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Chronic Phase Lipids in Sera of Chronic Fatigue Syndrome (CFS), Chronic Ciguatera Fish Poisoning (CCFP), Hepatitis B, and Cancer With Antigenic Epitope Resembling Ciguatoxin, as Assessed With MAb-CTX

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Clinical reports and descriptions of chronic fatigue syndrome (CFS) and chronic ciguatera fish poisoning (CCFP) show great similarities in clinical symptomology. These similarities in the literature suggested the exploration of lipids in sera of CFS, CCFP, and other diseases with the membrane immunobead assay (MIA), which is typically used for screening ciguateric ocean fish. Sera from patients with other diseases, including hepatitis B, cancer, and diabetes, were included to assess the degree of specificity involved. Sera were treated with acetone in a ratio of 1 part serum to 4 parts acetone. The suspension was centrifuged, and the acetone layer was evaporated. The residue was weighed and redissolved in 1.0 mL methanol and tested by the MIA, undiluted and titered to 1:160. The undiluted acetone fraction of the 37 normal showed $\pm$ activity to $+$ activity with 16 no titer, 15 with 1:5 titer and two with 1:10 titer, and four with $\geq$1:40 titers. One hundred fifteen CFS sera showed 1 with 1+ and 114 with 2+ activity in the undiluted samples, 1 with 1:10 titer, 3 with 1:20 titer, 31 with 1:40 titer, 50 with 1:80 titer, and 30 with 160 titer. Thus 95.6% of the samples had $\geq$1:40 titer. Eight hepatitis B sera samples had $\geq$1:40 titers. Four CCFP samples had $\geq$1:40 titers. Three of 16 cancer samples had 1:40 titer. These data are summarized in Fig. 1. As shown in Table 1, a significant increase ($P < 0.001$) in the chronic phase lipids (CPLs) was shown relative to the normal group. A preliminary chemical study in C18 octadecylsilyl columns showed all fractions (100% chloroform, 9:1 chloroform : methanol, 1:1 chloroform : methanol, and 100% methanol) to contain lipids reactive to MAb-CTX with different intensities. Prostaglandins were shown in 100% methanol fraction. Competitive MIA with crude fish ciguatoxin and CFS with synthetic JKLM ciguatoxin epitope suggested similarities in structure with ciguatoxin. This was compatible with the neuroblastoma assay demonstrated in the C_{18} column fractions 9:1 and 1:1, chloroform : methanol solvents. J. Clin. Lab. Anal. 17:132–139, 2003.

Key words: chronic phase lipids; CFS; CCFP; hepatitis B; cancer; ciguatera; membrane immunobead assay (MIA)

INTRODUCTION

This study was initiated on the premise that patients with chronic fatigue syndrome (CFS), myalgic encephalomyelitis, and chronic ciguatera poisoning (CCFP) show similar clinical symptoms (1–3). The persistent occurrence of an endogenous lipid similar in structure to ciguatoxin (4) is one of the clinical features of these diseases.

CFS is caused by a variety of factors, including exposure to chemicals and other organic pollutants (for example, as experienced by Gulf War veterans), ciguateric fish, viruses (Epstein virus in infectious mononucleosis), allergenic antigens triggering immune hypersensitivity states, and fungal antigens causing asthma-type problems (5).

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In this study we examined CFS and CCFP sera with the membrane immunobead assay (MIA) using MAb-CTX (monoclonal antibody-ciguatoxin) to analyze their lipids in sera (6). The MIA titers were obtained with each acetone-extracted serum from healthy individuals and patients with various diseases. Significant increases were shown by CFS, CCFP, and acute hepatitis B, and moderate responses were obtained in sera from patients with cancer and other diseases.

According to the MIA test, CFS, CCFP, and acute hepatitis B patients appeared to have high titers in the acetone-soluble lipids. Cancer and other diseases showed low to moderate titers. Normal sera showed generally no titer, with two samples showing 1:10 titers and four with 1:40 and higher titers. Since the lipids that react with MAb-CTX are present in several diseases, we suggest that these lipids be designated as "chronic phase lipids" (CPLs). CPLs are comparable to the "acute phase proteins" found in many inflammatory diseases (7). The results of our initial study are presented in this report.

