Gambierdiscus toxicus from the Caribbean: A Source of Toxins Involved in Ciguatera

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ABSTRACT—Gambierdiscus toxicus, *an epibenthic dinoflagellate, was collected with detritus from the surfaces of several genera of macroalgae (*Acanthophora, Caulerpa, Dictyota, Halimeda, *and* Laurencia*) in the Caribbean Sea on the south side of St. Thomas, U.S. Virgin Islands. The detritus was fractionated by sieving and then processed in the laboratory to give samples which were 99 percent* G. toxicus. *Extraction yielded lipid (PPT-A) and water-soluble (PPT-B) toxic components that were heat-stable and precipitated in cold acetone. Intraperitoneal injection of PPT-A or PPT-B caused signs in mice (hypothermia, reduced locomotor activity, reduced reflexes, cyanosis, breathing difficulties, convulsions, and death) very similar to those produced by chromatographically purified extracts of fish remnants implicated in human ciguatera intoxications, particularly the marked lowering of body temperature. The ciguatoxic fish extract (CTX), however, was water insoluble and did not precipitate in cold acetone. In several chromatographic systems, PPT-A and PPT-B showed strong similarities but both differed markedly in comparison to fish CTX. The toxins of* G. toxicus *thus may undergo structural transformation when passed through the food web to ultimately become the CTX in fish that causes ciguatera poisoning in humans.*

Introduction

Dinoflagellates are responsible for the biosynthesis of many toxic compounds, some of which cause fish kills during red tides and, after transmission through the food chain, toxic shellfish poisoning and ciguatera in humans (Steidinger and Baden, 1984). The postulation by Randall (1958) of a benthic microorganism as the source of toxin that caused ciguatera was followed by studies on detrital feeders and herbivorous fishes (Yasumoto et al., 1971, 1976; Yasumoto and Kanno, 1976).

An examination of gut contents of surgeonfish, *Acanthurus* sp., from the Pacific led to work on benthic detritus and the discovery of a previously undescribed benthic dinoflagellate (Bagnis et al., 1977; Yasumoto et al., 1977b), later identified as a new genus and species, *Gambierdiscus toxicus* (Adachi and Fukuyo, 1979; Taylor, 1979). Indirect evidence such as the similarities in the fish species involved and the symptomatology of fish poisoning victims suggests ciguatera is the same problem circumtropically (Banner, 1976; Withers, 1982), but this has yet to be shown unequivocally. A parallel situation appears to exist for the toxicology of the putatively causative organism, *G. toxicus* (Ragelis, 1984).

We present here our results on the toxicity in natural populations of *G. toxicus* collected from the Caribbean Sea. These findings are compared with our data on ciguatoxic extracts from Caribbean fish and with reports on the toxicology of *G. toxicus* and reef fish from the Pacific Ocean.

Materials and Methods

Field Collection and Laboratory Processing of *Gambierdiscus toxicus*

Gambierdiscus toxicus was collected from the surfaces of macroalgae (*Acanthophora*, *Caulerpa*, *Dictyota*, *Halimeda*, and *Laurencia* spp.) at depths of 0.5-1.0 m near Range Key in Brewer's Bay on the south side of St. Thomas, U.S. Virgin Islands. The site is a sandy-bottomed, well-protected baylet adjacent to the College of the Virgin Islands where sea action is gentle.

We took periodic survey samples of macroalgae, carefully engulfing plants with plastic jars or bags to avoid dislodging surface material, to monitor the *G. toxicus* population levels via stereoscopic (40X) examination. For large-scale collection we employed a hand-held plastic bilge pump to draw up detritus from the algal surfaces while avoiding bottom sediment. The seawater-detritus mixture was separated by filtering through 106 and 45 μm stainless steel sieves. The residue from the latter was taken to the lab, mixed with seawater at 30°C (since field temperatures ranged from 28 to 33.5°C) and allowed to settle in white plastic trays (34 × 45 × 12 cm) under 15,000 lux of light.

In 8-12 hours, *G. toxicus* sorted themselves from the other settled detritus and formed visible, mucilagenous assemblages along the tray sides and floating in the seawater. Assemblage formation seemed to be enhanced by 1-2 hours of darkness before being easily siphoned with a Pasteur pipet and plastic tubing. The harvests were concentrated on a 45 μm sieve and the cells washed off with and stored in absolute methanol.

