Toxin Production by *Gambierdiscus toxicus* Isolated from the Florida Keys

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Introduction

Ciguatera is a tropical fish-borne disease in which the lipid-soluble neurotoxin, ciguatoxin, is believed to be transferred through the food chain and bioconcentrated primarily in carnivorous reef fish to levels toxic to humans. Since ecological research in the South Pacific implicated *Gambierdiscus toxicus* as the probable cause of ciguatera (Yasumoto et al., 1977), this dinoflagellate had been collected from the wild, grown unialgally, and extracted for toxins that have been analyzed principally by mouse bioassay.

Evidence that *G. toxicus* produces the fish-extracted ciguatoxin is based on reports in which either wild cells (Yasumoto et al., 1977, 1979; Bagnis et al., 1980; Shimizu et al., 1982) or cultured cells (Yasumoto et al., 1979; Tindall et al., 1984) were extracted and partitioned between solvents of different polarities. The biological and chemical nature, however, of the limited quantity of lipid-soluble dinoflagellate toxin obtained by these nonstringent extraction, partitioning, and purification techniques can only be conjecture. Cultured *G. toxicus* readily produce a toxin distinguished from ciguatoxin on the basis of higher molecular weight and lower polarity. This toxin has been tentatively identified as maitotoxin (Yasumoto et al., 1979), a toxin first isolated from the gut of surgeonfish, *Acanthurus* sp. (Yasumoto et al., 1976).

Unialgal cultures, initiated with up to 30 cells, are used in most studies on toxin production by cultured *G. toxicus*. The objectives of this study were to isolate and culture clonal *G. toxicus* collected concurrently from a single site in the Florida Keys, and compare the quantity and nature of the toxins produced. Toxicity, as measured in mice (LD_{50}), was determined using whole cells or nonfractionated extracted toxins, and the relative polarity of the extracted toxins was analyzed by high performance liquid chromatography (HPLC) (Higerd et al., 1986). The toxic characteristics of the Florida isolates are compared with a highly toxic clonal *G. toxicus* isolated in Hawaii (Sawyer et al., 1984).

ABSTRACT—The toxicities of six clonal Gambierdiscus toxicus *cultures collected concurrently from Knight Key, Fla., were compared with the toxicity of the Hawaiian* G. toxicus *strain, T39. LD_{50} values obtained using mouse bioassay demonstrated a hundredfold range in whole-cell toxicity. The Hawaiian and two Floridian strains had comparable mouse toxicity (LD_{50}) of about 2.5×10^4 cells/kg. Two additional groups of Floridian strains had toxicities of about 2×10^5 and >1 million cells/kg, respectively. Fractionation of methanol extracts by high-performance liquid chromatography suggests that toxins produced by different clones of* G. toxicus *are indistinguishable from each other but are more polar than fish toxin. Isolates of* Ostreopsis heptagona, *also isolated from Knight Key, had relatively low toxicities ($LD_{50} > 5 \times 10^6$ cells/kg).*

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Materials and Methods

Benthic dinoflagellate samples were collected from the Florida Keys at a site previously involved in ecological studies on benthic dinoflagellates associated with ciguatera (Bomber, 1985). Samples were collected in December 1983, and in February, May, July, and December 1984, from Knight Key, which consistently had a high *G. toxicus* population. This site, station 3 of Bomber (1985), is located in Florida Bay, 0.8 km east of seven-mile bridge (lat. 42°44′20″N, long. 81°07′16″W). The area consists of an algal reef depauperate in coral with strong tidal currents and up to 95 percent cover by *Halimeda* spp. (Bomber, 1985). Pieces of macroalgae were collected in plastic jars with seawater, shaken, and the seawater filtered through a 250 μm Nitex[1] sieve. The filtrate was then refiltered through a 25 μm Nitex sieve and the retentate analyzed microscopically for *G. toxicus*. Samples with high populations of *G. toxicus* were suspended in 1 liter polycarbonate bottles containing 700 ml filtered (20 μm) seawater for shipment to the laboratory.

The medium used for isolation and growth of *G. toxicus* was Provasoli's ES enriched seawater medium as modified by J. West (McLachlan, 1973). Seawater was collected either at the Florida Keys

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

sampling site, or at two sites from 7 to 10 miles off Charleston, S.C., and filtered onsite through a 20 μm Nitex sieve into 10 to 20 liter polycarbonate carboys. Upon arrival at the laboratory, the water was refiltered through 0.45 μm cellulose nitrate membranes in a Nuclepore radial flow cell into sterile polycarbonate carboys and refrigerated until used to prepare media. The vitamin mixture (stored frozen) and enrichments were prepared in concentrated stocks, filter-sterilized and added aseptically to the seawater. The prepared medium was filter-sterilized through 0.2 μm cellulose nitrate membranes and used immediately or refrigerated.

