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AN IMMUNOLOGICAL APPROACH FOR CTX

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INTRODUCTION

The application of immunological techniques for the detection of low molecular weight, non-immunogenic compounds has increased markedly in the past decade. The ability to conjugate these haptenic small molecules covalently to appropriate immunogenic carriers via their functional groups has led to the production of specific antibodies to the haptenic molecules following administration into appropriate animals. Primary functional groups or moieties amenable to conjugation, provided they are accessible to chemical coupling, include amino, hydroxyl, and carboxyl groups. Some common methods for coupling include carbodiimide, nucleophilic substitution, diazo and azide coupling. In some cases, for example with lipid antigens, noncovalent complexes have been utilized using methylated bovine serum albumin as the carrier (Butler et al., 1973; Becher, 1976).

The production of a specific antibody to the low molecular weight compounds can then be utilized for the development of sensitive and specific immunological procedures such as radioimmunoassay (RIA) and enzyme-linked immunoabsorbent assay (ELISA) for detection of minute amounts of antigens or antibodies. In addition, the antibodies are useful in examining other areas of study such as tissue distribution, synthesis and in some cases the structure and function of the haptenic molecule.

The application of the sensitive immunological methods for the detection of marine toxins, such as saxitoxin, ciguatoxin, and tetradotoxin, merits strong consideration in light of the minute amounts of these toxins in the natural environment. The minute amount in tissues has constituted serious hazards to the consumer and has created anxiety within the fishing and shellfish industries.

This study discusses the immunological approaches for the development of a radioimmunoassay procedure for the detection of ciguatoxin (Hokama et al., 1977). The methodology presented is applicable for the development of immunological procedures for the other low molecular weight marine toxins such as saxitoxin, tetradotoxin, and *Gymnodinium breve* toxins. In addition, for correlative analysis, the guinea pig atrium procedure (Miyahara et al., 1979) for quantitation of ciguatoxin and maitotoxin from crude extracts of fishes is also included in this study. Comparison of the RIA procedure with the mouse M.U. test is also presented in this study.

METHODS FOR CIGUATOXIN ANALYSIS

The development of the RIA procedure for the direct detection of ciguatoxin (CTX) from

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fish tissues has been reported previously (Hokama et al., 1977).

Purified ciguatoxin (Scheuer et al., 1967) isolated from livers of toxic eels was conjugated by the carbodiimide procedure (Oliver et al., 1968) to human serum albumin (HSA). The latter is the carrier for the lipid hapten-ciguatoxin. Precipitation of the ciguatoxin-HSA (CTX-HSA) with cold acetone indicated that CTX was in the precipitate (CTX-HSA) and not in the acetone phase. This suggested that most if not all CTX was bound to HSA.

The antibody to CTX-HSA was raised in sheep given three weekly subcutaneous injections of a CTX-HSA-Freunds complete adjuvant mixture (1:1). A total of 500 μg of CTX and 50 mg of HSA (CTX-HSA) was administered to the sheep in a period of 7 weeks. The sheep was bled 10 days after the last injection and the serum removed and stored in 30 ml aliquots at -20°C.

The details of the determination for the presence of anti-CTX-HSA by immunoelectrophoresis and mouse studies and for the development of the RIA procedure have been described (Hokama et al., 1977). In brief, the RIA method consists of the following steps: (1) sample tissues from the dorsal and ventral muscles, tails, or gonads of the fish; (2) dehydrate samples in oven at 70°C for 1 hour; (3) after cooling, weigh 15-mg samples; (4) rehydrate with buffered saline; and then remove buffer; (5) add 0.2 ml of ^{125}I-labelled sheep anti-CTX-HSA (sp. act., 1.03 mCi/mg protein) containing 50,000 cpm/0.1 ml; (6) shake for 3 hours at room temperature; (7) add 3.0 ml buffered saline; (8) aspirate buffer thoroughly; and (9) count the sample in gamma-counter. After background correction, the cpm/tube is divided by 15 mg to give cpm/g tissue. Samples are determined in triplicates, or duplicates in some instances. Toxic and nontoxic fish tissue samples are examined with each set of unknown determinations.

The present study includes examination by the RIA procedure of fishes recovered from clinically diagnosed ciguatera poisoning cases and nontoxic fishes from commercial sources. The toxic fishes were obtained through the courtesy of the Hawaii State Department of Health. The nontoxic fish samples were obtained from various commercial and noncommercial sources. Reef fishes from Tahiti were obtained through the courtesy of Dr. Bagnis of the Institut de Recherches Medicales Louis Malarde.

