciguatera (fish poisoning) in French Polynesia. Haw. Med. J. 28:25-28
3. Bagnis, R., J. Bennet, M. Barsinas, M. Chebret, G. Jacquet, I. Lechat, Y. Mitermite, Ph. Pérolat and S. Rongeras. 1985. Epidemiology of ciguatera in French Polynesia from 1960 to 1984. Proc. 5th Inter. Coral Reef Cong. 4:475-482.
4. Bagnis, R.J. and A.M. Legrand. 1987. Clinical features on 12,980 cases of ciguatera (fish poisoning) in French Polynesia. In: Progress in Venom and Toxin Research. (P. Gopalakrishnakone and D.K. Tan, eds.). National University of Singapore and International Society of Toxinology, Asia-Pacific Section. pp. 372-377
5. Bagnis, R., A.M. Legrand, Ph. Cruchet, F. de Dekker, J.N. Genthon and H. Pascal. 1988. Human intoxications by ciguateric phycotoxins in French Polynesia: incidence, clinical and epidemiological features in 1987. In: Mycotoxins and Phycotoxins. (S. Natori, K. Hashimoto and Y. Ueno, eds.) Elsevier, Amsterdam.

Ciguatera: Clinical, Epidemiological and Anthropological Aspects

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Abstract

This work presents the preliminary results of an interdisciplinary study comprising clinical/ epidemiological, anthropological and laboratory aspects of ciguatera conducted in Santo Domingo and some fishing communities of the Dominican Republic. The epidemiological surveillance, utilizing sentinel centers, allowed the study of thirteen outbreaks and six isolated cases in which 81 cases (confirmed by laboratory analysis) out of 127 underwent intoxication. Information regarding "popular knowledge" about ciguatera, collected by the anthropological team from five communities belonging to different areas of the country is also summarized. A thorough analysis of the therapeutic and preventive measures employed as "home medicine" in these communities is also reported. The advantages of a multi-disciplinary team and the effort of building an interdisciplinary framework are discussed.

Introduction

Among the diseases related to consumption of fresh marine species, ciguatera is perhaps the one having the highest incidence in the Dominican Republic. Even though this disease formed a part of our popular culture and generated a number of beliefs, it had not yet been studied locally. Existent data came from research done in other Caribbean areas, particularly the Virgin Islands and Puerto Rico, as well as the tropical Pacific.

After the occurrence of an important ciguatera outbreak in the Dominican Republic, an inter-disciplinary and inter-institutional group was organized at the Dominican Institute of Industrial Technology (INDOTEC). This group had as objectives the study of clinical, epidemiological, fisheries and cultural aspects of ciguatera intoxication in this country. The first stage of the study program was limited to the identification and characterization of cases occurring in the city of Santo Domingo and surrounding areas. It also aimed at the

rectification of popular beliefs in four important fishing communities located in different parts of the country. All these activities were performed as part of three subprograms: clinical/epidemiological, anthropological and laboratory studies. The latter, besides its research activities would assist the other two concerning outbreaks and confirmation of cases.

There is a law in our country banning the commercialization of four different species of fishes. However, many indications suggested that other species were also involved and that the forbidden species were still being consumed. This work summarizes how each subprogram targeted the ciguatera problem. The results point out the advantages of a multi-disciplinary study and the value of this group in building a system aimed at creating a better knowledge of the problem in our country. This effort will promote development of a more integrated plan regarding the future prevention of ciguatera.

Materials and Methods

In order to get a basic description of the intoxication, the clinical-epidemiological group established a surveillance network utilizing five sentinel centers located at the most important public and private medical care facilities, including the medical staffs of the top rated hotels in Santo Domingo. The medical personnel were trained and oriented by interviews, meetings and printed data, including a poster showing the clinical signs and symptoms of ciguatera. In order to gather data a notification card was devised to record the personal record of each patient. Each case was subsequently surveyed by questionnaire. This information was computer processed, incorporating clinical and epidemiological data given by the patient and his physician. Using all this information the ciguatera cases were diagnosed. The diffusion media (newspapers, community informants) and the sentinel centers contributed to case identification. However, evolution of the recorded cases after diagnosis was not followed.

Whenever possible, samples of the suspected fish were sent to the laboratory. Once there, they were identified and maintained at 10°C until processing. Toxin was extracted by using the McMillan *et al.* method developed in 1980[1]. Toxicity tests were done following methods developed by Sawyer et al., using mice of both sexes, weighing 19-21 grams[2]. Degree of toxicity was assessed following the method developed by McMillan, modified by our laboratory staff[1]. In this method a range from 0 to 5 is established, where 0 means no toxicity and 5 maximum toxicity (death of experimental mice before six hours)[1].

Popular beliefs related to ciguatera were explored by direct and indirect observation plus non-structured, targeted interviews, supplemented by photographic and audio records. Indirectly, information was provided by key

informers, members of the community with enough experience to recognize and communicate pertinent information concerning this topic. The four fishing communities under research were Puerto Plata, La Romana, Palenque and Pedernales (Figure 1). These communities were chosen because of their fishing production[3].

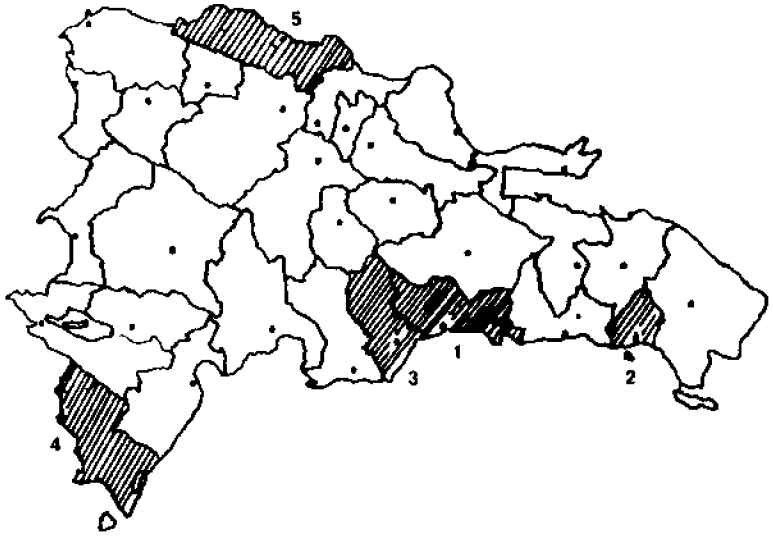

Figure 1. Communities Studied by the Anthropology and Epidemiology Subprogram. 1) Santo Domingo (Epidemiological Surveillance) 2) La Romana. 3) San Cristóbal 4) Pedernales 5) Puerto Plata

Results

A total of thirteen outbreaks and six isolated cases of intoxication were studied in which there were 81 (63.8%) cases of intoxication out of 127. Forty fish samples (82%) out of a total of forty nine suspected samples were found to have varying degrees of ciguatoxicity after being analyzed by the laboratory. In only one of these outbreaks could the presence of ciguatoxin not be confirmed. Table 1 summarizes this information. In 74.6% of the cases the fishes were consumed at home while the rest were eaten at restaurants. The confirmed species were "meros" (*Serranidae* spp.), "jurel" and "cojinua" (*Caranx* spp.), "chillo" (*Lutjanidae* ayas), "puerco" (*Balistes* spp.) and "loro" (*Scarus* sp.) (Figure 2). It was impossible to identify the species in two of the outbreaks. The toxicities of the 49 analyzed samples were as follows: 20.5% were grade 0, 10.2% grade 1, 2.0% grade 3, 18.4% grade 4 and 26.5% were grade 5. No organoleptic characteristics (appearance, flavor, odor) were detected as indicative of ciguatoxicity.

Table 1. Ciguatera Cases Studied in the Dominican Republic, 1989.

Case No.	Reported by	Location	Exposed People	Consumed at	Persons Intoxicated	Persons Interviewed	Lab Results
1	Press	Moca	40	Home	32	16	+
2	S. C.	S. Domingo	2	Home	2	1	+
3	S. C.	Santiago	11	Home	9	6	+
4	S. C.	S. Domingo	4	Home	3	3	+
5	S. C.	S. Domingo	2	Restaurant	1	1	+
6	Family	S. Domingo	2	Home	2	2	+
7	S. C.	S. Domingo	3	Home	1	1	+
8	S. C.	S. Domingo	27	Restaurant	3	27	+
9	S. C.	S. Domingo	4	Home	3	3	+
10	S. C.	S. Domingo	2	Restaurant	1	1	+
11	S. C.	S. Domingo	2	Home	2	2	+
12	S. C.	S. Domingo	5	Home	1	4	-
13	S. C.	S. Domingo	3	Home	3	3	+
14	S. C.	S. Domingo	2	Home	2	2	+
15	S. C.	S. Domingo	4	Restaurant	4	3	+
16	S. C.	S. Domingo	5	Home	4	2	+
17	S. C.	S. Domingo	2	Restaurant	1	2	+
18	S. C.	S. Domingo	6	Home	6	4	+
19	Physician	S. Domingo	1	Restaurant	1	1	+

Interviews of 67 patients out of the 81 involved in the ciguatera cases were collected for clinical and epidemiological characterization of the disease. From the interview data, 38 (57%) of the patients were males and the average age of all patients was 33 years (range 6-77; st. dev. 15.5). Nine of these patients declared they had other medical disorders prior to the intoxication. In two of these cases the disease was of a chronic nature, such as arterial hypertension and diabetes mellitus. Alcohol drinking combined with fish consumption was reported for 15 patients (23.1%). However, synergistic or antagonistic action could not be evaluated in such cases.