MATERIALS AND METHODS

The criteria for selection of CFS patients in this study were based on the Centers for Disease Control (CDC) studies of CFS, as described in Refs. 8–10.

Materials

CFS samples

CFS sera samples were obtained primarily from New York state through the sponsorship of the National CFIDS Foundation. Blood was withdrawn by venipuncture. The serum was separated and then shipped frozen with dry ice to the Department of Pathology, University of Hawaii–Manoa, by Federal Express. The serum was thawed upon receipt and immediately precipitated with acetone (see Methods).

The patients were selected after they were determined to meet the criteria for a diagnosis of CFS. CFS (also termed "myalgic encephalomyelitis" in the U.K.) is a debilitating and complex disorder characterized by profound fatigue that is not improved by bed rest, and may be worsened by physical or mental activity. Persons with CFS often function at a substantially lower level of activity than they did before the onset of illness. In addition to these key defining characteristics, patients report various nonspecific symptoms, including weakness, muscle pain, impaired memory, lack of mental concentration, insomnia, and post-exertional fatigue lasting more than 24 hr. In some cases, CFS can persist for years. The cause or causes of CFS have not been identified, and no specific diagnostic tests are available. Moreover, since many illnesses have incapacitating fatigue as a symptom, care must be taken to exclude other known and possibly treatable conditions before a diagnosis of CFS is made. Data concerning gender distribution are presented for 100 cases of CFS. For 15 samples these data were not available.

Acute hepatitis B samples

Eight sera samples were obtained from patients with acute hepatitis B who were being treated with α-interferon. The samples were stored at –76°C until use. Normal sera

Thirty-three sera samples were obtained from normal subjects of both sexes and various ethnicities and ages. The samples were kept frozen at −76°C until they were used in the MIA. Four samples from recent individuals with no CFS were also examined.

Cancer and other diseases

Sera were obtained from subjects of both sexes and various ethnicities and ages, with cancer or other diseases. The samples were kept frozen at –76°C until they were thawed. They were then examined by the MIA.

Chronic ciguatera fish poisoning (CCFP)

Four sera samples from patients with CCFP were examined by the MIA. The patients had eaten ciguateric fish and CCFP had been confirmed in their clinical history (1,2).

Methods

The immunological procedures used in this study was developed primarily for the assessment of ciguatoxin in contaminated fish tissue involved in ciguatera fish poisoning (6). Monoclonal antibody (MAb-CTX) was prepared from purified ciguatoxin in 1987. The hybridoma was maintained frozen at −75°C. It was occasionally recloned in culture and tested for immunological activity. The clones were refrozen. The hybridomas have been maintained over the years and have been consistently producing MAb-CTX, which has been used to determine the presence or absence of ciguatoxin (see Fig. 2A) in toxic fish (4). The MIA consists of assaying a piece of fish (50 ± 0.5 mg) soaked in methyl alcohol (0.5 mL) for 20 min with a hydrophobic membrane attached to a plastic template. The membrane is air-dried and then soaked in antibody coated with colored polystyrene beads for 10 min. The membrane is then washed in tap water, dried, and examined for the intensity of blue color (6). The

procedure for testing lipids in serum is similar, except for the preparation of the lipid. First, 1 mL of serum is treated with 4 mL of absolute acetone. The precipitate (mainly proteins) is centrifuged at 1,000 rpm for 10 min at 20°C. The yellowish acetone supernatant is decanted into a tared 16 × 100 mm test tube. The acetone is then removed by a jet airstream in a hood overnight. The sample in the test tube is weighed, and the sample weight is determined by subtracting the test tube weight.

Procedure for assessment of the acetone-soluble serum fractions

The air-dried lipid residues are dissolved in 1 mL of methanol in a soluble solution, by shaking. The plastic end of the membrane is inserted into this solution for 10 min and then air-dried. The membrane is then immersed into the blue latex beads coated with MAb-CTX, for 10 min. Then it is removed and dried, and the color intensity is recorded. Titration is carried out by diluting 0.2 mL of the undiluted lipid solution with 0.8 mL methanol (1:5 dilution). This is then diluted 0.5 mL with 0.5 mL methanol and subsequently titrated out to 1:160 dilution. Each dilution is tested by the MIA with latex MAB-CTX. The blue color intensity on the membrane is read as 2, +, ±, or –, and the results are recorded for each sample. The end-point titer is the last ± scored.

