

# Acute Phase Phospholipids Related to the Cardiolipin of Mitochondria in the Sera of Patients With Chronic Fatigue Syndrome (CFS), Chronic Ciguatera Fish Poisoning (CCFP), and Other Diseases Attributed to Chemicals, Gulf War, and Marine Toxins

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This study examined 328 CFS sera in a study with 17 CCFP, 8 Gulf War Veterans (GWV), 24 Prostate Cancer (PC), and 52 normal sera in the modified Membrane Immunobead Assay (MIA) procedure for CTX. Three hundred and twenty-eight CFS patients' sera were examined by the modified MIA with purified MAb-CTX and 91.2% gave a titre  $\geq 1:40$ . 76% of the 17 CCFP sera samples and 100% of the 8 GWV sera samples also had a titre  $\geq 1:40$ . 92.3% of 52 normal sera showed titres of 1:20 or less, while 4 gave titres of  $\geq 1:40$ . In addition, 41 sera were examined for Anti-Cardiolipin (aCL) by a commercial ELISA procedure with 87.8% demonstrating IgM, IgM+IgA, or IgM+IgG aCL antibodies. These results showed mostly the IgM aCL antibody alone in the sera samples. In addition, 41 serum samples were examined for aCL, with 37 showing positive for aCL, representing 90.2% positive for the three

disease categories examined: CFS, CCFP and GWV. Examination for antiMitochondrial-M2 autoantibody (aM-M2) in 28 patients (CFS (18), CCFP (5), and GWV (5)) was negative for aM-M2. Inhibition analysis with antigens, CTX, CFS "Acute Phase Lipids", commercial Cardiolipin (CL) and 1,2-Dipalmitoyl-sn-Glycerol-3-[Phospho-L-Serine] (PS) and antibodies, MAb-CTX and aCL from patients' serum show that the phospholipids in CL and CTX are antigenically indistinguishable with antibodies MAb-CTX and CFS-aCL. Preliminary chemical analyses have shown the lipids to be phospholipids associated with CL of the mitochondria. We designate this "Acute Phase Lipid" comparable to "Acute Phase Proteins" (C-reactive protein (CRP) and Serum Amyloid A (SAA)) in inflammatory conditions. *J. Clin. Lab. Anal.* 22:99–105, 2008.  
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**Key words:** chronic fatigue syndrome; anticardiolipin; cardiolipin; acute phase phospholipids