After accumulating two or three harvests each from several field collections the cells were counted before extraction.

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Six aliquots were taken after thoroughly mixing the methanol cell pool, diluted with water until a 30 μl sample could be scanned microscopically (100×) without difficulty under a 25 mm cover slip and slide, and the cells totaled with a hand counter. Each diluted aliquot was sampled and counted twice, the highest and lowest aliquot counts discarded, an average determined, and the total cell number estimated by back calculating to the total methanol volume in the cell pool. In our experience this technique of lab processing yielded dinoflagellate samples that were more than 99 percent *G. toxicus*, as verified during microscopic quantification, and very few cells remained behind in the detritus.

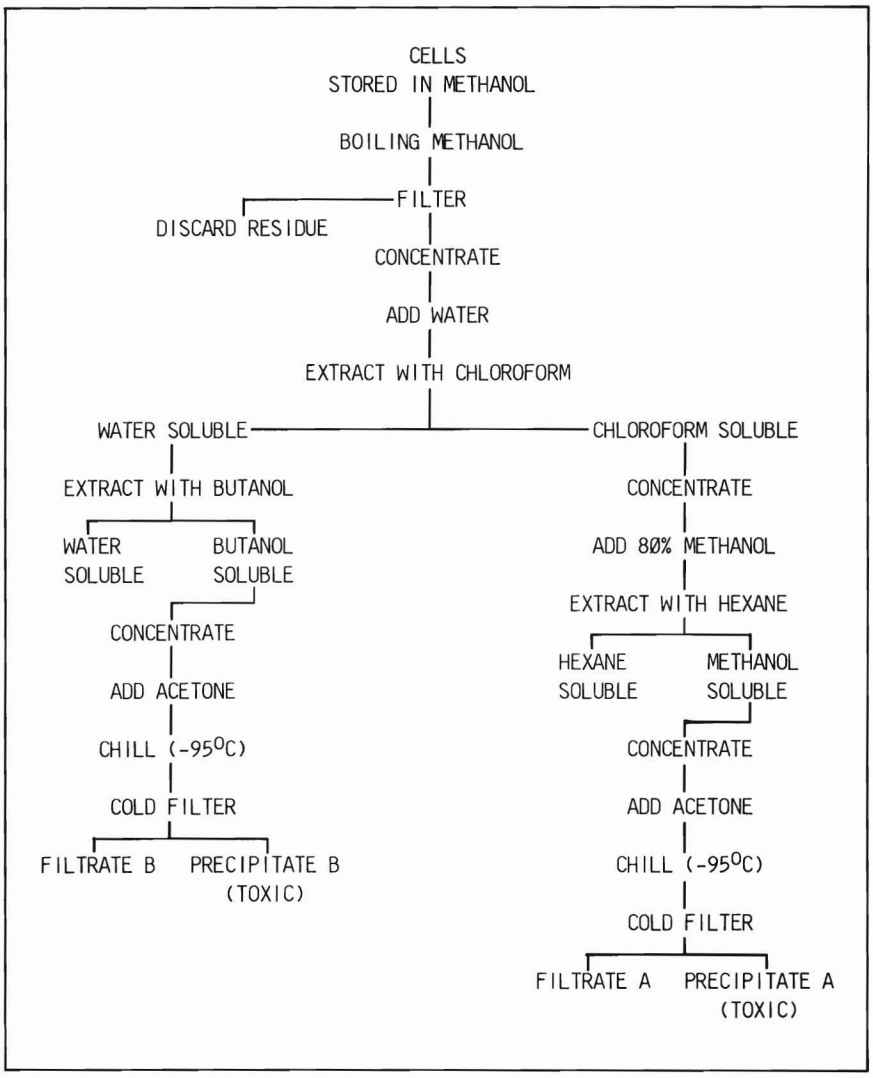

Figure 1.—Extraction and preliminary purification procedure for lipid-soluble (PPT-A) and water-soluble (PPT-B) toxins of *Gambierdiscus toxicus*.