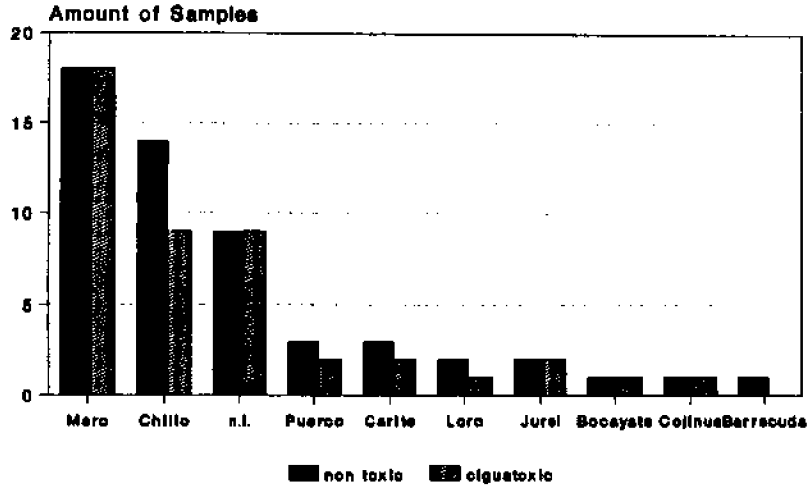

Figure 2. Suspected Ciguatoxic Fish Samples Analyzed by the Laboratory, April-November, 1989, Dominican Republic.

The average incubation period of the poisoning was 6 hours (range 0.5-27; st. dev. 4.4). Gastrointestinal symptoms were present, nausea (52.8%), diarrhea (74.6%) and vomiting (50.1%) being their first manifestations. Nervous system disorders were pain in the joints (64.1%), myalgia (56.7%), pruritus and/or paresthesis (47.8%), and mainly temperature sensibility inversion (63%). Therapy utilized was fairly diverse including steroids, antihistaminics, analgesics and vitamins, accompanied in some cases with fluids and electrolytes by parenteral and oral administration.

The fishermen as well as the communities under investigation differed greatly from a socioeconomic standpoint. Puerto Plata has the highest fisheries production rate of the country, exploiting not only Dominican coasts, but also nearby extraterritorial waters. This zone is the center of the biggest

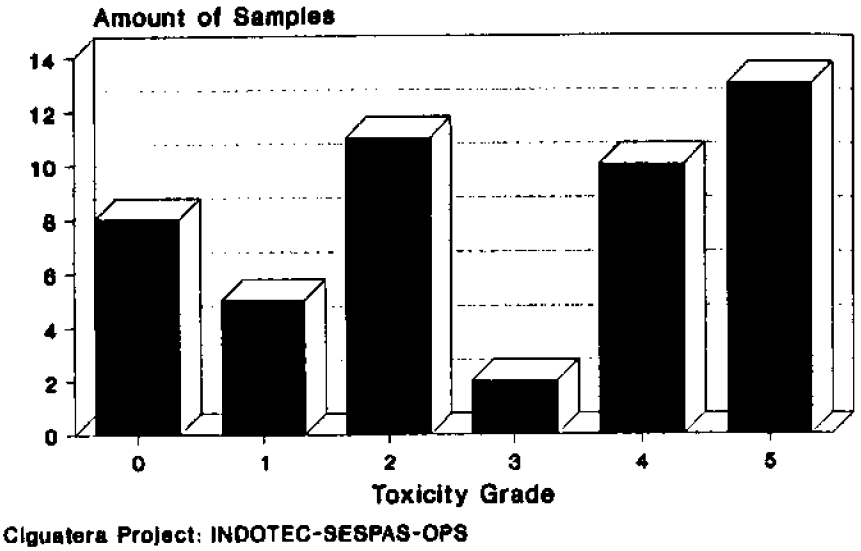

Figure 3. Toxicity of Analyzed Fish Samples.
See text for explanation of Toxicity Grade.

fishing companies of the country, owning ships and equipment dedicated to deep sea fishing. On the contrary, La Romana, Palenque and Pedernales exploit just the coastal platform. In the first two locations the fishermen, working mostly on a part time basis, combine their activities with other related efforts such as tourism and agriculture. Even though Pedernales has the highest fisheries production rate among the three towns, these fishermen were at the lowest socioeconomic level. They work on a full time basis, doing different kinds of fishing, predominantly using SCUBA equipment.

Some reticence in the fishing communities to talking about ciguatera was evident, even though the visits of the anthropology team were welcomed. This reaction prompted an investigation in order to find the hidden causes of this negative attitude. The adherence of fishermen and commercial concerns to the law of 1975 that banned the sale of thirteen species of fish considered to be ciguatoxic was investigated. Although the number of banned species was reduced to four in 1986, "barracuda" (*Sphyraena barracuda*), "picúa" (*Sphyraena picudilla*), "medregal" (*Seriola riseliana*) y "peje rey" (*Alectis crinitus*), this law was frequently broken among fishermen. The apparent complicity between fishermen and fish markets permitted the latter to buy banned fishes at reduced prices, and subsequently sell them to consumers at the regular prices established by the commercial classification. These transgressions of the law did not bring about frequent confiscations by the authorities. Lack of enforcement may have been be due to:

1. Fishing inspectors, paid to enforce the law, shared commercial interests with fishermen in that they are ex-fishermen or part-time fishermen;

2. Among fishing inspectors, the interest in fishing activities and commercialization overcame the ciguatera banning regulations;

3. Low income encouraged fishermen to accept bribes;

4. Traders have developed strategies to catch the high risk species.

Ciguatera was widely known in the fishing communities, because of its high incidence. In all, the disease was considered to be simple and as common as a cold. Originally, fishermen believed that fishes caught the ciguatoxin when ingesting the copper left by sunken boats. Later, the information transmitted to them by the Fisheries Department of the Agriculture Ministry, made them transpose the copper concept to "a small herb" instead, obviously making reference to the microscopic algae (dinoflagellate) responsible for the toxin production. In Pedernales, fishermen also believed that ciguatera was caused by the "aguasvivas" (jellyfishes) floating on the sea. Informants believed ciguatoxicity was characteristic not only of species living in deep water, but also found in shallow species, and that this condition could occur at any time of the year. Temperature increases and lack of food were believed to obligate fishes to consume the small herb producing the toxin.

The following species were considered ciguatoxic by the fishermen: "albacora" (*Thunnus alalunga*) "barracuda" (*Sphyraena barracuda*) "picúa" (*Sphyraena picudilla*) "chícharo" (*Selar crumenophythadmus*), "carite" (*Scomberomorus cavalla*), "colirubia" (*Ocyurus chrysurus*) "doctor" (*Acanthurus* spp.), "casabito" (*Caranx* spp.), "meros" (*Serranidae*), "pejerrey" (*Alectis crinitus*), "jabón" (*Ripticus saponaceus*), "pargos" (*Lutjanus spp.*), "sardina" (*Harengula spp.*) y "tiburón" (*Squalidae*).

In all except two cases, informants believed they were able to detect ciguatoxic fishes by noting the following characteristics: bad odor, copper odor, red eyes, presence of a blood line in the eyes, easily detaching scales, low weight, fluffy and sluggish flesh. Similarly, a fish was considered ciguatoxic if the flesh turned black or purple when lemon was added, when boiled with potatoes or sweet potatoes or when a copper coin or a garlic slice was placed on it.