## INTRODUCTION

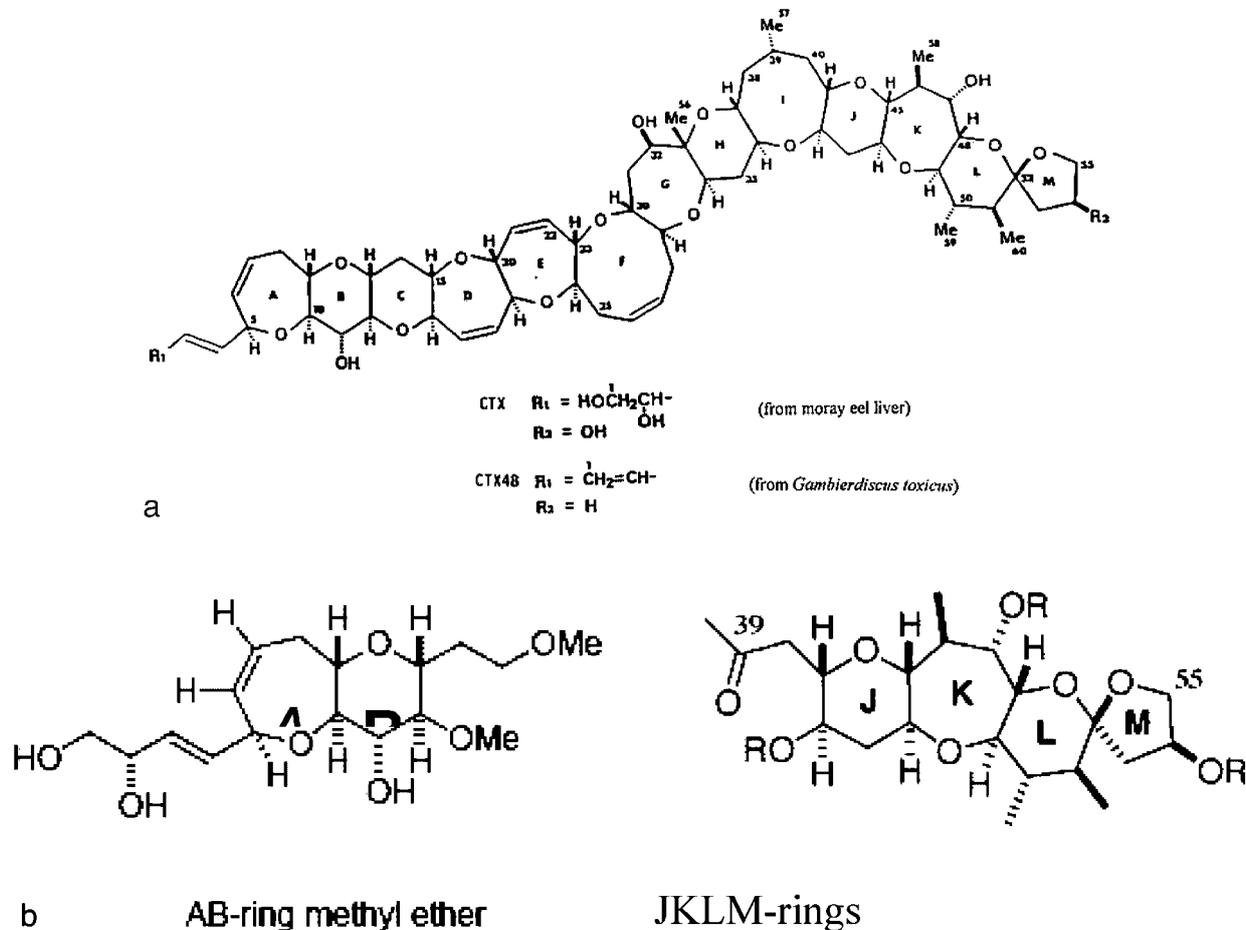
During a recent examination of the clinical history of chronic fatigue syndrome (CFS) and chronic Ciguatera fish poisoning (CCFP), it was shown that the clinical symptoms found in both diseases were remarkably similar, including recurrent fatigue, impaired memory and concentration, tender lymph nodes, muscle and joint pain, headaches, and other neurological impairments (1–5). This prompted our laboratory, with years of experience in the field of marine toxins including ciguatoxin (CTX), to explore serological similarities in serum lipids of patients with CFS, CCFP, and other traumatic diseases.

Serum was obtained from patients clinically diagnosed with both CFS and CCFP from various parts of the United States, specifically the eastern, central, and western states. The study found that the serum lipid fraction of patients with CFS has a structural epitope(s) similar to CTX, a polyether lipid with

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Received 6 December 2006; Accepted 30 August 2007  
DOI 10.1002/jcla.20217

Published online in Wiley InterScience (www.interscience.wiley.com).



**Fig. 1.** CTX molecule (a) showing the AB fragment (b) and the JKLM region (c).

neurotoxic properties produced by a marine organism (*Gambierdiscus toxicus*). Lipids extracted with acetone from patient serum were tested and shown to react positively with a monoclonal antibody (MAb-CTX) reacting with the JKLM fragment, or epitope, of the CTX structure, thus resulting in the data presented in this study. The structure of CTX, including both the ABCD and JKLM epitopes of the molecule, is shown in Fig. 1a–c (6).