Clonal cultures of *G. toxicus* and *Ostreopsis* sp. were established from single cells isolated with a micropipet, washed three or four times in sterile seawater, and inoculated into 16× 125 mm culture tubes containing 5 ml ES medium. *Ostreopsis* spp. occur with *G. toxicus* in the Florida sampling site (Bomber, 1985) and have been shown to be toxic by Nakajima et al. (1981). Growth was monitored with an inverted microscope, and if apparent within 30 days, the cultures were transferred to 25 ml mini-Fernbach flasks or 20 × 150 mm culture tubes containing 15 ml ES medium. After successful growth for 15-30 days, the cultures were placed in 250 ml polycarbonate Erlenmeyer flasks containing 100 ml ES medium. Stock cultures were maintained in 250 ml flasks and transferred to new medium every 30 days. These cultures were maintained at 27°C under an illumination of 30-50 $\mu E \cdot M^{-2} \cdot S^{-1}$ and a 16:8 hours light:dark cycle without aeration.

For toxin bioassay, 100 ml cultures were inoculated into 2.8 liter Fernbach flasks containing 1.5 liters ES medium. These cultures were grown for 30-45 days under incubation conditions described above. *Ostreopsis* clones were cultured for 10-21 days under the same conditions. Cell counts were determined in Palmer-Maloney chambers. To compare cell diameters with other reported *G. toxicus* isolates, cells were measured during their late log growth phase at 400× magnification with bright-field microscopy. Cells were harvested by filtering the culture medium through a 20 μm Nitex filter and resuspending the retentate in 30 percent aqueous methanol (v/v). This suspension was evaporated to dryness under a stream of nitrogen.

Toxicities of whole cells (LD_{50}) were determined by suspending the dried sample in phosphate buffered saline containing 5 percent Tween 80 and injecting 0.2 ml of appropriate cell concentrations intraperitoneally into female ICR mice weighing approximately 20 g (Kelly et al., 1986). In addition to determining whole cell toxicities, the seven *G. toxicus* clones were grown and harvested to obtain total cell densities greater than 1×10^6 cells. These samples were extracted in 80 percent aqueous methanol (v/v) for 48 hours at room temperature and bioassayed or fractionated by HPLC using a 50-100 percent methanol linear gradient. Each fraction was later assayed for toxicity by the mouse bioassay (Higerd et al., 1986).

Results and Discussion

Six *G. toxicus* clones were isolated concurrently from a dinoflagellate sample collected at Knight Key, 20 December 1983 (Bomber, 1985, station 3). The Hawaiian strain, T39, was hand-carried from Hawaii to the SEFC Charleston Laboratory. Five *Ostreopsis* clones represent a new species, *O. heptagona*, with cellular lengths >100 μm (Norris et al., 1985). *G. toxicus* clones were identified microscopically by their cellular shape and the characteristic "fishhook" apical pore slot (Adachi and Fukuyo, 1979; Taylor, 1979). Further taxonomic studies are in progress using a chloral hydrate-hydriodic acid staining method (Schmidt et al., 1978) and scanning electron microscopy.

Maximal cellular yields obtained for *G. toxicus* cultures grown for toxin bioassays were 1,000 cells/ml. Larger yields of 2,000-4,000 cells/ml have been reported for cultures of smaller *G. toxicus* (35-55 μm) (Bagnis et al., 1980; Carlson et al., 1984). On occasion, stored ES medium prepared with specific lots of natural seawater collected both from the Florida Keys and South Carolina coastal waters produced a precipitate that proved detrimental to growth

Table 1.—Whole-cell toxicity of cultured *G. toxicus* clones.

Clone	Source	Cell dia. (μm)[1]	LD_{50} (cells/kg)
T39	Tern Island, HI	76	2.5×10^4 (4)[2]
Cd20	Knight Key, FL	79	3.5×10^4 (4)
Cd4	Knight Key, FL	81	4.5×10^4 (3)
Cd8	Knight Key, FL	94	2.5×10^5 (2)
Cd10	Knight Key, FL	81	3.0×10^5 (2)
Cd9	Knight Key, FL	86	$>2.3 \times 10^6$ (2)
Cd13	Knight Key, FL	77	$>2.5 \times 10^6$ (2)

[1]Cellular measurements were obtained by measuring live individuals at 400× with bright-field microscopy (n = 30).
[2]Bioassay analyses (n).

and toxin production. This precipitate was prevented by eliminating the Tris buffer from ES medium.

Five *O. heptagona* clones isolated from Knight Key, prepared and bioassayed using the same procedure described for *G. toxicus*, had relatively low toxicity ($LD_{50} > 5 \times 10^6$ cells/kg. Mouse mortalities were observed at injections of 5×10^6 cells/kg, but higher dosages were not assayed so a definitive LD_{50} could not be calculated. Although *O. siamensis* and *O. ovata* were shown to be toxic by Nakajima et al. (1981), their toxicities were also $> 5 \times 10^6$ cells/kg. Besada et al. (1982) reported no toxicity in *Ostreopsis* cultures isolated from the Caribbean Sea.