It is evident that the prevalence of ciguatera in the fishing communities plus the existence of therapeutic measures contradict the popular beliefs about the recognition of ciguatoxic species. However, interviewed people rationalized the prevalence of the disease in different ways:

1. Intoxication was related to individual human conditions, weaker persons were more susceptible;

2. Ingestion of fish with "non-compatible food" increased the risk of intoxication;

3. Intoxication was more frequent if one ate stewed fish or fish soup, since it was more toxic than fried fish.

According to the fishermen, clinical manifestations of intoxication were: swollen mouth, redness, headache, dizziness, aching joints, tremors, diarrhea, vomiting, lack of motor coordination, hair loss and blurred vision. The available therapy in the four communities was targeted for two different situations: a) when a person had just ingested a suspected ciguatoxic fish, and b) when first symptoms appeared. In the first situation, the affected person was to drink milk, lemon juice, sugar in high amounts, sweetened water, warm water, a mixture of urine and ash (to provoke vomiting) or cooking oil (to prevent the toxin from entering the bloodstream). In the second situation, people were to drink extracts obtained from red mangrove (*Rhizophora mangle*), "uva de playa" (*Coccoloba uvifera*), black cane nodes (*Saccharum officinaru*) or sargassum (*Sargassum* sp.). It is important to point out the high amount of sugar utilized for prophylactic or therapeutic measures. The use of sugar is supported by the fact that according to our informants ciguatera never attacked diabetic people.

Discussion

The most important product of this research is the increased knowledge about ciguatera now available for health care facilities, since this poisoning is a serious problem for hotels, restaurant services, and the Dominican population in general. Clinical and epidemiological characteristics of the disease were the same as those cited in the literature[4-8]. Gastrointestinal and neurological symptoms, specially paresthesia, were the basis for clinical diagnosis in all the studied cases. Our clinical findings may reinforce the belief that cold temperature sensibility reversal is a very distinctive attribute of ciguatera. This feature may be eventually useful for differential diagnosis of this disorder[1,6]. It is important to note that 18 (95%) out of 19 episodes (single cases and outbreaks) were confirmed by ciguatoxin detection tests on fish samples. This fact illustrates the importance of a close link between laboratory, clinical and epidemiological investigators working together to give a prompt case determination, allowing for quick localization of affected people, accurate identification of causative fish samples and preservation of fish samples for subsequent processing. Early determination and localization of cases has also made it possible to start an investigation about the use of mannitol for treatment of early stages of the intoxication.

Results of anthropological and epidemiological studies suggested the need to examine carefully certain aspects of the clinical treatment of ciguatera. These results suggested we investigate some of the prophylactic and therapeutic measures found in the communities for their possible use in future prevention programs. Some of the epidemiological characteristics found in this descriptive study, such as concurrent alcohol consumption, previous chronic illness, previous medication use and fish cooking style, could be considered as factors for a hypothesis concerning protection or risk. They

are actually being evaluated by our research team by using analytical epidemiological techniques. These results will lead to studies of the correlation between severity of clinical symptoms and degree of toxicity. This information will be very useful in the future.

The anthropological study revealed an unexpected problem. Legal action banning suspicious fish species from sale influenced negatively the attitude of fishermen, generating a defensive behavior when asked about ciguatera. This may be due to their desire to protect their sources of income. Thus, they diminished the importance of ciguatera or hid its existence. Nevertheless, this defensive behavior is in contradiction with practices they developed to recognize ciguatoxic fish and their preventive and therapeutic measures. These practices are not just "beliefs" (as supposed at the beginning) but "popular knowledge", following the concepts of home medicine[9,10]. A "belief" supposes the absence of a logical principle, being simply an adherence to a statement considered true due to confidence in the source from which it came. "Popular knowledge" is on the other hand a response of common sense, originating in the observation of reality and experience that attributes events to cause-effect principles and not magic or religious beliefs.

If as we postulate the therapeutic practices found are part of home medicine, then they are not opposed to scientific medicine, but rather support its existence. Some of the comments of our informants supported this contention:

1. Cause-effect relationship.

The concept of food chain in fishes as responsible for ciguatera.

Particular fish features (smell, eye color, loss of weight, ease of scale detachment) as ciguatera indicators.

2. Susceptibility concept.

The belief that strong people will not get ciguatera.

3. Scientific medicine elements.

Use of substances to induce vomiting.

The use of sugar and its association with diabetes.

4. Home medicine elements.

Use of animal parts, like fish spines, and plant infusions, like the ones coming from mangrove, black cane and sargassum, as therapeutic agents.

It is important to establish a clear distinction between "belief" and "popular knowledge." An adequate educational process should be enough to modify popular knowledge. Since there are not magical or religious elements in the treatment of ciguatera, it is easy to replace empirical medication with a more scientific one. However, it is difficult to modify a "belief" for it requires knowledge of related cultural aspects. Practices utilized to avoid fish intoxication are related to prevention. These facts should be taken into consideration for the implementation of sanitary-educational programs concerning ciguatera.

The findings reported here indicate that the ciguatera problem in the Dominican Republic requires an in-depth interdisciplinary approach. This is necessary in order to define the complex relationship between fishermen, intermediary fish traders, people and facilities involved with processing and retailing of fish. These relationships should be taken into consideration for the successful development of control and prevention programs. This research has reinforced the belief that only by close interactions between sociologists, clinical physicians, epidemiologists, biologists, chemists and other laboratory specialists, will it be possible to develop better techniques that will lead in the future to a reduced risk of ciguatera among the population, and an inprovement in patient care.

Acknowledgements

The authors would like to express their sincere thanks to the Pan American Health Organization for its sponsorship and collaboration. Thanks also to the Dominican Ministry of Health for its collaboration. A great part of the laboratory work would not have been possible without the assistance of Rafaelina Tejada, Luis Sánchez and Juan Bonifacio. Rafael Alba, a very fine artist, was the designer of our information poster and brochure. Thanks. We are very indebted to Frank Richardson for his help with the English version of the manuscript, graphics and pictures, and to Fabián Tello and Fabiola Oviedo for their computing work.

Literature Cited

1. McMillan, J.P., H.R. Granade and P.A. Hoffman. 1980. Ciguatera fish poisoning in the United States Virgin Islands: Preliminary studies. J. Coll. Virgin Island. 6:84-107.
2. Sawyer, P., D. Jollow, P. Scheuer, R.York, J. McMillan, N. Withers, H. Fudenberg and T. Higerd. 1984. Effects of ciguatera-associated toxins on body temperature in mice. In: Seafood Toxins, (E. Ragelis, ed.). ACS Symposium Series #262, American Chemical Society. Washington, D. C. pp. 321-329.
3. Desarrollo Pesquero en la República Dominicana. 1980. Fisheries Development Limited and Instituto Dominicano de Tecnología Industrial (INDOTEC), 435 pp.
4. Bagnis, R., T. Kuberki and S. Lauyer. 1979. Clinical observation on 3,009 cases of ciguatera (fish poisoning) in the South Pacific. Am. J. Trop. Med. Hyg. 26: 1067-1073.
5. Bagnis, R. 1968. Clinical aspects of ciguatera (fish poisoning) in French Polynesia. Haw. Med. J. 28: 25-28.
6. Bruce, S., J. Anderson, J.K. Sims, N.H. Wiebenga and S. Mitsuto. 1983. The epidemiology of ciguatera fish poisoning in Hawaii, 1975-1981. Haw. Med. J. 42: 326-332.

7. Engleberg, N.C., J.G. Morris, J. Lewis, J.P. McMillan, R.A. Pollard and P.A. Blake. 1983. Ciguatera fish poisoning: A major common source outbreak in the U. S. Virgin Islands. Am. Intern. Med. 98: 336-337.
8. Frenette Ch., J.D. MacLean and T.W. Gyorkos. 1980. A large common-source outbreak of ciguatera fish poisoning. J. Infect. Dis. 58:
9. Menéndez, E. 1981. Poder, Estratificación y Salud. Ediciones de la Casa Chata. México, 590 págs.
10. Nader, F. F. 1951. Fundamentos de Antropología Social. Fondo de Cultura Económica. México. 461 pags.

How Ciguatera Affects Canadians

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INTRODUCTION

Ciguatera affects Canadians in two ways, those who are tourists to tropical or subtropical coastal regions and those who consume imported tropical fishes. Many hundreds of Canadians have been exposed to ciguatoxic fishes and many have developed symptoms. Unfortunately, these are rarely properly diagnosed in the country of origin and only those who persist in seeking medical help on their return to Canada are likely to know the cause of their chronic symptoms. There may be 500 persons returning to Canada each year with ciguatera, costing $2,950,000 in medical treatment and loss of work time. Because of this concern, the Health Protection Branch has alerted Canadian tour operators of the risks of locally consumed seafood and asked them to warn their customers, since law suits have been initiated by ill tourists who were not previously aware of the risks. The Branch has also worked with authorities in the Dominican Republic and the Pan American Health Organization to establish a National Ciguatera Commission to alert fishermen, market owners, restaurant and hotel operators, and tourist agencies of the best ways of limiting exposure to toxic fishes, and the most effective treatment for the poisoned.