A total of 328 CFS sera samples were received and examined, most of which showed peculiar or acute phase lipids similar in configuration to the JKLM epitope of CTX. We have found that the “acute phase lipid” in the CFS lipid fraction appears to be associated with phospholipids (1,3-bis(sn-3'-phosphatidyl)-sn-glycerol) of mitochondria found in many serum samples of diseases including viral infections (Epstein-Barr, hepatitis viruses, and human immunodeficiency virus [HIV]), parasitic diseases, marine toxins (CTX, okadaic acid, palytoxin, polyether lipids), and other microbial infectious diseases (7–16). It was recently reported in a National Chronic Fatigue and Immune Dysfunction

Syndrome (CFIDS) Foundation press release that CFS/myalgic encephalomyelitis (ME), multiple sclerosis (MS), and idiopathic epilepsy, and CFS were caused by a Cryptovirus (Zoonotic virus), similar to the parainfluenza virus-5, originally found in swine (17). These disease processes, particularly CTX, most likely affect the major organ, the liver. Since phospholipid release is suspected, it is the mitochondria of these cells in the liver that is thought to be affected during the detoxification or destruction of the toxic entity. We hypothesize that damage or stimulation of the mitochondria results in the release of phospholipids, which in turn induce production of an autoimmune antibody (antiphospholipids). Many of the disease states listed above have been found to involve antiphospholipids, including autoantibodies to cardiolipin (CL) of the immunoglobulin G (IgG), IgM, and IgA isotypes.

Thus, this work presents immunological data to show that these phospholipids are associated with the mitochondria and part of the CL, especially in CFS, CCFP, Gulf War veterans (GWV), and prostate cancer

(PC) sera. In addition, anti-CL antibodies (aCL) have been shown in the samples of these sera examined by commercial enzyme-linked immunosorbent assay (ELISA) kits and the results are also presented in these studies. We wish to designate these lipids as “acute phase lipids,” comparable to C-reactive protein (CRP) (18,19) and serum amyloid A (SAA) (20) symbolized in diseases triggered by cytokines in the inflammatory processes of these diseases in the liver (18–20). Data of CRP and SAA from the same samples are also presented.

CFS was based on Fukuda et al. (21) as accepted by the Centers for Disease Control (Atlanta, GA). GWV sera were obtained through Dr. Kathleen Hannan (Lakewood, FL). PC (15) sera samples were obtained from Dr. Karen Halliday (Albuquerque, NM). Diagnosis of PC was verified by pathological findings. A total of 49 normal sera samples from various sources were obtained without any clinical diagnosis of illness. Normal sera were obtained from various students and staff from the John A. Burns School of Medicine.