A correlative study of the RIA procedure and the *in vitro* guinea pig atrium assay was carried out for several samples of toxic and nontoxic fish tissues. The details of the guinea pig atrium analysis were reported recently by Miyahara et al. (1979). The crude extracts of the fish tissues were prepared according to a previously described procedure (Yasumoto et al., 1977). A 20-g tissue is homogenized and extracted with 200 ml hot methanol. The residue is removed by filtration and the methanol phase concentrated to 10 percent of the original volume. The concentrated solution is diluted with distilled water (5 volumes) and the lipid solutes extracted with diethyl ether. The ether is evaporated and the crude lipid residue dissolved in minimal methanol and stored at -20°C until ready for study. Assay for the mouse toxicity procedure has been described previously (Yasumoto et al., 1971).

Based on the examination of fishes from human ciguatera poisoning and previous results obtained by RIA (Hokama et al., 1977) the following arbitrary criterion has been established relative to the cpm/gm tissue values.

Cpm/gm Tissue	Toxicity Index
< 350,000	Negative
350,001 to 399,999	Borderline
> 400,000	Toxic

The diagnosis of ciguatera poisoning of patients following consumption of suspected toxic fishes is based on the clinical symptoms described previously (Okihiro et al., 1965; Bagnis, 1968).

RESULTS

RIA Examination of Fish Samples. The results of the examination of individual cases of fish samples recovered from clinically diagnosed ciguatera

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Table 1. RIA Examination of Fish Samples From Clinically Documented Ciguatera Poisoning

No. of Samples	Fish Species	Common/Hawaiian Name	RIA Results CPM/GM Tissue	Remarks
3	*Scarus* sp.	Parrotfish/uhu	463,039	Toxic
	Scarus sp.	Parrotfish/uhu	524,106	Toxic
	Scarus sp.	Parrotfish/uhu	421,064	Toxic
1	*Elagatis bipinnulatus*	Rainbow runner/kamanu	463,636	Toxic
6	*Seriola dumerilii*	Amberjack/kahala	415,870	Toxic
	Seriola dumerilii	Amberjack/kahala	549,534	Toxic
	Seriola dumerilii	Amberjack/kahala	412,600	Toxic
	Seriola dumerilii	Amberjack/kahala	447,045	Toxic
	Seriola dumerilii	Amberjack/kahala	436,778	Toxic
	Seriola dumerilii	Amberjack/kahala	296,667	Toxicity not detected by RIA
13	*Caranx* or *Carangoides* sp.	Jack/ulua	414,489	Toxic
	Caranx or *Carangoides* sp.	Jack/ulua	420,667	Toxic
	Caranx or *Carangoides* sp.	Jack/ulua	492,634	Toxic
	Caranx or *Carangoides* sp.	Jack/ulua	505,779	Toxic
	Caranx or *Carangoides* sp.	Jack/ulua	497,406	Toxic
	Caranx or *Carangoides* sp.	Jack/ulua	400,867	Toxic
	Caranx or *Carangoides* sp.	Jack/ulua	711,346	Toxic
	Caranx or *Carangoides* sp.	Jack/ulua	448,936	Toxic
	Caranx or *Carangoides* sp.	Jack/ulua	351,400	Borderline
	Caranx or *Carangoides* sp.	Jack/ulua	387,553	Borderline
	Caranx or *Carangoides* sp.	Jack/ulua	362,920	Borderline
	Caranx or *Carangoides* sp.	Jack/ulua	370,597	Borderline
	Caranx or *Carangoides* sp.	Jack/ulua	300,300	Toxicity not detected by RIA
2	*Mugil cephalus*	Mullet/ama'ama	449,967	Toxic (viscera sample)
	Mugil cephalus	Mullet/ama'ama	462,753	Toxic
1	*Aprion virescens*	Grey-snapper/uku	482,133	Toxic
1	*Monotoxis grandoculis*	Porgy/mu	660,556	Toxic
1	*Priacanthus cruentatus*	Red big eye/aweoweo	382,584	Borderline
1	*Lutjanus gibbus*	Paddle-tail snapper	442,000	Toxic
3	*Epinephelus mario*	Sea bass	479,339	Toxic
	Epinephelus mario	Sea bass	490,392	Toxic
	Epinephelus mario	Sea bass	375,189	Borderline

poisoning are indicated in Table 1. A variety of species has been implicated, including the following species: *Scarus* sp., parrotfish/uhu; *Elagatis bipinnulatus*, rainbow runner; *Seriola dumerilii*, amberjack/kahala; *Caranx* and *Carangoides*, sp., jack/ulua; *Mugil cephalus*, mullet/ama'ama; *Aprion virescens*, grey snapper/uku; *Monotoxis grandorulis*, mu; *Priacanthus*, sp., red big eye/aweoweo; *Lutjanus gibbus*, paddle-tail snapper; and *Epinephelus mario*, sea bass. Two samples, a *Seriola dumerilii* and a *Caranx* sp., gave RIA values in the nontoxic range. The mean ± 1 S.D. (434,654 ± 110,184) value for the 32 toxic samples is summarized in Table 2. This value is significantly ($p < 0.001$) different from the mean ± 1 S.D. (264,202 ± 44,291) value of the 91