Immigrants to Canada have consumed toxic fishes in Canada through gifts from overseas friends or through the local fish markets. Many of these immigrants are unwilling to initiate an investigation and their illnesses remain undetected. Tropical fishes are now being served in restaurants more frequently than in the past and, therefore, more consumers are being exposed to potentially hazardous fishes. Control is limited, however, because the fishes are not always correctly labeled as to species or country of origin. Thus, in this category there may be as many as 100 cases of ciguatera a year in Canada, at a cost of $247,000 to the national economy. To reduce the number of cases occurring in the future, recommendations are proposed here for medical authorities, tourists, tour operators and fish suppliers.

Ciguatera Arising From Fishes Eaten In Endemic Regions

Canada has no part of its coastline in subtropical or tropical waters yet some Canadians are at risk from ciguatera poisoning associated with tropical fishes. These include the increasing number of tourists going to the Caribbean. An estimated 99,000 tourists (mostly Canadians) visited Puerto Plata in the Dominican Republic in 1985, compared with 22,000 in 1981. In 1988, 153,000 Canadians visited the Dominican Republic as a whole[2]. Reports of individual cases of ciguatera were first made in the 1960's and 1970's by physicians who had treated patients on their return to Canada, but little was published until the 1980's[3]. With the development of group tours to specific resorts, several outbreaks occurred that could be properly documented on their return. The largest and most recent of these took place in a resort area on the southwest coast of Cuba at the end of March, 1987[4]. A fish casserole was served 4 hours before a group departed for Montreal, and 57 of the 61 eating this dish became ill (93% attack rate) during the flight or shortly after arrival. Once local health authorities became aware of a problem, the travel agency booking the tour was notified and all passengers were advised to contact the McGill University Centre for Tropical Diseases, Montreal General Hospital. Symptoms ranged from very severe to mild, with some of the neurological problems lasting 25 weeks. Ninety-one percent (91%) of cases had neurological and gastrointestinal symptoms, 5% had neurological symptoms alone and 4% had gastrointestinal symptoms alone. No cardiovascular symptoms were observed in any of the patients. Twelve cases were seen and examined at the Centre for Tropical Diseases, and two of these had persistent diarrhea apparently caused by *Giardia lamblia* which was found in their stools. This could have resulted from an independent infection in Cuba or from germination of cysts in the gut through the gastroenteritis brought on by the ciguatera poisoning. Most cases (61%) consulted a physician (mean of 1.7 visits), one person was hospitalized and 26% were off work for a mean of 7.8 days. There was no correlation between those who vomited extensively and those with less severe illness, indicating that vomiting did not have a protective effect (gastrointestinal illness began 1-24 hours, mean of 5.7 hours, after consuming the fishes). Presumably the ciguatoxin had already been absorbed or was in the intestine by the time vomiting commenced. Cold sensitivity was the most distinctive symptom, manifested by a burning sensation on touching cold surfaces, disagreeable feeling in cold air, and an oral burning sensation when consuming cold foods. The symptoms are generally similar to those described in other outbreaks except for the extent of insomnia (58% of patients, lasting up to 150 days, mean 45 days). Without the follow-up made on their return to Canada, it is likely that very few cases would have been reported as ciguatera poisoning victims. A control group of tourists who did not eat the fishes were also questioned, but none had distinctive ciguatera-like

symptoms. However, seven other members of the group had typical ciguatera illness three days preceding the outbreak. The source of fishes may have been the same as for the casserole, but the history of the type of fish served at the resort was not known, as the outbreak was not investigated by Cuban authorities, although ciguatera was previously known from this country[5]. It is possible some of the milder cases ate a mixture of toxic and non-toxic fishes in the casserole.

A total of at least 11 incidents of illness affecting Canadians have been reported, ranging from a few cases to the above episode of 57[3,4,6-10]. Freudenthal also records 13 incidents from 1978 to 1987 in Nassau County, New York[11]. She considered that only a small percentage of tourists visiting the Caribbean, who were victims of ciguatera poisoning, were investigated by New York health personnel, mainly because cases did not seek assistance from the appropriate authorities. Outbreaks and cases in the United States and its territories including areas in tropical or subtropical localities where ciguatera fishes are locally caught (Florida, Puerto Rico, U.S. Virgin Islands, Guam, Hawaii) show two peaks for incidents, one in 1979 and the other in 1985 (Table 1). Since underreporting is widespread and details of the outbreaks are not given, the use of such data to determine overall trends may be misleading[12,13]. However, it is apparent that more people are ill from fishes consumed at home than from fishes served in food service establishments.

Table 1. Ciguatera in the United States

a) Outbreaks and Cases

Year	1979	1980	1981	1982	1983	1984	1985	1986	1987
Outbreaks	18	15	15	8	13	18	27	18	11
Cases	85	52	152	37	43	78	106	70	35

b) Outbreaks and Site of Toxic Fish Consumption

Year	1979	1980	1981	1982	1983	1984	1985	1986	1987
Homes	12	14	3	3	9	13	20	16	11
Foodservice	4	0	2	5	2	0	2	0	0
Unknown	2	1	10	0	2	5	5	2	0

Table 2. Ciguatera Poisonings in Canada

Onset Date	Province	Location	Hospital time	Food	Country of Origin
January, 1983	Toronto, Ontario	home	overnight, recovered quickly	dried barracuda	Jamaica
April 26-27, 1984	Toronto, Ontario	home	0 day, 1 1 day, 7 5 days, 1	grouper	Florida
November 5, 1986	Toronto, Ontario	restaurant	a few days	grouper	?
April 24-26, 1987	Toronto, Ontario	home	?	mongol snapper	?
October 24, 1989	Hull, Quebec	home	overnight	mahi-mahi	?
June 7, 1989	Kitchener, Ontario	home	overnight	grouper	Spain
February 26, 1990	Vancouver, British Columbia	home	child, hospital for 6 days	grouper (Fiji red salmon)	Fiji

† N=nausea, C=abdominal cramps, H=headaches, V=vomiting, D=diarrhea
‡ By immunoassay using antibody to partially purified ciguatoxin (Y. Hokama, University of Hawaii, personal communication).

Ciguatera Arising From Fish Eaten in Canada

In recent years, the demand in Canada for tropical fishes has increased. This is partly because there are more immigrants (particularly Vietnamese refugees) from tropical countries where such fishes are traditionally eaten, and also because Canadians, travelling more widely to the Caribbean and Pacific, are developing a taste for such fishes. The demand for ethnic food as a whole in Canada is expected to rise 8.1% each year until 1996. For instance, in 1985/86, 625 lots of snapper (153,676 kg), 488 lots of grouper (238,126 kg) and 282 lots of kingfish (52,307 kg) were imported to

Cases (at risk)	Incubation period (hrs)	Duration (hrs)	Symptoms †	Mouse toxicity rating
2 (2)	1.5	18	D., weakness, numbness of tongue and lips	5
9 (9)	3-6	1 day - 16 months	N.V.C.D., weakness, tingling or numbness of tongue, jaw, face, hands, feet	1-2 (5) ‡
1 (3)	1	days	hypertension, bradycardia pruritis, reversed hot-cold sensation. Case claimed he had ciguatera through his knowledge of the disease, but no samples were available for mouse bioassay	not done
7 (?)	?	?	no details available	not done
1 (2)	1.5	24	pruritis of torso, flushing of the face and neck, swelling of the face, difficulty breathing	5
2 (2)	5.5	12	N.V.D., weakness, numbness, in arms, legs tongue	1-2
4 (4)	5.5-7	up to 45 days	N.V.C.H., sore joints, itching tongue	4-5

Canada with a total of 10 lots rejected, none for ciguatoxin presence[14]. This increased demand may explain the documentation of apparent ciguatera incidents occurring in Canada. These have been reported since 1983 with an average of one incident and 3.7 cases a year (Table 2). In the seven incidents, all fishes were eaten at home, except one which was eaten in a high-class restaurant. Five incidents were in Ontario, and one each in Quebec and British Columbia. The types of fishes were grouper (four incidents), snapper, mahi-mahi and barracuda (one incident each). The origin of the fishes was known in only four of the incidents (57.1%): Florida, Jamaica, Spain and Fiji. The latest

episode occurred in February, 1990, caused by a grouper imported from Fiji. There were four cases, two of which were mild and two more severe[15]. Table 3 indicates that the dose was clearly a factor in the severity of the illness. The 5-year old boy was the most seriously ill. The mouse bioassay (modified McMillan extraction procedure followed by intraperitoneal injection of the extract into mice to give a toxicity rating of 0 to 5 based mainly on rectal temperature) was performed on the samples associated with illness that were available[16]. Toxicity ratings ranged from 1 to 5 (Table 3). These numbers do not necessarily reflect the amount of ciguatoxin present, for it is known that toxins other than ciguatoxin will cause a fall in temperature and death in mice. However, whether these toxins, mostly undefined, will cause human intoxication by ingestion, as opposed to mouse intoxication by intraperitoneal injection, is not known. Ideally, these results should be confirmed by chemical or immunological procedures that are specific for ciguatoxin. A grouper causing 9 cases in Toronto in 1984 was the only sample that had some form of verification. This had a mouse toxicity rating of between 1 and 2, but also had

Table 3. Dose and Severity of Symptoms†

Sex	Age	Body Weight (kg)	Dose ‡ (pieces eaten)	Dose (g/kg)	Symptoms
M	34	61.25	5 curried (1125 g)	18.4	severe for 3 d, off work for 2 wk, rash lasted >3 months
F	33	56.75	1 pan fied (225 g)	4.0	mild, some itching lasting >3 months
M	12	38.5	1 pan fried (225 g)	5.8	mild, no continuing symptoms
M	5	18.15	2 pan fried (450 g)	24.8	hospitalized for 6 d, ill for 2 months, stiff and sore joints after excersice for >3 months

† A family outbreak. Information from A. Hamade, Environmental Health Division, Health Department, Richmond, British Columbia.
‡ Fish cut into 15 approximately equal pieces, either pan fried or curried. Each piece weighed about 225 g.

a rating of between 4 and 5 for an ELISA test with polycyclic ether antiserum and sheep anti-ciguatoxin, both for the water-soluble fraction in butanol and the ether-soluble fraction in methanol (Y. Hokama, University of Hawaii, personal communication). However, in 1984 the ELISA methodology had not been perfected and the ciguatoxin was not pure.