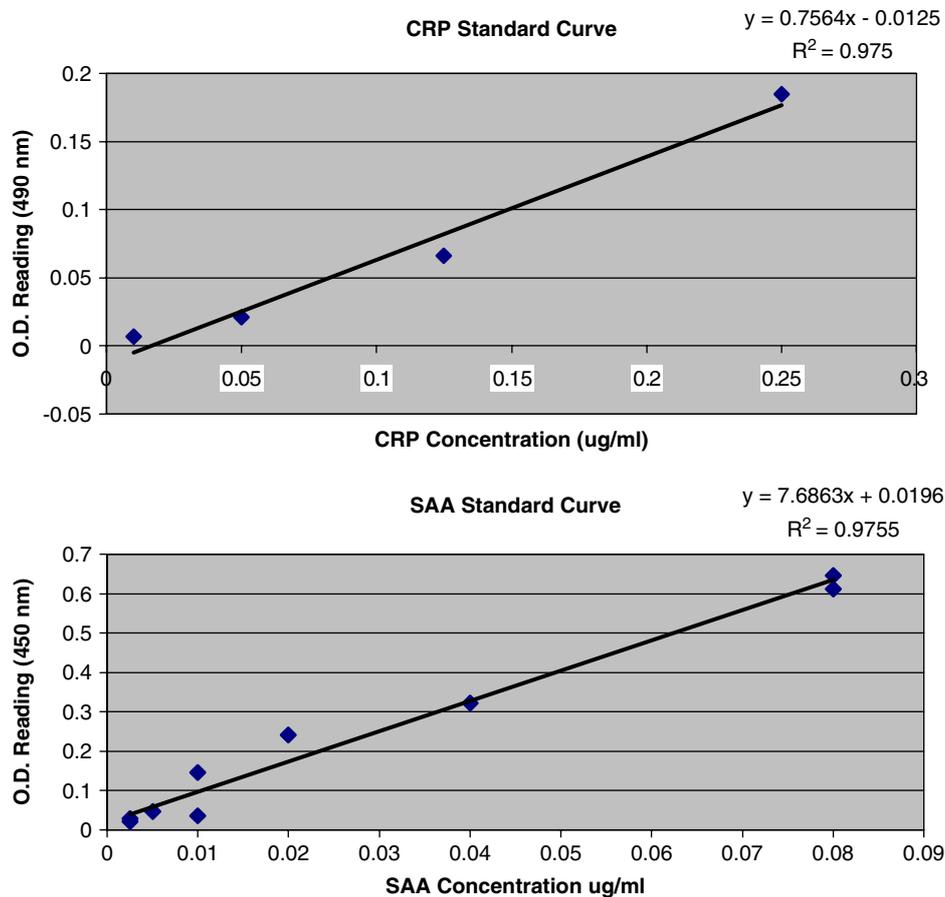
**MATERIALS AND METHODS**

**Sera Samples**

A total of 328 CFS sera samples were obtained from patients’ physicians from various parts of the United States. CCFP samples examined included 18 sera samples from areas of fish poisoning outbreaks diagnosed as Ciguatera. The initial clinical diagnosis used was that of Hokama et al. (1), Palafox et al. (2), Pearn (3,4), and Bagnis (5). CFS, including fibromyalgia, included 328 sera samples. The clinical diagnosis for

**Immunological Studies Using Patient Sera With the Modified Membrane Immunobead Assay**

The modified membrane immunobead assay (MIA) (1,2,22,23) was initially established for the detection of CTX from ciguateric fish. A total of 1 mL of patient serum is mixed with 4 mL of absolute acetone. The solution is shaken thoroughly and the suspension is centrifuged at 1,000 rpm for 10 min. The acetone phase is decanted into a clean-tared 10-mL test tube and the acetone evaporated by a stream of air or nitrogen gas in a hood for 18 hr. The dried lipid phase is weighed and



**Fig. 2.** The CRP standard curve (a) and the SAA standard curve (b).

the weight of lipid is recorded. Studies to date show no correlation between weight and immunological MIA titer. The dried lipid is dissolved in 1 mL of absolute methanol and is tested using the modified MIA procedure (22,23).

### ELISA: CRP, SAA, and aCL

CRP and SAA are synthesized in the liver and commonly found in an inflammatory condition in diseases (18,20). The ELISA procedures were based on the classical sandwich method utilizing two antibodies initially presented by Voller et al. (24). The first antibody, usually a goat, horse, or rabbit sera, is coated onto 96-well Co-Star plates (Cole-Parmer, Vernon Hills, IL) followed by the addition of serum samples containing the antigen to be examined. It is then washed thoroughly and followed by the second antibody to the antigen conjugated with the enzyme (horseradish peroxidase) used as the detection step. After washing, the substrate, *o*-phenylenediamine (OPD; Sigma Chemical Company, St. Louis, MO) is added to give the color reaction, which is then recorded by the ELISA reader. The color intensity is proportional to the concentration of the antigen. A linear gradient standard curve is plotted and the unknown determined from this standard curve.

The aCL (INOVA Diagnostics Inc., San Diego, CA), CRP, and SAA (Antigenix America, Inc., Huntington Station, NY) data for the sera examined were derived by this ELISA procedure.

Results of standard and samples are read in the Automated Microplate Reader (Bio-Tek Instrumental, Inc., Highland Park, Winooski, VT) at 450 nm. The standard curve (Fig. 2a and b) for CRP and SAA were calculated by statistical software. Figure 2a presents the standard curve for (protein concentration vs. optical density) CRP by protein analysis of various concentrations as scored by the Automated Microplate Reader. The unknown sera were determined with the same ELISA procedure. The concentrations of CRP in samples extrapolated from the standard curve are presented in Table 1. Similarly, the standard curve for SAA (Fig. 2b) is derived by the ELISA procedure,

**TABLE 1. CFS sera samples examined**

Acute phase protein	Number of samples	µg/mL serum	%
CRP <sup>a</sup>	13	<1.5 µg/mL	27.7
	34	>1.5 µg/mL	72.3
SAA <sup>b</sup>	47	≤5 µg/mL	20.9
	185	≥5 µg/mL	79.1

<sup>a</sup>A total of 47 CFS sera were examined for the presence of CRP.