Table 2. Summation of RIA Values for Fishes From Clinically Defined "Ciguatera Poisoning" Cases and Nontoxic Fishes

Fish	CPM/GM Tissue (Mean ± 1 S.D.)		
	Nontoxic	Toxic	p*
Several Species†	264,202 ± 44,291 (n = 91)	434,654 ± 110,184 (n = 32)	< 0.001

*p value based on two-tailed analysis.
†See Table 1 for the individual species listed for the toxic group; the nontoxic fishes in this category were sampled from the same species implicated in the "ciguatera poisoning" or the toxic group.

non-toxic samples of essentially the same species shown in Table 1.

Comparison of the RIA with the Mouse Toxicity, M.U. Figure 1 summarizes a comparative

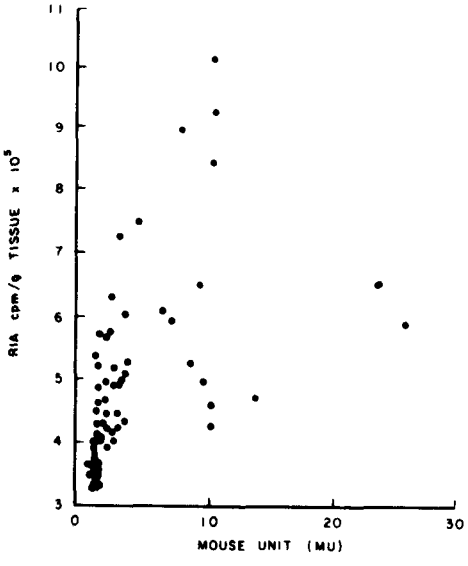

Figure 1. Comparison of RIA procedure for ciguatoxin detection and the mouse toxicity based on M.U.; each circle represents a sample. (RIA values are average of triplicate samples.)

study of the RIA with the mouse toxicity of reef fish tissues obtained from Tahiti. The incidence of ciguatera poisoning is high in French Polynesia in contrast to other areas of the Pacific. The coefficient of correlation, r, is moderate between RIA:M.U. The r values are summarized in Table 3. The RIA:Mongoose r value from a previous report (Hokama et al., 1977) is also shown in Table 3.

Comparison of RIA with Guinea Pig Atrium Assay. This correlative study between the RIA and inotropic effect of ciguatoxin on guinea pig atrium is presented in Figure 2 with the r value shown in Table 3 (r = 0.88). Although the sample number is small, a significantly good correlation is shown between the RIA and guinea pig atrium assay. The latter assay appears to be a valuable method for ciguatoxin studies in correlation with the RIA and other biological procedures.

Table 3. Summation of the Comparison of the RIA Procedure with the Other Biological Methods for the Evaluation of Ciguatoxin

No. of Samples	System	Coefficient of Correlation (r)	Reference
66	RIA:M.U. (mouse)	0.56	Hokama et al., 1980
66	RIA:Mongoose	0.56	Hokama et al., 1977
13	RIA:Guinea pig atrium	0.88	Hokama et al., 1980

M.U. = Total weight in gm of mice killed within 48 hours by a 1 ml extract from 20 gm of fish tissue (Yasumoto et al., 1971).

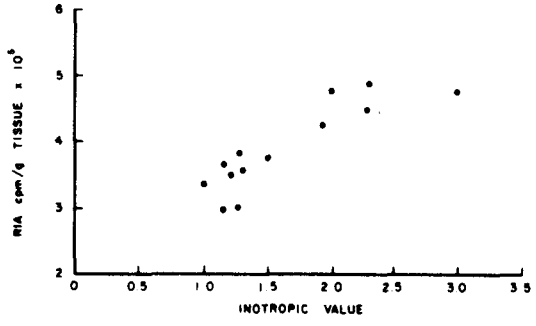

Figure 2. Comparison of the RIA procedure for ciguatoxin detection and the inotropic effect on guinea pig atrium. The inotropic values indicated are obtained by dividing the increase in amplitude (inotropic effect) by the base line contraction.

DISCUSSION

A general approach for the use of immunological procedures for application to small molecules is presented elsewhere (Hokama et al., 1980). The significant factors include: (1) the importance of coupling of the low molecular weight compounds to appropriate antigenic carriers, that is, presentation in positions of accessibility, without gross changes in the haptenic molecule; and (2) selection or choice of the antigenic carrier. Of equal importance is the choice of animal for the production of antibody and the mode of administration of the hapten-carrier conjugate. The analysis and development of the immunological methods for the antibody raised will depend on the levels of the antibody and the haptenic molecule to be examined (Landsteiner, 1945; Rose and Friedman, 1976).