Estimates of Ciguatera Cases and Their Cost in Canada

There is approximately one incident per year of ciguatera poisoning, with an average of three cases from persons eating toxic fishes in Canada (Table 2). It has been estimated that most foodborne illnesses in Canada and the United States are underreported by a factor of 350 to 1[17,18]. This would mean that there are approximately 1,000 cases each year in Canada. This figure may seem high, but it is known that most of the victims are immigrants, some of whom are reluctant to contact a health agency because of language problems or fear of an authority figure. Until surveys are done of certain segments of the population, this figure will be difficult to determine with accuracy. However, because most Canadians do not eat tropical fishes, it might be wiser to be more conservative and select a figure of 100 cases a year. Costs have been determined for one episode in 1990 dollars (Table 4) with an average cost per case of $2,470. Hospitalization expenses were the main factor in this figure. The total annual costs of ciguatera arising from fishes eaten within the country, therefore, is $247,000.

The number of tourists returning ill to Canada is more difficult to estimate, but certainly exceeds those at home. In 1988, there were 570,000 visits to Bermuda and the Caribbean for an average of 10.0 days, 244,000 visits to Hawaii for an average of 14.8 days, and 141,000 visits to Oceania (Australia, New Zealand and the Pacific islands) for an average of 24.0 days[2]. Therefore, there are about 1,000,000 Canadians visiting areas where ciguatera is endemic, for an average time of 10-24 days. If it assumed that 10% of these eat tropical fishes during their stay and only one in 200 of these has some kind of illness from ciguatera, then the number ill would be 500. Costs for these are higher than for resident cases (Table 4) with an average cost per case of $4,875-$6,920 (mean $5,900). This results in a cost of $2,950,000 for tourist-associated ciguatera. Time off work or lost vacation time seems to be a major component of these costs because of the long-term debilitating effects of the disease. Total cost for both types of ciguatera is about $3,200,000.

Treatment For Ciguatera

Until recently, effective treatment had eluded medical researchers, although the use of a variety of drugs had been attempted in the 1970s and 1980s. In 1988, Palafax *et al.* published data that indicated intravenous manni-

Table 4. Cost of Ciguatera Poisoning to Canadians†

Location	Jamaica		Dominican Republic		Toronto	
Year	1983[3]		1985[3]		1984	
Cases	2		3		9	
	Actual Cost	Percent Total	Actual Cost	Percent Total	Actual Cost	Percent Total
Medical Costs	410	4.2	550	2.7	1,380	6.2
Hospitalization	NA	NA	NA	NA	17,610	79.2
Investigation	NA	NA	NA	NA	1,150	5.2
Value of time off work or vacation	9,340	95.8	20,210	97.3	2,060	9.3
Loss to fish retailer	0	0	0	0	30	<1
Total Costs	9,750	100	20,760	100	22,230	100
Costs per case	4,875		6,920		2,470	
Mean cost per case for incidents involving tourists in Jamainca and the Dominican Republic				5,900		

†Actual cost given in 1990 Canadian dollars.

tol, when given 1 g/kg over 30 min, had successfully lessened symptoms[19]. All neurologic and neurosensory manifestations were resolved completely, usually within 48 hours, although improvements were noticed in minutes. Gastrointestinal symptoms disappeared more slowly. All 24 patients from the Marshall Islands treated gave noticeable improvement. Pearn *et al.* confirmed the efficacy of intravenous mannitol in a study of 12 Queensland cases, although some responded better than others[20]. They used the same concentration of mannitol, after rehydration therapy, over a 45 min period. For two cases this was repeated within 24 hours. A lower dose of 0.5 g/kg infused over 1 hour did not seem to be effective, suggesting that the rapid increase in osmolality at higher dosages may be important. The mechanism of the therapeutic action is not yet known.

In summary, intravenous mannitol infusion seems to be a most promising form of treatment for cases in the acute stages if given at a concentration of 1 g/kg over 30-45 min. Improvements were rapid even for one patient 8 days after ingestion of toxic fishes. It is a cheap, simple procedure that can be used in areas with limited medical facilities. Previous experience with intravenous mannitol for therapy in other clinical syndromes has caused few problems, but patients should be properly hydrated (after vomiting and diarrhea) before the

mannitol is given. Pearn *et al.* are planning a single-blind clinical trial and other researchers should be attempting the same before a whole-hearted recommendation can be made[20]. If this style of therapy is justified, then patients who are still ill on their return to non-endemic areas, could also be given this treatment. Therefore, centers for tropical disease should be aware of this type of therapy and advice given to those who have the symptoms to seek this form of treatment as soon as possible.

Ciguatera Control in the Dominican Republic

As a result of complaints by tourists ill in the Dominican Republic in the mid 1980s, I was asked to work with representatives of the Pan American Health Organization and the Dominican Republic to determine the extent of the problem and recommend possible control measures.

The following was the position in the Dominican Republic in 1987 concerning ciguatera.

1. Tourists are generally unaware of ciguatera and are more likely to be familiar with danger of non-potable water and infections from shellfish. In Mexico, in contrast, tourists are usually well aware of the potential for gastroenteritis and may even expect to be ill, but it does not stop them from going there.

2. The demand for fishes by tourists and local residents and for export is increasing, and is being encouraged by the government, with some retailers and processors expanding rapidly. Fish costs, however, are relatively high and this is one reason local people do not eat as much fish as in other Caribbean countries. Also the fear of ciguatera or other forms of illness is recognized. The high price also limits exports to many countries. Freshwater *Tilapia* farming is being encouraged by the government as another source of fish protein.

3. Species considered to be potentially toxic are great barracuda (*Sphyraena barracuda*), southern sennet (*Sphyraena picudilla*), Atlantic moonfish (*Selene setapinnis*), Atlantic lookdown (*Selene vomer*), almaco jack (*Seriola rivoliana*), greater amberjack (*Seriola dumerili*), yellow jack (*Caranx bartholomaei*), African pompano (*Alectis ciliaris*), tiger grouper (*Mycteroperca tigris*), black grouper (*Mycteroperca bonaci*), yellow fin grouper (*Mycteroperca venenosa*), green moray eel(*Gymnothorax funebris*) and frigate tuna (*Auxis thazard*).

4. Knowledge of ciguatera illness is limited because a) there is no reporting system for monitoring ciguatera cases, b) the methods used by fishermen to recognize toxic fishes have not been scientifically confirmed. In addition, some markets are selling potentially toxic fishes (included in the old list of prohibited fishes but dropped from the new, revised list).

This new ban prevents year-round catching of 4 species of non-commercial fishes (*Sphyraena barracuda, S. picudilla, Seriola rivoliana, Alectis*

ciliaris). It replaced one that prevented the harvesting of many more species but only from May to October. The rationale for this change was not explained, but it is apparent that some commercially important fishes are allowed to be sold that were previously considered to be seasonally toxic.

Suggested Control Strategy

I. Immediate Action (3-6 months)

1. Tourists should be advised by tour operators of the potential of illness from eating tropical fishes, and resort operators should be responsible for obtaining fishes least likely to be toxic, i.e., small individuals of species not known to be locally toxic, and imported fishes and shellfish from temperate seas. An educational leaflet or notice could be designed locally, explaining the problem and that every precaution has been taken to minimize illness.

2. Physicians attending resorts, hotels and in hospitals in tourist regions should become familiar with ciguatera and other seafood toxin problems to give advice for care and treatment. They should also counsel cases that most of them will recover within a few days to weeks, although weakness may persist and recurrences may be triggered by exercise, alcohol or eating non-toxic fishes. Physicians should also report incidents to the appropriate government authority(ies) and fish samples should be retrieved for testing.