Competitive binding assay with crude JKLM fragments of ciguatoxin, with ciguatoxin and CFS lipids

Fragment JLKM is a synthetic fragment (Fig. 2B) of whole ciguatoxin (Fig. 2A) representing rings J, K, L, and M of the east sphere of ciguatoxin. This is the epitope, which reacts with MAb-CTX.

In this study we used a modified competitive MIA procedure in which fragment JKLM (Fig. 2B) is synthetically prepared (12) at varying concentrations and mixed with a constant concentration of crude ciguatoxin from ciguateric fish extracts. At high concentrations the synthetic JKLM fragment diminishes the color intensity of the ciguatoxin present in the crude extract. On the other hand, in some cases, lower concentrations of JKLM increase the color intensity of the constant crude ciguatoxin extract, demonstrating an additive effect rather than a blocking effect (Fig. 3).

A similar test with a constant concentration of CFS and varying concentrations of JKLM fragments was performed by the MIA with MAb-CTX latex beads. Any interference from JKLM would suggest structural similarities between CFS lipids and ciguatoxin via the JKLM epitope employing the MAb-CTX from the hybridoma (Fig. 5).

C18 column extraction with organic solvents (polar and nonpolar)

Solid phase extraction of the acetone-soluble lipids from serum was separated by use of a C18 500-mg tube (SPE; Alltech Associates, Inc., Deerfield, IL) phase type: Octadecysilyl column.

The 500-mg tube (column) was conditioned with 50 mL of chloroform. Then 0.5 mL of lipids in 1 mL of 100% chloroform were added to the column, followed by 20 mL of chloroform in 20-mL consecutive increments. The solutes were then collected and air-dried. The chloroform elution was followed by 9:1 chloroform : methanol in 20-mL increments, and the eluates were air-dried. The third fraction collected was two 20-mL increments of 1:1 mixture of chloroform : methanol and and the final three 20-mL increments of 100% methanol. All eluates were jet-stream dried in a hood. The weights of each pooled fraction (designated as fractions 1–4 (Table 2)) were determined and recorded. Prior to pooling, each fraction was tested with the MAB-CTX (Table 2).

Neuroblastoma cell assay

This experiment was carried out to determine the effects of the CPL fractions from the C18 (octadecysilyl) separation. Fractions 9:1 and 1:1 were examined in the NB assay, since they corresponded best with the ciguateric fish extracts relative to ciguatoxin activity.

Mouse neuroblastoma cells (neuro-2a, ATCC, CCL 131) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Atlanta Biologicals). In addition, 2 mM glutamine, 1 mM sodium pyruvate (Sigma), 50 μg streptomycin, and 50 U/mL penicillin cultures were maintained at 37°C in humidified 5% CO_2, 95% air atmosphere. With the exception of the bovine serum, all of the reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture bioassay

Cell bioassays in microtiter plate format, and the preparation of the CPL dilution were performed as described previously (13). Briefly, N2a cells were seeded into antibody well microtiter plates at a density of 5×10^5 cells in 200 μL complete medium per well. They were maintained for 24 hr at 37°C in the humidified incubator, with 5% CO_2 : 95% air atmosphere before further additions. Subsequently, 10 μL each of 10 mM ouabain, 1 mM veratridine, and dilutions of CPL (0, 125, 250, 500, and 1,000 ng) were added to the N2a cells. The wells were incubated for 16 hr and the viable cells were assessed with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) (Sigma) as described previously

(13). The end results were read in the ELISA reader microplate at 540 μm. N2a cells with dilutions of CPL alone, ouabain alone were also carried auto quadruplicated wells were set up for each analysis. Figure 6A and B show the average of each dilution of the CPL C18 fractions.