<sup>b</sup>Results of 185 CFS sera samples examined for SAA.

showing a straight line at variable protein concentrations vs. optical densities. Unknowns of patient sera were extrapolated from this curve and the results are indicated in Table 1.

### Inhibition Analysis Using Modified MIA Procedure

The inhibition analysis shows antigenic epitope configuration similarity using three different antigens (epitopes) and two different antibodies. The antigens include, CTX, “acute phase lipids” of CFS sera and CL (bovine heart tissue lipids—commercial), while the antibodies include MAb-CTX and patient serum auto-antibody, aCL. The procedure is the modified MIA as follows: The lipid antigens are solubilized in absolute methanol and the blank membrane is immersed in the alcohol-lipid solutions for 10 min. The membrane is removed, air dried, and then immersed in the MAb-CTX-coated beads for 20 min. The membrane is then removed, rinsed in water to remove excess MAb-CTX beads, dried, and the color intensity is scored.

Inhibition is determined by treatment of the lipid bound membrane with the unbeaded antibodies (MAb-CTX or aCL) before immersion into the labeled or colored beaded antibodies. The results of these studies are presented in Table 2a, b, and c for CTX, “acute phase lipids” of CFS, commercial bovine CL, MAb-CTX, and CFS diluted sera (aCL-containing).

The phospholipid, 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-L-Serine] (PS) was similarly examined with the MAb-CTX. The results are shown in Table 2d.

## RESULTS

### Modified MIA Data

Table 3 presents the data by the modified MIA procedure of the acetone soluble lipid fraction of sera containing “acute phase lipids” (phospholipids) in four categories of diseases: CFS, CCFP, GWV, and PC. Of the 328 CFS examined, 91.2% (299 samples) had titers of ≥1:40 and 9.0% (29 samples) had titers of ≤1:20. CCFP serum samples of patients exposed to the marine toxin, CTX, had 13 with titers ≥1:40 (76.5%) and 4 (23.5%) with titers of ≤1:20. A total of 17 CCFP sera samples were examined. Of the eight GWV serum samples examined, 100% had titers of ≥1:40. Serum samples (15) of PC showed 73.3% with a titer of ≥1:40 and 26.7% with a ≤1:20 titer.

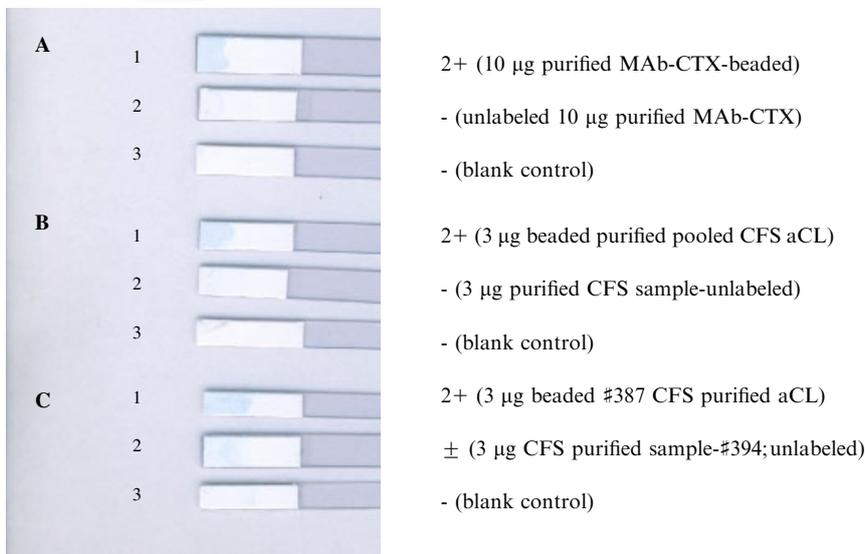
### ELISA Results

It was of interest to examine the CFS sera for the presence of “acute phase proteins” to establish an inflammatory status in CFS patients. Both CRP and SAA were determined. The results of this survey are shown in Table 1. A total of 47 randomly selected CFS