3. Designated laboratories should be able to test fishes for the presence of ciguatoxin, and they should be familiar with the mouse bioassay with positive and negative samples.

4. Literature on ciguatoxin and other toxins should be collected and filed for reference.

5. A National Ciguatera Commission should be established to coordinate these activities.

II. Short-range Action (6-12 months)

1. An educational strategy should be developed not only for tourists but also for fishermen, retail shop and restaurant operators.

2. Epidemiological teams should be set up to link with government departments to obtain a better understanding of the true nature of the ciguatera problem in the Dominican Republic. Bureaucratic responsibilities may have to be altered to allow one person (or group of persons) to monitor and summarize the information. A phone survey could be attempted and emergency room hospital records reviewed for the nature and seasonality of illnesses involving consumption of fishes, as has been done in the Virgin Islands.

3. A limited survey should be conducted to test representative samples of the main economic species of fishes (ones eaten most often). If fishes, however, are identified as toxic by the mouse bioassay no immediate ban is suggested unless large numbers of the same species are involved or fishes are associated with human illness. It is also important to make sure that the

laboratory methodology is accurate and at least one other laboratory should confirm the toxicity if enough sample remains. Extracts could also be sent to laboratories in other countries for confirmation by the best current methodology. Trimmings such as tails, heads, intestines, as well as whole fishes obtained by inspectors could be used as samples. Also, fishes considered to be toxic by fishermen should be tested to see if their local knowledge is valid. (These fishes may turn out to be non-ciguatoxic but may still be inappropriate to eat because of disease, spawning conditions or spoilage.)

4. The laboratories should also learn to detect scombroid and possibly other fish toxins, as well as ciguatera.

5. Contacts with other Caribbean countries should be established or strengthened to help determine the most useful strategies for dealing with the ciguatera problem

III. Long-term Action (1-5 years)

1. The monitoring of ciguatera cases in tourists and residents should continue to help determine what fishes are involved, locations apparently toxic for specific species, seasonal changes and yearly changes. The supply and toxicity of fishes obtained from beyond Dominican waters need to be carefully noted so that there is no confusion with local sources.

2. More extensive surveys based on the limited surveys done earlier should be attempted with resources obtained from various agencies. Samples taken from specific locations at certain times of the year, based on the previous survey and epidemiological data, may define more precisely where ciguatoxic fishes are likely to occur. Additional sampling should also be done following hurricanes, shipwrecks, construction, dredging, dumping, where coral reef damage is expected or new rock surfaces are available for dinoflagellate colonization. A scientific project to determine the presence of *Gambierdiscus toxicus*, *Prorocentrum* spp. and other potentially toxic dinoflagellate species, and associated symbiotic algae, may be attempted but this should be done with biologists having research experience (university and institution teams, including experts from other countries).

4. The use of a simplified "stick" immunoassay test should be available within this year. This could be used by inspectors at fishing communities, sports fishermen, processors, food service operators and laboratory analysts.

5. The National Ciguatera Commission should convene at regular intervals to control the whole process and should be vigilant concerning changes because ciguatera toxicity seems to be a variable phenomenon.

Action Taken in the Dominican Republic and Canada

A National Ciguatera Commission was established in the Dominican Republic which initiated Project Ciguatera to make the local public and tourists aware of the ciguatera problem. The Commission:

- produced posters and pamphlets in Spanish and English;
- alerted physicians and nurses of the symptoms and supplied appropriate illness investigation forms;
- established Instituto Dominicano de Tecnología Industrial (INDOTEC), responsible for testing and tracing suspected fishes;
- informed visitors that fish caught between November and April may be toxic, even though fishermen consider this the least toxic period;
- publicized the ban on barracuda, southern sennet, jacks and African pompano;
- organized scientific conferences on ciguatera.

More details of the activities carried out by the Dominican Republic are published in these proceedings.

The Health Protection Branch in Canada has also become more involved in the following areas.

1. The Health Protection Branch is cooperating with the Pan American Health Organization in attempts to develop control strategies in the Caribbean.

2. A Health Advisory notice has been issued describing the disease and its origins and has been sent to tour organizers. It concludes that "ciguatera poisoning is not new and has been recognized in returning travellers for many years. It is likely that many undiagnosed cases have occurred in the past in tourists to the Caribbean. Cases have also occurred after eating toxic fishes imported into Canada. Tour organizers and resort operators should be aware of this problem. Steps should be taken to ensure potentially toxic species of fishes are excluded from menus served to vacationers, especially when little choice of food is offered in a package vacation. Travellers should be advised to avoid eating potentially toxic fishes, as defined by local authorities for the area of travel. Further information is available from Dr. E. Todd (613) 957-0887, Health Protection Branch, Food Directorate."

It is interesting to note that this advisory notice was translated into Spanish and circulated in the Dominican Republic where it was drawn to the attention of some United States tourists who were affected by ciguatera.

3. There is interest in pursuing methods permitting the detection of ciguatera in individual fish before they are marketed.

Other Recommendations

As a result of the occurrence of a large number of ciguatera cases (103) on the small West Indian island of Antigua in September-October, 1980, specific recommendations were made (J. Andrews, Centers for Disease Control, Atlanta, GA. USA, personal communication). These included improving surveillance, especially after storms and other factors disturbing coral reefs, education of consumers, fishermen and exporters, and exchange of informa-

tion by countries that have a ciguatera problem. It was noted that the fishes causing the illness had been bought from the fish market in the main town one month after a hurricane had passed through the area. Also, at least some of the fishes had been caught off Redonda Rock, an area known to contain toxic fishes. One reason for fishing in areas that are generally recognized as off-limits is the ease of catching fishes, which are often large and give a better price per pound.

Because of ciguatera occurring in tourists from the New York area, various recommendations were suggested by Freundenthal[11]. These included better epidemiologic information, holding of fish carcasses for two days to allow for testing, preferably by the "stick" immunoassay test, education of consumers and tourists, counseling of victims, and overall coordination of the various groups involved.

To improve awareness and ultimately reduce the prevalence of ciguatera it is recommended to:

1. Make physicians and nurses more aware of ciguatera, particularly in areas where the disease is most likely to be encountered. These include tropical communities where seafood is eaten, and hospitals with tropical disease centers in large cities where tourists returning from overseas vacations may seek help. In addition, medical personnel associated with ethnic communities where tropical fishes are imported, and pharmacists who may be asked for drugs to help lessen the symptoms, should also be alerted.

2. Promote the use of any proven therapeutic measures, including the use of intravenous mannitol, at least in the acute stages of illness.

3. Inform the public of the possible dangers of eating potentially toxic fishes through posters and warning notices at fishing communities, fish markets, travel agencies, and tourist information booths. Multilingual notices may be required in certain areas. It may also be desirable to alert specifically those at highest risk (young children, pregnant and nursing women, and possibly those with underlying diseases).

4. Acquire better epidemiologic data from acute and chronic cases, e.g., type and duration of symptoms, time of year, fish species, and amount of fish eaten. Hotlines can be set up for ill persons to contact physicians for counseling as well as reporting.

5. Publicize any bans on specific fishes that have been appropriately derived, including information on specific seasons, if any, to avoid consuming potentially toxic fishes.

6. Encourage contact between countries which have an endemic ciguatera problem, through international organizations like CAREC. Information on outbreaks, areas where toxic fishes occur, seasonality, and adoption of specific control measures should be shared. Standardized forms for assisting the investigation of outbreaks and follow up of cases

should be agreed upon. Although these should be as complete as possible, they also must be practical for investigators to fill out. Investigations of outbreaks and single cases should be reported to a national agency and made available to public health officials of other countries.

7. Correctly label fishes at markets and for export so the species can be accurately determined if it is involved in an illness. Consider the possibility of storing frozen fish parts (heads, tail viscera) for at least two days at restaurants after the remainder of the fish has been prepared into a meal and served. This would allow the possibility for toxin detection if illness occurs.

8. Make the best methodology available to analysts wishing to test fishes for ciguatoxin or other fish toxins. Existing bioassay techniques should be replaced by specific chemical or immunological procedures once they are validated and standards are available.