RESULTS

MAB-CTX Antibody Assessment of Various Sera Samples by the MIA

Figure 1 shows the distribution in numbers, and the titers of the CPL in sera of normal individuals and patients with CFS, hepatitis B, CCFP, cancer, or other diseases. Thirty-seven samples from normal subjects showed titers less than 1:10 (89%), while four samples had titers of 1:40 and 1:80, representing 11.0% of the samples examined. In contrast, of the 100 CFS samples, 95.6% had titers of 1:40–160, while five samples had 1:20 titers and less, representing 4.4% of the samples examined. Sera from 96 CFS patients (65 females and 31 males) was examined, representing a gender ratio of 2:1, F:M. No gender data are presented for the other 19 CFS samples recorded in the early phase of this study. Nevertheless, this 2:1 ratio has been reported by other investigations as well (11).

Weights of Acetone-Soluble Lipids

Weights in milligrams of the solid lipids in 1 mL of serum are represented for each category shown in Table 1. The weight represents the mean ± S.D. of each category, including the normal sera. An examination of the weight of each sample in each category revealed no significant differences in the mean ± S.D. between each category, including normals. However, the disease categories showed a few higher values in the upper range.

Competitive Inhibition of Crude Ciguatoxin Fish Extracts and CFS Lipids With Synthetic Ciguatoxin Fragment JKLM

A competitive reaction occurred in the MIA when ciguatoxin (Fig. 2A) was mixed with a synthetic epitope of ciguatoxin, JKLM (Fig. 2B), as summarized in Fig. 3; as little as 5 pg JKLM showed inhibition in the MIA with 1 mg of crude ciguatoxin. Complete inhibition with pure CTX3C as low as 0.001 pg P-CTX3C was shown with 3 mg crude CTX fish extract (Fig. 4).

The same procedure was used to assess the immunological relationship between CFS lipids and the synthetic epitope of ciguatoxin, JKLM. Various concentrations of synthetic epitope JKLM of CTX 50, 100, 250, 500, and