**TABLE 2. Inhibition study\***

Antigen	Unbeaded Ab (Inhibitor)	Beaded Ab	Reaction	Results
a. Inhibition study with CTX as the antigen				
CTX		MAb-CTX	(+)	(2+)
		control		
CTX	MAB-CTX	MAB-CTX	Inhibition	(-)
CTX	MAB-CTX	Patient-serum (aCL) diluted (1:3,200)	Inhibition	(-)
		control		
CTX		Patient-serum (aCL) diluted (1:3,200)	(+)	(2+)
		control		
CTX	Patient-serum (aCL) diluted (1:3,200)	Patient-serum (aCL) diluted (1:3,200)	Inhibition	(-)
		control		
CTX	Patient-serum (aCL) diluted (1:3,200)	MAB-CTX	Inhibition	(-)
b. Inhibition study with CFS lipids as the antigen				
CFS AP Lipids		MAB-CTX	(+)	(2+)
		control		
CFS AP Lipids	MAB-CTX	MAB-CTX	Inhibition	(-)
CFS AP Lipids		Patient 363 (aCL) diluted (1:3,200)	(+)	(2+)
		control		
CFS AP Lipids	Patient 363 (aCL) diluted (1:3,200)	Patient 363 (aCL) diluted (1:3,200)	Inhibition	(-)
		control		
CFS AP Lipids	Patient 363 (aCL) diluted (1:3,200)	MAB-CTX	Inhibition	(-)
c. Inhibition study with CL as the antigen				
Cardiolipin (CL)		MAB-CTX	(+)	(2+)
		control		
Cardiolipin (CL)	MAB-CTX	MAB-CTX	Inhibition	(-)
Cardiolipin (CL)		Patient 363 (aCL) diluted (1:3,200)	(+)	(2+)
		control		
Cardiolipin (CL)	MAB-CTX	Patient 363 (aCL) diluted (1:3,200)	Inhibition	(-)
		control		
Cardiolipin (CL)	Patient 363 (aCL) diluted (1:3,200)	MAB-CTX	Inhibition	(-)
Cardiolipin (CL)	Patient 363 (aCL) diluted (1:3,200)	Patient 363 (aCL) diluted (1:3,200)	Inhibition	(-)
		control		
d. Mitochondrial membrane lipid, 1,2-Dipalmitoyl-sn-Glycero-3-(Phospho-L-Serine) as antigen vs. MAB-CTX in the modified MIA				
Results		Treatment		

**Results**



AP, acute phase.

sera were examined by the sandwich ELISA procedure. A total of 72.3% showed a relatively moderate increase in levels of CRP, while 79.1% of 185 CFS sera demonstrated moderate to high levels of SAA.

Table 3 represents the result of the ELISA analysis of aCL in CFS, CCFP, and GWV patient sera. A total of 20 CFS sera contained aCL of which three sera contain IgG + IgM; one serum had IgA + IgM, and 20 sera had IgM alone. The mean and range of these isotypes concentrations are presented for CFS. CCFP and GWV showed an absence of IgA isotype. The mean and range of isotypes are shown for all these diseases in Table 4. In general, the antibody levels appear to be low. The common isotype of aCL present in the diseases examined appeared to be IgM.

### Inhibition Analysis

Table 2a–c show both that MAb-CTX and aCL in patients' sera at high dilutions react strongly with epitopes CTX, CFS "acute phase lipids", and bovine CL, and that these reactions can be inhibited. These immunological reactions suggested that CTX, bovine CL, and CFS "acute phase lipids" have chemical structural configurations similar to phospholipids associated with CL. The immunological results strongly suggest that phospholipids associated with CL are involved in these observations of CFS, CCFP, GWV, and PC sera as has been shown for many other viral, infectious diseases, and other ailments reported in the literature (7–16). Thus, we strongly believe that these lipids are "acute phase lipids" in diseases, synonymous with "acute phase proteins" in diseases.