The LD_{50} values using whole cells of *G. toxicus* are shown in Table 1. The Hawaiian clone, T39, was similar in toxicity to Cd20 and Cd4 from the Florida Keys and to T39 cultures grown in Hawaii. Florida Key clones Cd8 and Cd10 were 10 times less toxic, while the toxicities of Florida Key clones Cd9 and Cd13 were just evident with more than 2×10^6 cells/kg. As with *O. heptagona*, mortalities were observed, but higher dosages were not assayed so a definitive LD_{50} could not be calculated. LD_{50} values for nonfractionated methanol extracts of two Cd20 cultures were 1×10^5 cells/kg, or about 70 percent less toxic, than whole cells.

This is the first report of comparative toxicities using clonal cultures of *G. toxicus* isolated concurrently from a single site. Two unialgal cultures isolated from the Florida Keys have been reported in a previous study (Bergmann and Alam,

Table 2.—Relative HPLC elution times for toxic fractions of extracted *G. toxicus*.

Source of extracted material	^1R_t
G. toxicus (Hawaii) T-39	2.2
G. toxicus (Hawaii) T-39	2.1
G. toxicus (Florida) Cd-4	2.8
G. toxicus (Florida) Cd-4	2.6
G. toxicus (Florida) Cd-10	2.6
G. toxicus (Florida) Cd-20	2.7
Fish (St. Thomas, U.S.V.I.)	4.0

[1]Ratio of toxic activity elution time relative to elution time for phenol standard, where phenol = 1.

1981) and these cultures had toxicities in mice of 3.1×10^5 cells/kg and 1.2×10^5 cells/kg.

To compare polarities of dinoflagellate toxins, the ratio between the toxic fraction elution times from a HPLC column for extracted dinoflagellates and a phenol standard was calculated. Results were expressed as a relative retention time (R_t), with phenol equivalent to 1.00 (Table 2). Only a single toxic component was detected in each of the four *G. toxicus* cultures and they exhibited similar R_t values. In contrast, the dinoflagellate toxins were far more polar than the fish toxin, which eluted much later in the linear methanol gradient. Since toxic fractions were detected by mouse bioassay, this technique may have missed toxic fractions since LD_{50} values $\geqslant 1.0 \times 10^6$ cell/kg were not assayed. Cells extracted from the remaining three *G. toxicus* samples did not provide enough toxic activity to be detected with this procedure. The chromatographic details of this HPLC technique can be found in an accompanying conference paper (Higerd et al., 1986).

This study demonstrated a hundredfold variation in toxicity of clones isolated concurrently, which would suggest that toxin production may vary in natural populations of *G. toxicus*. Unialgal cultures, initially containing several competing clones, might produce variable toxin profiles until a single clone became dominant and the other clones were lost through transfer dilution. Cultural parameters can also influence the relative toxicity of *G. toxicus* (Bergmann and Alam, 1981) since maitotoxin, the primary and most potent *G. toxicus* toxin, is produced late in the growth phase (Yasumoto, et al., 1979). Insufficient data in other studies and the lack of a standardized mouse bioassay made it difficult to compare quantitatively the levels of *G. toxicus* toxicity reported in this study with previous investigations. However, approximate toxicities reported for cultured *G. toxicus* range from 6×10^3 cells/kg (Yasumoto et al., 1979) to 1×10^6 cells/kg (Tindall et al., 1984). Toxicity quantitation presently is limited by the nonspecific nature and lack of precision and accuracy of the mouse bioassay.

Toxins extracted with aqueous methanol were 70 percent less toxic than whole cells as measured by mouse bioassay, indicating that the toxic moieties of extracted nonfractionated toxins and whole cells may differ or that extraction efficiency requires improvement. Tindall et al. (1984) reported ciguatoxin, one ciguatoxin derivative, and maitotoxin present in fractionated extracts of cultured unialgal *G. toxicus*. Their limited data, however, could not exclude the possibility of multiple toxins derived from carryover of maitotoxin during the extraction and separation procedures as observed by Yasumoto et al. (1979) or interchangeable toxin forms like that reported for purified ciguatoxin (Nukina et al., 1984). The HPLC system used in the current study permitted distinct separation of several toxins, but because of limited amounts of extracted material, toxins present in minor quantities may have gone undetected.

There is a need to identify and quantitate toxins present in cultured benthic dinoflagellates associated with ciguatera. An extremely valuable analytical method for determing total toxin profiles has been developed for paralytic shellfish poisoning (PSP) toxins (Sullivan and Wekell, 1984; Sullivan et al., 1985). This HPLC analysis uses derivatives of PSP toxins to fluorometrically detect toxic fractions separated in an HPLC column at concentrations four times more sensitive than mouse bioassays. A technique similar to the HPLC procedure for PSP toxins has been developed for detecting toxin extracted from *G. toxicus* (Sick et al., 1986). Such a technique may be capable of quantitating and comparing the chemical nature of toxins from clonal *G. toxicus* cultures and determining the effects that cultural parameters have on toxin profiles, similar to a study reported for PSP toxins (Boyer et al., 1985).

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