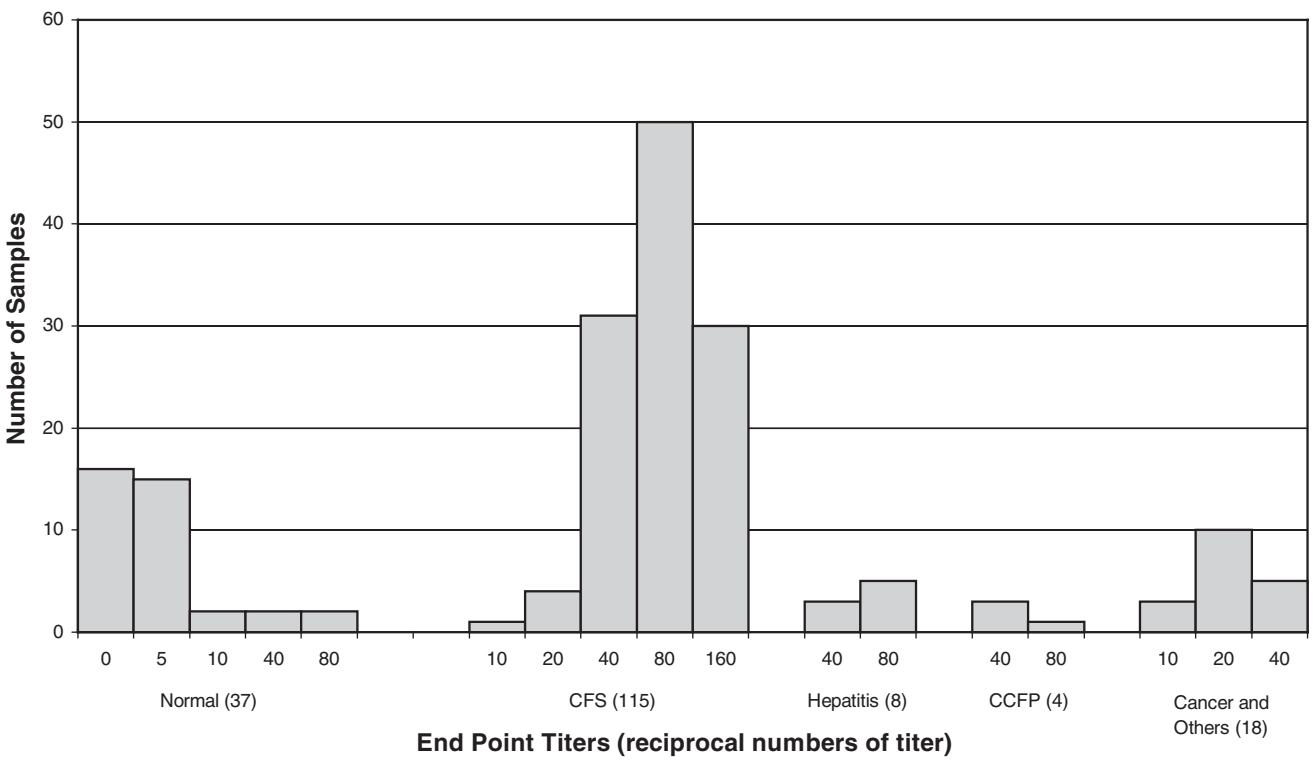

Fig. 1. Summary of each category of diseases, showing titers and number for each titer.

TABLE 1. Comparison of total lipids in mg/ml serum sample, acetone soluble fractions in disease sera samples vs. normal samples

Category	n	Weight ± SD (mg)	Range (mg)
CFS	115	10.5 ± 3.17	7.0–25.0
Hepatitis B	8	11.9 ± 3.37	7.4–8.0
Cancer	16	12.8 ± 2.28	8.40–16.0
Normal	37	11.8 ± 2.50	3.0–15.4

1,000 pg were added to 2.75 mg CFS lipid in methanol, and the mixtures were allowed to stand at room temperature for 2 hr. The mixtures were then immersed with the membrane for 10 min and were subsequently examined by the MIA. An example is shown in Fig. 5. Complete inhibition by JKLM was shown at 100 pg concentration and higher.

Preliminary Characterization and Fractionation of the CFS and Normal Acetone Fractions From the C18 Column

Seventy-two milligrams of pooled CFS acetone-soluble lipids were extracted with a prepared C18 column, with organic solvents ranging from nonpolar to polar. The initial phase was 100% chloroform (30 mL), 9 parts chloroform : 1 part methanol mixture (30 mL), then 1 part chloroform : 1 part methanol (30 mL), and finally an elution with 100% methanol (30 mL). Table 2 shows the distribution, % recovery, and immunological activity of the fractions from the CFS acetone-soluble lipids. The immunological activity with MAb-CTX showed all fractions to be similarly active, with the exception that CFS fraction 3 (1:1, chloroform: methanol) was less active. The yield in fraction 4 (100% methanol) was high in CFS (46%). This may be significant, since preliminary studies (data not shown) revealed prostaglandins 6-keto-PGF1a, PGE2, PGF2a, and leukotriene B4 in fraction 4 (100% methanol).

CFS C18 Column Fraction Assessment in the Neuroblastoma Assay

CFS fraction samples at 50 ng, 125 ng, 250 ng, 500 ng, and 1,000 ng were added to NB cells in the presence of ouabain, ouabain plus veratridine, and NB cells only. In this assay only fractions 2 and 3 of the C18 extraction were examined, since these are the regions where ciguatoxins appear in the toxic fish extracts. The effects of the 9:1 fraction (fraction 2) on the NB cells