Toxin Extraction

Our procedure for the extraction and preliminary purification of toxins from *G. toxicus* (Fig. 1) is similar to that of Bagnis et al. (1980). After twice boiling under reflux in methanol, filtration of cell debris, and concentration by rotary evaporation, the extract was fractioned into chloroform and water soluble phases. The chloroform-soluble fraction was concentrated, 80 percent methanol added, extracted with hexane, the methanol concentrated, and acetone added. The acetone was twice chilled overnight at −20°C and cold filtered (Whatman No. 50[1]) to yield Filtrate A (FLT-A) and Precipitate A (PPT-A). FLT-A was then repeatedly ultrachilled overnight at −95°C and cold filtered until either precipitate ceased forming or was of marginal toxicity. The water soluble fraction was extracted with butanol, the butanol concentrated, and acetone added. The acetone was cold treated as above to yield Filtrate B (FLT-B) and Precipitate B (PPT-B). After concentration and weighing, PPT-A and FLT-A were stored in chloroform, PPT-B in water, and FLT-B in acetone at −20°C. Extracts from the flesh of Caribbean fish that had caused human ciguatera intoxications were prepared using a procedure different from Figure 1 and published elsewhere (McMillan et al., 1980; Hoffman et al., 1983).

Toxin Chromatography

The chloroform-soluble (PPT-A) and water-soluble (PPT-B) toxins from *G. toxicus* extraction and fish flesh ciguatoxin extract (CTX) were chromatographed in several systems. A silicic acid column (Mallinkrodt 100 mesh, activated at 100°C, 1.2 × 15 cm) was poured in chloroform and later a PPT-A or CTX sample in chloroform was added. PPT-B samples were dissolved in 0.5 ml of water with 0.5 g of silicic acid and freeze-dried. The silicic acid with the adsorbed PPT-B was then placed on the column. The column was washed with 90 ml chloroform and eluted with 90 ml each of chloroform: methanol at 95:5, 9:1, and 1:1 ratios, and methanol. The eluates were concentrated, dried under anhydrous N_2, weighed, and stored as above. For preparative thin-layer chromatography, glass plates precoated with 1.0 mm silica gel (Brinkman SIL G-100 UV 254 or Whatman PLK5F preadsorbent plates) were activated for 1-hour at 80°C and samples spotted in chloroform or water

[1]Reference to trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

(PPT-B). Four solvent systems were employed for development (see Table 2). Several R_f bands were scraped and eluted with chloroform:methanol at 3:1. *G. toxicus* sample plates were re-eluted with methanol. Bands from plates developed by partition TLC (System 4) were eluted with chloroform:methanol: water at 60:35:8.

Toxin Bioassay

A mouse bioassay, developed to screen fish flesh extracts for ciguatoxicity (Hoffman et al., 1983), was used to test cell and fish extracts and chromatographic fractions for toxicity. Test materials were dissolved in water or emulsified with 3 percent Tween 60 in water, injected i.p. into 16 to 24 g female mice (I.C.R., Medical University of South Carolina) and the minimum lethal dose determined in two to five mice.

Results

The yields and toxicity to mice of extracts from three samples of *G. toxicus* are given in Table 1. The variability of yield and toxicity per cell among the samples is probably due to unknown but natural causes since the same location, collection, and extraction procedures were used in all instances. We must emphasize, however, the importance of adequately ultrachilling and cold filtering the acetone filtrates to obtain the acetone precipitates (PPT-A and PPT-B, see Fig. 1). Sample 3 FLT-A, for example, was chilled (−95°C) overnight 11 times before it was only nominally toxic and PPT-A (toxic) ceased forming. Failure to chill cold enough and repeatedly may give the erroneous impression of appreciable toxicity in the filtrates. We interpret our results with this extraction procedure as yielding two toxic components, PPT-A and PPT-B. No toxicity was found in the hexane soluble or water soluble (after butanol extraction) fractions. A comparison of the biochemical properties of toxins from *G. toxicus* and reference ciguatoxic fish flesh is presented in Table 2.

PPT-A and PPT-B evoked signs in mice that were virtually indistinguishable from those caused by fish CTX (Hoffman et al., 1983), except there was infrequent salivation, only occasional lacrimation, and no diarrhea. Lower rectal temperature (normal 35°-38°C, toxin-treated 30°-31°C), reduced locomotor activity, reduced reflexes (pain, pinnal, corneal, withdrawal), cyanosis, breathing difficulties, convulsions, and death were manifest consistently. Convulsions, however, were observed much less frequently with PPT-B. Also, in contrast to fish CTX, cell toxins caused, within 1 hour after injection, a loss of body tone and intense vasodilation, which later progressed to cyanosis.