Literature Cited

1. Anon. 1986. Turismo en cifras, 1985. Secretaría de Estado de Turismo, República Dominicana.
2. Statistics Canada. 1989. 1988 International travel. Travel between Canada and other countries. 66-201, Ottawa, Canada.
3. Todd, E. 1985. Ciguatera poisoning in Canada. In: Toxic Dinoflagellates (D.M. Anderson, A.W. White, and D.G. Baden, eds.). Elsevier, New York. pp. 505-510.
4. Frenette., C., J.D. MacLean and T.W. Gyorkos. 1988. A large common-source outbreak of ciguatera fish poisoning. J. Infect. Dis. 158:1128-1130.
5. Muñoz, E.V. 1980. Toxicidad y alimentación de algunos peces sospechosos de provocar la ciguatera. Informe Científico-Técnico No. 123. Academia de Ciencias de Cuba, La Habana.
6. Bourgault., A.-M., and E. Todd. 1985. Ciguatera poisoning acquired in the Dominican Republic - Quebec. Can. Dis. Weekly Rep. 11:151-152.
7. Cohen, L., J.D. MacLean and R. Lalonde. 1986. Ciguatera fish toxin poisoning: An outbreak in Canadian tourists in the Dominican Republic. Can. Dis. Weekly Rep. 12:73-75.
8. Heimbecker, R.O. 1979. Ciguatera poisoning - snowbirds beware. Can. Med. Assoc. J. 120:635.
9. Philpott, J.P. 1987. Ciguatera poisoning in travellers to the Dominican Republic. Can. Dis. Weekly Rep. 13:71.
10. Wittes, R.C., and J.D. MacLean. 1984. Ciguatera poisoning with neurologic findings: four more probable cases. Can. Dis. Weekly Rep. 10:202-204.
11. Freudenthal, A. 1990. Public health aspects of ciguatera poisoning contracted on tropical vacations by North American tourists. In: Toxic Marine Phytoplankton (E. Graneli, B. Sundstrom, L. Elder, and D.M.

Anderson, eds.). Elsevier, New York. pp. 458-463.
12. MacDonald, K., and P.M. Griffin. 1986. Foodborne disease outbreaks, annual summary, 1982. Morbid. Mortal. Weekly Rep. 35 (SS-1):7-16.
13. Bean, N.H., P.M. Griffin, J.S. Goulding, and C.B. Ivey. 1990. Foodborne disease outbreaks, 5-year summary 1983-1987. Morbid. Mortal. Weekly Rep. 39 (SS-1):15-57.
14. Fisheries and Oceans Canada - 1987. Canadian Fisheries Annual Statistical Review. 1986. Vol. 19. Ottawa, Canada.
15. Garry, J.D., K. Higo and A. Hamade. 1990. Ciguatera fish poisoning. British Columbia Disease Surveillance. In press.
16. Hoffman, P.A., H.R. Granade and J.P. McMillan. 1983. The mouse ciguatoxin bioassay: a dose-response curve and symptomology analysis. Toxicon 21:363-369.
17. Todd, E. 1989a. Preliminary estimates of costs of foodborne disease in Canada and costs to reduce salmonellosis. J. Food Protect. 52:586-594.
18. Todd, E. 1989b. Preliminary estimates of costs of foodborne disease in the United States. J. Food Protect. 52:595-601.
19. Palafax, N.A., L.G. Jain, A.Z. Pinaro, T.M. Gulick, R.K. Williams, and I.J. Schatz. 1988. Successful treatment of ciguatera fish poisoning with intravenous mannitol. J.A.M.A. 259:2740-2742.
20. Pearn, J.H., R.J. Lewis, T. Ruff, M. Tait, J. Quinn, W. Murtha, G. King, A. Mallett and N.C. Gillespie. 1989. Ciguatera and mannitol: experience with a new treatment regimen. Med. J. Austral. 151:77-80.

Report on Ciguatera Fish Poisoning, Owia[1], November 23, 1985

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On the morning of November 24, 1985, I was asked by the Honourable Minister of Health, Mr. E.G. Griffith, to visit the Georgetown Hospital along with himself and the Senior Medical Officer. Several people had been admitted to that hospital the night before after having consumed a barracuda fish (*Shpyraena barracuda*).

On arrival at the hospital I spoke with the District Medical Officer, Dr. Krishna Hari, as well as with several patients. The symptoms, which included vomiting, diarrhoea, abdominal pains, temperature reversals, metalic taste, itching and tingling hands and feet indicated that they were victims of ciguatera poisoning. The DMO informed me that he had treated two similar cases in Bequia in 1983, while he was District Officer of the Grenadines. One patient had a very weak pulse and was experiencing difficulty in breathing. She was transferred to the Kingstown General Hospital. The latter symptom was probably due to the effect of the ciguatoxin (a haemotoxin) on the parasympathetic nervous system causing paralysis or restricted movement of the diaphragm.

Discussions with the nurses aid on duty at Owia on the day of the event revealed that from 6:00 p.m. that evening victims started pouring into the clinic in search of relief from vomiting, diarrhoea and abdominal pains. Her response in addition to administering medication was to inform the authorities at the Georgetown Hospital eight miles away who advised her that she should send all victims to that hospital as soon as possible. The Owia clinic is linked to the Georgetown Hospital and Sandy Bay clinic by solar powered VHF radio. There is no ambulance service available so transportation by commercial vehicle was the only way. By 7:00 p.m. the buses "Sun Arrow" and "Carib B" had left Owia for Georgetown laden with people overcome by diarrhoea and vomiting. Some had to be carried to the buses as they were already dehydrated and very weak in their legs.

Normally, symptoms usually make their first appearance within six hours after eating and some patients had eaten the meal at noon on that day. Of the more than 105 people who were affected most of them had eaten the fish between 12:00 noon and 7:00 p.m. that evening, so that upon arrival at the

Georgetown Hospital (10:30 p.m.) most of the symptoms associated with the poison were already very much evident.

The time of appearance of first symptoms after consumption varied from half an hour to forty-eight hours. It must also be mentioned that many victims sought medical attention (out of caution) even though they experienced no symptoms at all. When one considers the time, distance and transportation problems involved it was very prudent of the victims not to sit and wait for the first symptoms to appear, hoping that they would never appear.

From all accounts the fish was fresh and not preserved in any way, and preparation was done in the normal ways. The fish had been speared by a thirty-seven year old fisherman, with some twenty years spear fishing experience, from Fancy (a nearby village to the west) who was on the government payroll and who should have been on the job at that time. Three other barracudas were caught by the same method on the same day by the same man and at the same location (Quashie Head), one being larger than the ciguatoxic one. The fish was estimated to be about thirty pounds (30 lbs) in weight by the fisherman himself and by those who saw the whole fish. It was caught at about 7:00 a.m. and by 8:30 a.m. was on sale in Owia by the fisherman himself. Selling price was two dollars and fifty cents ($EC) per pound. The fisherman confirmed that the fish looked no different from any other barracuda he had ever caught, or he would not have sold it. He did acknowledge hearing that barracuda were sometimes poisonous.

The predominant symptoms experienced by the victims were vomiting and diarrhoea but most of the classical symptoms were present. Later symptoms (appearing one week after consumption) included the shedding of pubic hairs in females and a burning sensation during micturition in both males and females (Personal communication with medical staff and patients at the Georgetown Hospital).

Of the more than one hundred and five people who sought medical attention 52.38% were female, 47.62% were males and ages ranged from 1 1/2 years to 78 years of age. The age distribution of the patients was;

0 - 5 years	12.38 %
6 - 9 years	14.28 %
10 -15 years	17.14%
16+ years	56.20 %

The average weight of fish consumed per person was 1.41 ozs. Some individuals had no fish but drank only the 'sauce' in which the fish was boiled. The type of fish preparation made no difference with respect to the time of appearance or nature of the symptoms observed. Most people had the fish boiled, and some had more than one 'serving' of different fish preparations. The breakdown of patients with respect to the type of fish preparation they consumed was as follows;

Boiled	70.48 %
Stewed	2.85 %
Sauce only	18.09 %
Fried	7.63 %
Roasted	0.95 %

Many of the victims reported experiencing their first (mild) symptoms about one to two hours after consumption. At about six (6) hours after consumption most of the victims had already experienced the symptoms. A total of fifteen people ate the flesh of the fish and/or drank the 'sauce' yet experienced no symptoms. Out of precaution however they sought medical attention at the Georgetown Hospital. Of this group, 40% were in the 1.5 - 5 year old range, 27% between 6 - 9 years, 6% between 10 - 15 years and 27% sixteen years of age or over.

An undetermined number of small domestic animals died after consuming the remains of food that was thrown away by their respective owners after they had determined that the food was the cause of their poisoning. These domestic animals were cats, dogs and chickens, the last of which are often utilized as a source of meat protein. In the cases of the dogs and cats, ulcers appeared on the skin and they became dehydrated from diarrhoea and vomiting. They also lost their appetite. In some cases there was muscular weakness as their limb muscles were unable to support their body weight. In the case of the chickens there was prolapse of the rectum through the anus due to frequent defecation. The feces was black due to internal haemorrhaging in the large intenstine and rectum. (Personal communication with Chief Veterinary Officer, Dr. N.C. Raninga).