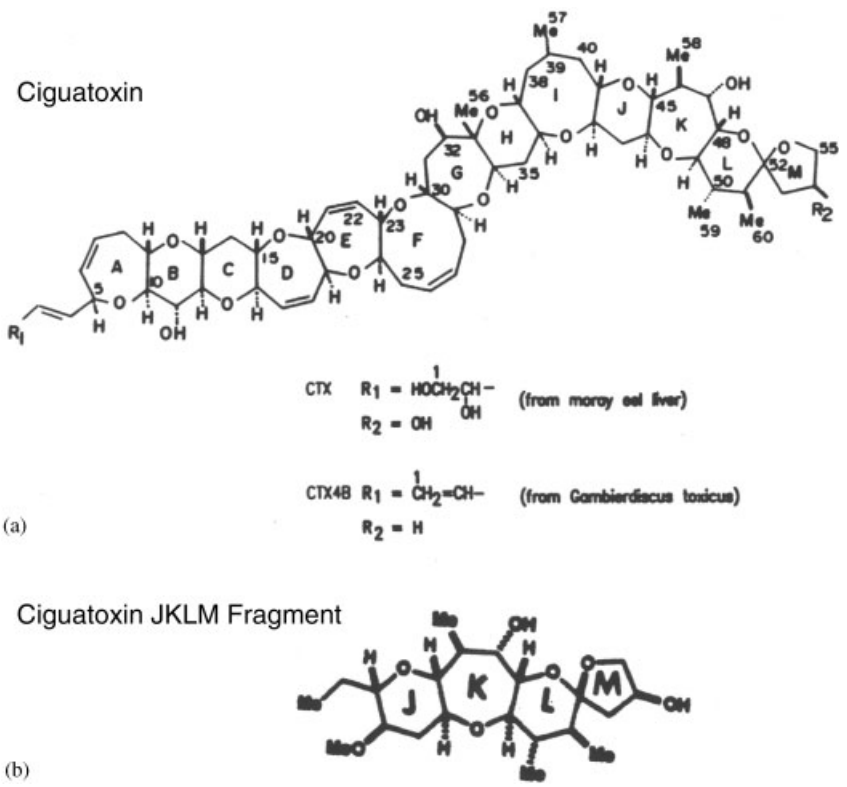

Fig. 2. **A:** Structure of complete ciguatoxin (moray eel and *G. toxicus*), showing differences. **B:** Fragments of ciguatoxin, which reacts with MAb-CTX labeled JKLM.

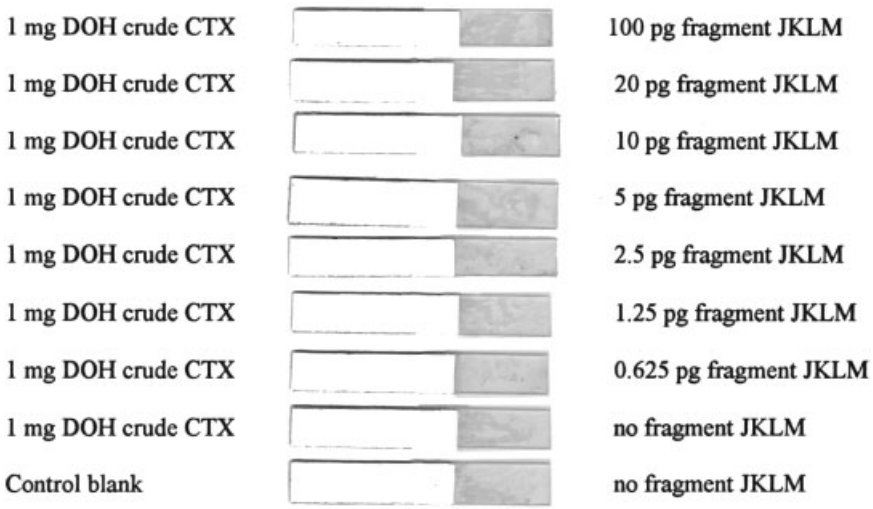

Fig. 3. Competitive MIA test, crude CTX from fish extract with added JKLM at various pg concentrations.