Table 1.—Yield and bioassay data of extracts from three samples of *Gambierdiscus toxicus* from the Caribbean Sea.

Extract and sample number[1]		Yield		Lethality in mice[2]	
		Total (mg)	ng per cell	mg per 20 g	Cells × 10^4 per 20 g
PPT-A	1	73.6	1.6	0.1	6.3
	2	80.4	1.6	0.1	6.3
	3	163.6	2.8	0.04	1.4
FLT-A	1	204.5	4.5	30.0 NL	666.6
	2	259.2	5.0	25.0 NL	500.0
	3	244.7	4.2	15.0	357.1
PPT-B	1	67.2	1.5	1.0	66.6
	2	36.7	0.7	1.0	142.8
	3	250.3	4.3	4.0	93.0
FLT-B	1	57.5	1.3	30.0 NL	2,307.7
	2	54.3	1.0	30.0	3,000.0
	3	93.7	1.6	30.0 NL	1,875.0

[1]See Figure 1 and Materials and Methods section for extract designations. Sample 1 = 44.9 × 10^6 cells, Sample 2 = 51.0 × 10^6 cells, and Sample 3 = 58.0 × 10^6 cells.
[2]NL = not lethal.

Table 2.—Comparison of solubilities and chromatographic properties of toxins from *Gambierdiscus toxicus* and ciguatoxic fish flesh from the Caribbean Sea.

Biochemical property	Reference fish CTX	PPT-A (fat-soluble)	PPT-B (water-soluble)
Solubility			
Water	−	−/+	+
Methanol	+	+	+
Butanol	+	+	+
Acetone	+	−	−
Chloroform	+	+	−
Hexane	−	−	−
Column chromatography			
Silicic acid, column eluent (chloroform:methanol)	95:5	1:1	1:1
TLC system[1]:			
Toxic bands			
1	0.6-0.8	0.0-0.1[2]	0.0-0.1[2]
2	0.1-0.4	0.0-0.1[2]	
3	0.3-0.5	0.0-0.15[2]	
4	0.65-0.85	0.0-0.4	0.1-0.3

[1]Thin layer chromatography: Glass plates precoated with 1.0 mm silica gel (Brinkman SIL G-100 UV_{254} or Whatman PLK5F), activated 1 hour at 80°C, eluted after development with chloroform:methanol (3:1). Solvent systems: 1) chloroform:methanol (8:2), 2) benzene:butanol (75:25), 3) chloroform:methanol:6N ammonium hydroxide (90:9.5:0.5), and 4) chloroform:methanol:water (60:35:8).
[2]Bands re-eluted with methanol.

Discussion

Our toxicological results with field-collected *G. toxicus* are the first from a natural population in the Caribbean. Similar findings have been obtained from laboratory cultures of Caribbean *G. toxicus* (Tindall et al., 1984) with toxicity limited to the equivalents of our PPT-A and PPT-B. Methanolic extracts of cultures of *G. toxicus* from Puerto Rico were nontoxic (Tosteson et al., 1986), and unialgal cultures of the dinoflagellate from the Pacific Ocean often showed diminished toxicity (Bagnis et al., 1980), particularly under axenic conditions (Yasumoto et al., 1979a). To facilitate a comparison of our results (Tables 1 and 2) with published data obtained from wild Pacific material, a compendium of those reports is presented in Table 3. There seems to be general agreement with respect to the presence and chromatographic properties of a water-soluble toxin ("MTX", our PPT-B) and the absence of or only slight toxicity (Yasumoto et al., 1977b) in FLT-B and their equivalent acetone-soluble fraction. The symptomatology in mice is very similar, including the lack of convulsions before death (Bagnis et al., 1980), although they do not report measurements of body temperature. This apparent unanimity fails, however, concerning the lipid-soluble toxic component. Bagnis et al. (1980) obtained significant toxicity in two fat-soluble fractions: An acetone soluble "CTX" (our FLT-A) and an acetone precipitable "MTX" (our PPT-A). The latter was combined with the water-soluble toxin ("MTX", our PPT-B). Yasumoto et al. (1977b, 1979a) report a diethyl ether soluble fraction (our FLT-A and PPT-A combined), which was interpreted as corresponding to ciguatoxin ("CTX"). Secondary fat-soluble toxins were also found that were chromatographically similar to PPT-A (Yasumoto et al., 1976). We find lipid-soluble toxicity vir-

Table 3.—Yield and bioassay data in reports on extracts from ciguatoxic fish and wild *Gambierdiscus toxicus* from the Pacific Ocean.