By far the most lamentable and regrettable incident to occur in the whole episode of this incidence of fish poisoning was the attitude of one of the bus drivers transporting the victims to the Georgetown Hospital. On his arrival at Sandy Bay the driver stopped the bus, ordered all passengers to disembark, said the fare was one dollar ($EC) per person and enquired of them as to how he was to be paid (Personal communication, victims). Here were people with a combined and unrelenting assault of vomiting, diarrhoea and temperature reversals in the dark of night nowhere near to any toilet facilities, some so weak they had to be lifted to and from the bus, some not knowing how far away they were from the end of their lives, being asked to pay for what could have been their last bus ride. Many had no money with them. All passengers from this bus were therefore transferred to a bus based at Sandy Bay to complete the journey to the Georgetown Hospital.

That thirty pounds of fish could be shared among some one hundred and twenty individuals is an indication of some of the socio-ecomomic problems of the area. If the fish were marketed in Kingstown for example, where families are smaller, fewer people would have eaten larger portions at any one

sitting. Restaurants in Kingstown serve a midday fish meal ($EC 9.00) consisting of a slice of stewed fish of about 4 ozs. in weight and barracuda is not excluded from the menu. Assuming a direct relationship between severity of symptoms and quantity of fish consumed, with the concentration of the toxin being constant, one can easily conclude that had the incident taken place in Kingstown the data collected would have been much different.

Notes

[1] A village on the extreme north east coast of St. Vincent and the Grenadines, population (1985) 1,035, 507 females and 528 males. There were 22 births and 7 deaths recorded for the year.

[2] Mr. Kerwin Morris, Chief Fisheries Officer of St. Vincent and the Grenadines, attended the Conference representing Mr. Davon Joseph, Development Officer, Organization of Eastern Caribbean States, Fisheries Unit, Kingstown, St. Vincent and the Grenadines. Mr. Morris submitted this report summarizing the events surrounding an incidence of ciguatera fish poisoning in St. Vincent and the Grenadines.

Legal Aspects of Ciguatera Fish Poisoning in Puerto Rico

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INTRODUCTION

In Puerto Rico for many years any person who became intoxicated with ciguatera by eating fish in a restaurant was certain of obtaining compensation for damages. The amount of compensation was dependent on the severeness of the intoxication, duration of symptoms and hospitalization. All the plaintiff had to prove was that he or she had eaten fish in the defendant's restaurant, and that as a consequence developed the symptoms associated with ciguatera fish poisoning. In order to receive compensation in Puerto Rico's system of law there are three basic elements that the plaintiff must prove:

1) A wrongful or negligent act or omission;

2) That the plaintiff suffered damages;

3) A causal relation between the damages and the wrongful or negligent act or omission.

The Supreme Court of the Commonwealth of Puerto Rico has applied the anglo-saxon theory of "implied warranty" or "absolute responsibility" in cases of food poisonings which involved establishments that manufactured food for human consumption on the precedents established in the cases of Castro vs. Payco, Inc., 75 DPR63 (1953), and Mendoza vs. Cervecería Corona, Inc. , 97 DPR499 (1969). This meant that the plaintiff did not have to prove a negligent act or omision on the part of the defendant in order to win his or her case.

The system of law in Puerto Rico is rather different from that in the continental United States, particularly in tort actions. In the common law system used in the United States, if a plaintiff claims damages for a tort action and the defendant can prove that the plaintiff contributed in any way to the cause of his or her damages, the plaintiff is barred from receiving damages. The basis of this judgement is that the plaintiff showed "contributory negligence."

The Supreme Court of Puerto Rico had ruled that the lower courts were bound to judge tort actions in the two cases mentioned above under the anglo-saxon doctrine of "implied warranty", however, the courts could not use the doctrine of "contributory negligence" to dismiss these cases. The Supreme

Court directed the lower courts to use the doctrine of "comparative negligence." Under this doctrine the plaintiff received compensation, however, the percentage of negligence which he or she incurred was deducted from the total compensation awarded.

Distinct from the Commonwealth of Puerto Rico, under federal or state laws, if the defendant could prove that at the time of eating the culprit fish the plaintiff knew that by eating fish he or she could become intoxicated with ciguatera, this constituted an assumption of risk, therefore "contributory negligence" was evident and the case would be dismissed. In the U.S. Virgin Islands where a vast majority of the people have had or knew of encounters with ciguatera, most of the legal cases filed in state or federal courts were dismissed (see Bronson vs. Club Comanche, Inc. 286 F. Supp. 21, 1968). However, in Puerto Rico even if the defendant could prove "contributory negligence", all he could expect would be a reduction in the compensation he would have to pay under the doctrine of "comparative negligence." As a result, most if not all legal cases claiming intoxication by ciguatera caused by fish consumption in public establishments brought to court in Puerto Rico were settled out of court. This was a rather unjust and unfair situation.

Recent Legal Progress

At the time this paper was presented at the Third International Conference on Ciguatera Fish Poisoning in May of 1990, this was the legal status in Puerto Rico of cases involving ciguatera fish poisoning. In this presentation I indicated that there were two test cases before the courts that could change the legal bases on which ciguatera fish poisoning cases were being judged. Such a change has in fact occurred.

In the first of these two test cases the defendant won by means of a Summary Sentence. This means that the case before the court presented no controversy of fact and that the court had only to resolve a matter of law. The defense accepted the fact that the plaintiff consumed fish at the defendant's restaurant, and that as a consequence the plaintiff developed symptoms associated with ciguatera intoxication and suffered damages. All the court had to decide was whether the "implied warranty" or "absolute responsibility" doctrine applied in cases involving ciguatera fish poisoning. Some of the many scientific and legal arguments which were used to convince the court that this doctrine could not apply in ciguatera cases were:

1. Ciguatera fish poisoning is a unique and exceptional kind of intoxication where human hands (mishandling of food) play no part. The neurotoxin that affects humans that consume ciguatoxic fish is accumulated in the fish by means of a natural process, through the food chain;

2. The other cases resolved by the Supreme Court (Castro vs. Payco Inc., 1953, and Mendoza vs. Cervecería Corona, Inc., 1969, cited above) dealt with

human illness caused by the consumption of **manufactured** food products. Fishes are not manufactured food products;

3. There is no way at this time to prevent the damages that a person who eats toxic fish might suffer;

4. There is no possible way in which the defendants could have been negligent, since human hands (mishandling of food) play no part in ciguatera fish poisoning;

5. If there was anyone responsible for the damages caused by ciguatera fish poisoning, it was the GOOD LORD who created the oceans and all the organisms that thrive in it. As a result we would have had to bring HIM into the case as a third party defendant, however, it would be impossible to find anyone who could or would deliver the subpoena.

The defendant won the second test case in trial. The plaintiff asked the Supreme Court to reverse the decision of the Superior Court, which had judged in favor of the defendant, the restaurant that served the fish from which the plaintiff contracted ciguatera. The Supreme Court denied the request and confirmed the decision of the lower court (Méndez Corrada vs. Ladi's Place, 90 JTS 125, 1990).

In deciding this issue the Supreme Court found that the evidence before the court incontrovertibly showed that:

1. Ciguatera is a serious problem around the world, specially in tropical areas. This intoxication is distinguishable from other types of fish poisonings which are caused by the growth of bacterial contaminants due to inadequate handling and processing practices;

Scientific investigations indicate that ciguatera is caused by small micro-organisms (dinoflagellates) which are eaten by small fishes, which in turn are eaten by larger predators. The toxin is transmitted through the food chain to the larger fishes which are consumed by human beings;

2. The toxin or toxins are not destroyed by conventional means of cooking such as: frying, boiling or broiling. Neither are they eliminated by conventional means of processing and handling such as: drying, salting, freezing or smoking;

3. The toxin is not detectable by the appearance, odor or taste of the fish. At the present time there is no adequate method of detecting the toxin. The origin and identity of the toxin or toxins involved in ciguatera fish poisoning, the organisms that generate and transmit it, as well as the ecological factors involved are not completely known or understood;

4. More than four hundred (400) species of fish are implicated in this type of poisoning. The outbreaks of ciguatera are sporadic and unpredictable, both in time and geographic distribution. Not all members of the same species captured in the same place, at the same time are toxic;

5. Medical treatment is symptomatic and there is no known antidote;

6. There is no known practical method to detect a toxic fish when it is captured, nor before or after its commercial distribution. One knows that the fish is toxic only after having eaten it.

These realities, said the Supreme Court, can only support one conclusion: "There is no way to prevent the damage that a consumer might suffer. The only way to prevent the damages would be total abstinence. The doctrine of 'absolute responsibility' does not apply in cases involving ciguatera fish poisoning, this doctrine developed only to protect the consumer from careless manufacturers."

In summary, ciguatera cases represent fortuitous events which do not generate responsibility. As long as the product (fish) is well handled and preserved, fishermen, wholesalers, distributors, restaurant owners and all persons involved in the fishing industry can rest assured that they will not be held legally responsible if their product happens to have eaten the wrong kind of dinoflagellate.