Essentially following the general procedures presented elsewhere (Hokama et al., 1980), development of an immunological procedure for the detection of ciguatoxin (a lipid hapten) has been initiated earlier (Hokama et al., 1977). This study presents further evaluations of the RIA procedure by examining various species of fishes including those specimens implicated in ciguatera poisoning.

The comparative examination of fish samples from Tahiti with the RIA and mouse (M.U.) procedure gave an r value which appeared to be similar (Table 3) to that demonstrated earlier for RIA and mongoose. The lack of a better correlation may be due to the highly sensitive nature of the RIA test as discussed previously (Hokama et al., 1977). The capability and limitations of the biological procedures have been discussed earlier by other investigators (Banner et al., 1960; Banner et al.,1961; Kosaki et al., 1968).

The present state of the RIA procedure is significantly sufficient to distinguish toxic from nontoxic fishes. Furthermore, the test appears to be helpful for the examination of various species of fishes from different geographical areas for the evaluation of the presence or absence of toxic fishes. For example, coral reef fishes from Tahiti tended to have a higher incidence of toxic levels and cpm/gm tissue than similar species caught in Hawaii. (Compare data on fishes from Tahiti in Figure 1 with data on fishes in Hawaii in Table 2.) Thus, in its present state the RIA procedure can be utilized for the exploration of the levels of toxicity and distribution among various fish species in different geographic areas, especially where ciguatera poisoning is endemic. This would aid in the development of the fishing industry.

The limitations of the RIA procedure for wide-scale use at present include: (1) restrictions on the number of sampling per day by a technologist; and (2) a minimum of approximately 6 hours required for the completion of the test. Other difficulties include: (1) sampling error, that is, the uncertainty of toxin distribution or localization in various parts of the tissue; (2) a tendency for false positive reactions due to nonspecific binding; and (3) reproducibility due to the fact that tissues are utilized, and even within close proximity "hot spots" of ciguatoxin may vary.

Efforts are currently being directed towards decreasing the test time, devising an ELISA procedure using homogenous crude lipid extracts from large tissue areas, minimizing the cost of the test, and increasing specificity by attempting to isolate and concentrate the specific antibody involved. In addition, the development of immunofluorescent procedures would be helpful in assessing the presence of ciguatoxin in *Gambierdiscus toxicus* (Figure 3) (Yasumoto et al., 1977).

The need for a specific, sensitive, and simplified procedure for the detection of ciguatoxin is evident in a recent report (Morris, 1980) from the Center for Disease Control, Atlanta, Georgia. Ciguatera poisoning has been reported as the leading cause of poisoning from marine foods in the United States, surprisingly even more so than paralytic shellfish poisoning (Morris, 1980).

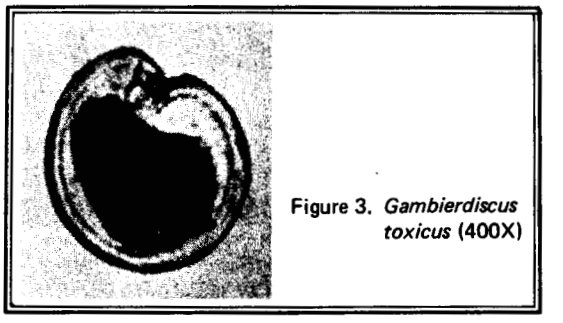

Figure 3. *Gambierdiscus toxicus* (400X)

SUMMARY

The steps involved in the utilization of immunological procedures to study small molecules including marine toxins are as follows: (1) conjugation of the small molecule to appropriate antigenic carriers; (2) immunization of animals for the production of antibodies; and (3) development of an immunological method for analysis of the small molecule. The development of the radioimmunoassay for ciguatoxin was carried out accordingly. The RIA procedure for detection of ciguatoxin showed moderate correlation with the mouse M.U. ($r = 0.56$) test. These results were similar to that reported for the RIA and mongoose ($r = 0.56$) test. A good correlation of the RIA with the *in vitro* guinea pig atrium assay was demonstrated ($r = 0.88$). Examination of nontoxic fishes and fishes recovered from patients with clinically defined ciguatera poisoning with the RIA procedure demonstrated that the test clearly differentiated toxic from nontoxic samples. It is suggested that the RIA test in its present state is useful for the assessment of toxic from nontoxic fish tissue in a variety of fish species from various endemic areas. Currently work is being carried out to develop a simpler enzyme-linked immunoabsorbent assay with increased specificity.

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