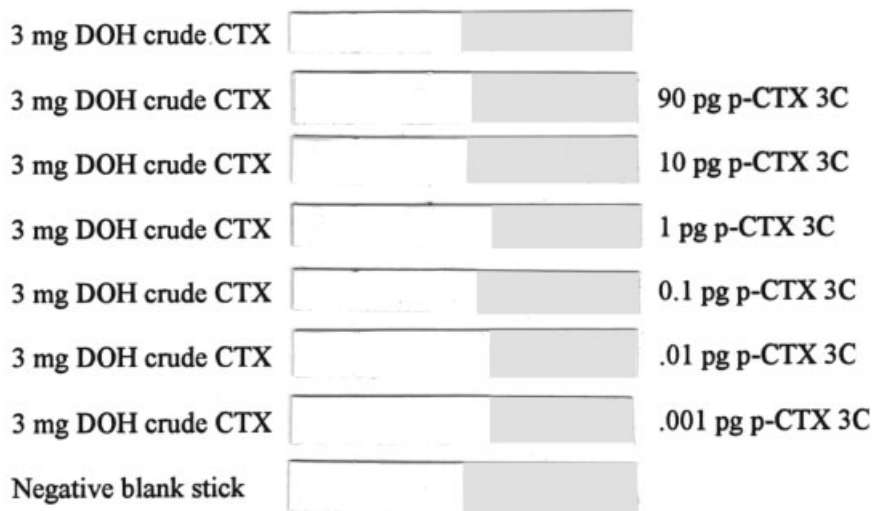

Fig. 4. Competitive MIA test, crude CTX from fish extract with added pure CTX3C from *G. toxicus* with various pg concentrations.

are presented in Fig. 6A. The control (fraction plus NB cells only) showed inhibition at 250 ng with no dose response. The addition of ouabain caused a greater inhibition at 250 ng and 500 ng, with stimulation at 1,000 ng. The combined effect of CFS lipid fraction ouabain and veratridine on the stimulation of the NB cells in Fig. 6A is significant. This is characteristic of paralytic shell toxins (PSPs) and tetrodotoxin (TTX), in which dose-response stimulation is seen under these experimental conditions. This stimulation was also previously demonstrated in ciguateric fish extracts (11).

CFS fraction 3 (1:1, chloroform : methanol; Fig. 6B) also showed activity in the NB cell assay comparable to ciguateric fish extracts. This is especially evident in the CFS + ouabain + veratridine mixture, in which a characteristic NB cell inhibition is shown in a dose-responsive manner with initial stimulation at 125–250 ng to inhibition at 500 and 1,000 ng CFS fraction.

DISCUSSION

The data obtained with the MIA assay for ciguatoxin with MAb-CTX clearly showed high levels of acetone-soluble lipids resembling ciguatoxin in CFS, CCFP, and hepatitis B sera. The MIA showed moderate activity or lower titers with serum lipids from sera of patients with cancer or other diseases. This observation is intriguing.

- Used 1:4 titration of Sample 6A
- JKLM Fragments incubated in sample 6A for 2 days
- Latex Solution used: 1:20 2D1 MAb-CTX, 0.02% Tween 60, 0.01% Azide, Blue Latex
- Test Date: 6/17/02
- Solution Date: 6/12/02

Amount of JKLM Fragment Added	Result	MIA Stick Data
No JKLM Fragment added	+	
50 pg JKLM Fragment added	+/-	
100 pg JKLM Fragment added	+/-	
250 pg JKLM Fragment added	-	
500 pg JKLM Fragment added	+/-	
1000 pg JKLM Fragment added	-	

Fig. 5. CFS serum lipids (2.75 mg) in competitive MIA tests with CTX fragment in pg concentrations.

TABLE 2. CFS sera pooled acetone fraction C18 column, MAb-CTX

Fraction number	Solvent fraction (eluates)	Weight of fractions recoverd (mg)	% of total sample recovered	MIA results (undiluted)
1	100% Chloroform	4.7	8.1	+
2	9:1 Chloroform/MeOH	8.0	13.7	+
3	1:1 Chloroform/MeOH	6.8	11.7	$\pm$
4	100% Methanol	26.8	46.0	+
	Total recovered	58.3 mg		
	Sample applied to column	72.5 mg		
			% recovered: 80%	

The immunological interaction between MAb-CTX and CFS lipids may be merely a cross-reaction, but nevertheless suggests a common structural epitope in CFS and ciguatoxin from moray eels and other ciguateric reef fishes. This was especially demonstrated by the use of the JKLM synthetic fragment, which showed inhibition of MAb-CTX with crude ciguatoxin and CFS acetone-soluble lipids in the competitive MIA.