Report	Sample	Extract designation and equivalent[1]	Average lethality in mice (cells × 10^4/20 g, range)	Chromatography[2] System	Chromatography[2] Toxic band or eluent
Bagnis et al., 1980	WGT: A-E	"CTX" (FLT-A)	4.8, 1.8-8.8	TLC-2 TLC-3 C.C.	0.2-0.6 0.2-0.4 9:1
		"MTX" (PPT-A and PPT-B combined)	0.7, 0.4-1.2	TLC-4 C.C.	0.1-0.3 4:6
	Reference fish CTX	"CTX" (CTX)		TLC-2 TLC-3 C.C.	0.0-0.3 0.25-0.4 9:1
Yasumoto et al., 1979a	Wild dinoflagellate (*Diplopsalis*, sp.) '75, '78	"CTX" (FLT-A and PPT-A combined)	31.4, 25.3-37.5	TLC-3 C.C.	0.5 9:1
Yasumoto et al., 1977b	*Diplosalis* sp. Fractions 2-4	"CTX" (FLT-A and PPT-A combined)	17.9, 8.1-25.4	TLC-2 TLC-3 C.C.	0.5-0.7 0.35-0.5 9:1
		"MTX" (PPT-B)	4.6, 0.8-8.4	C.C.	6:4 and 4:6
Yasumoto et al., 1980	Reference fish CTX	"CTX" (CTX)		TLC-2 TLC-3 C.C.	0.1-0.3 0.3-0.5 9:1
Yasumoto et al., 1976	Surgeonfish guts and contents	"MTX" (PPT-B) "Secondary toxins" fat soluble (PPT-A?)		TLC-4 C.C. C.C.	0.17-0.3 6:4 1:1

[1]Designations in reports: CTX = ciguatoxin, MTX = maitotoxin. See Figure 1 and Materials and Methods section for equivalents.
[2]TLC systems 1-4; silicic acid column chromatography (C.C.), eluent (chloroform:methanol); see Table 2.

tually limited to PPT-A, which behaves chromatographically as PPT-B, and no evidence of a toxin with chromatographic properties like reference Caribbean CTX. In fact, the chromatographic data cited to support the identity between Pacific *G. toxicus* "CTX" and reference Pacific fish "CTX" is less than conclusive (Table 3, compare particularly TLC-2 in Bagnis et al. (1980) and in Yasumoto et al. (1977b, 1980)). Interestingly, our reference Caribbean fish CTX seems remarkably similar to Pacific fish CTX in essentially the same chromatographic systems (compare Tables 2 and 3) and in HPLC (Higerd et al., 1986).

Pacific scientists offer much evidence, including food chain and gut-contents studies and surveys on the abundance of the dinoflagellate in ciguatera-endemic areas, to support the contention that *G. toxicus* is a biogenitor of toxins involved in ciguatera (previous references in Introduction, Table 3, and Bagnis et al., 1985; Yasumoto et al., 1977a, 1979b). We have also observed *G. toxicus* in the gut contents of herbivorous fish (unpubl.). And, even though the toxins of Caribbean *G. toxicus* appear to differ chemically from CTX, it would seem highly unlikely that PPT-A, PPT-B, and CTX could evoke essentially the same bioassay signs in mice, particularly the pronounced effect on body temperature (Sawyer, et al., 1984), and not be related. Considering all of the above from a circumtropical perspective, a substantially more uniform view of the ciguatera problem emerges. Unravelling the metabolic relationships among the toxins from *G. toxicus* and other dinoflagellates possibly involved (Yasumoto et al., 1980; Nakajima et al., 1981; Murakami et al., 1982; Bagnis et al., 1985; Tindall et al., 1984) as they are accumulated, transformed, and transferred through the foodweb represents a most promising but challenging aspect of understanding ciguatera.

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