This increase of lipids in CFS, CCFP, hepatitis B, and cancer is an interesting phenomenon. At this time it is not known whether the lipids are abnormal in structural conformation, or there is merely an increase in normal lipids of significant physiological function creating the clinical symptomology shown in these diseases. Future studies will investigate the chemistry of the acetone-soluble lipids. It is speculated here that the lipids involved may merely be CPLs, comparable to "acute phase proteins" such as C-reactive protein, which increases in diseases associated with inflammation and trauma (7). In addition, further MIAs will be carried out with as many disease sera as possible to determine the extent of these CPLs. Patients with CPLs will be examined over a long period of time to determine how long the CPLs are present, and the rate of clearance from blood.

The observation of high levels of CPLs in hepatitis B infections and CCFP indicates that the liver is a possible source of CPLs, since it is the major organ in the detoxification process. We propose that liver function tests be conducted when CFS, CCFP, and hepatitis B are clinically diagnosed, in addition to a serum MIA for CPL.

Finally, we suggest that the MIA procedure with MAb-CTX should be used in clinical laboratories to supplement the clinical diagnosis of CFS.

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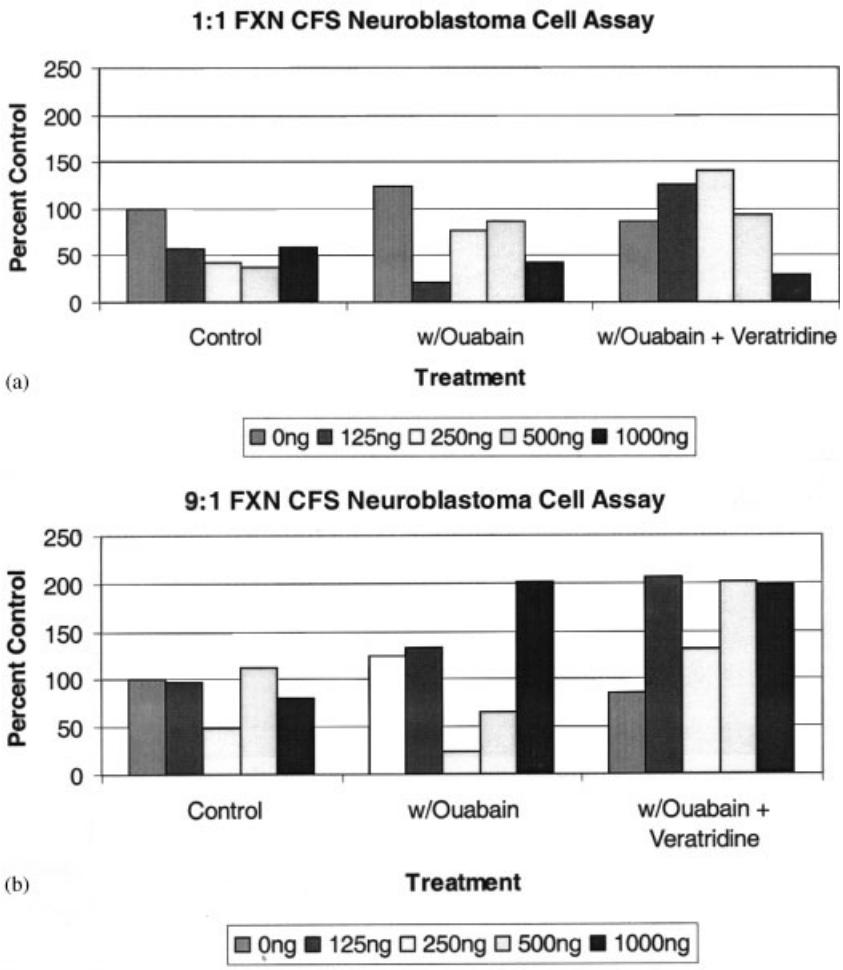

Fig. 6. **a:** Neuroblastoma cell assay treated with C18 column fraction 2 (9:1 chloroform : methanol) from CFS serum lipids. **b:** Neuroblastoma cell assay treated with C18 column fraction 3 (1:1 chloroform : methanol) from CFS serum lipids.

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