**TABLE 3. Acute phase lipids (phospholipids) in diseases listed per category as assessed with the MAb-CTX**

Disease category	Total number	Titer		Titer	
		≤1:20	%	≥1:40	%
Normal	52	48	92.3	4	7.7
CFS	328	29	8.8	299	91.2
CCFP	17	4	23.5	13	76.5
Gulf War	8	0	0	8	100
Prostate cancer	15	4	26.7	11	73.3

**TABLE 4. Data of ELISA examination of anticardiolipin antibody in sera from various patients\***

Disease category	Number of samples		IgG isotype	IgG mean concentration (GPL)/IgG range	IgM isotype	IgM mean concentration (MPL)/IgM range	IgA isotype	IgA mean concentration (APL)/IgA range
	Total	Negative						
CFS	26	2	3	13.83/9.79–15.99	24	18.74/15.52–25.61	1	12.88/12.88
CCFP	9	1	3	13.66/8.46–20.23	8	21.21/15.47–36.2	0	0/0
Gulf War	6	2	1	17.28/17.28	5	15.68/15.2–16.56	0	0/0

\*IgG absorbance <8 considered negative; IgM absorbance <15 considered negative; IgA absorbance <13 considered negative.

### Phospholipids Associated With Mitochondria and Its Immunological Interaction With MAb-CTX and Patients' aCL

The following results are shown in Table 2d (A, B, and C):

- A. 1) 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-L-Serine] (PS) + beaded (0.12%) MAb-CTX gave a 2+ reaction; 2) PS treated with unbeaded MAb-CTX, washed, then treated with beaded MAb-CTX showed a negative reaction; and 3) is a blank control.
- B. 1) Pooled CFS sera diluted 1:500 with PBS and colored beads (0.12%) gave a 2+ color reaction; 2) PS on the membrane treated with 3μg unlabeled CFS diluted pooled sera samples, then washed, and followed by 3μg beaded CFS diluted pooled sera showed no color indicating inhibition; and 3) is a blank control with no reaction.
- C. 1) 3μg of beaded #387 purified CFS sample; 2) 3μg beaded #394 purified CFS sample showed a weak reaction when tested with 3μg of beaded #387. This suggests that the reactive aCL in sample #394 was less in concentration, hence a partial block; and 3) is a blank control.

The data suggest that the antibodies in MAb-CTX, pooled purified CFS, individual CFS, (#387 and 394) are reacting with PS and are aCL as revealed in several CFS sera shown by the standard ELISA procedure (Table 4).

### DISCUSSION

This study has examined "acute phase proteins" (CRP and SAA) together with lipids designated "acute phase lipids" in sera of lipids of CFS, CCFP, GWV, and PC patients. CRP, associated with inflammation in diseases, has been prominently related to cardiovascular diseases causing narrowing of blood vessels contributing to blockage of blood circulation in heart diseases (18,19). CRP has been noted to react with a variety of compounds in the presence of Ca<sup>2+</sup> to produce flocculating aggregates, especially with lipids and mucopolysaccharides (18). SAA has been associated

with amyloidosis, especially as deposits in tissues and organs commonly found in diseases of the heart and kidney (20).

The “acute phase lipids” found in the sera of CFS, CCFP, GWV, and PC in this study are part of the phospholipids of the mitochondrial membrane associated with CL, which appears in many or all diseases, notably, viral infections (7–16,25). The presence of CL is associated with aCL titers in most diseases (7–16). In this study, aCL was shown in a few normal sera but of lower titer. The nature of aCL function is unclear. Data show, in this study, a strong reaction with the “acute phase lipids” found in CFS, GWV, and CCFP.

The blocking or inhibition studies demonstrated that CTX, CL, and CFS lipids and phospholipids had an antigenic epitope with a structural configuration that reacted with the MAb-CTX and CFS serum aCL antibodies, thus immunologically indistinguishable.

In Ciguatera studies by Terao et al. (26), it was demonstrated that purified CTX injected into inbred mice produced structural changes in the tissue of mitochondria. Swelling and destruction of the inner membrane in the mitochondria of heart and liver tissue was demonstrated by electron microscopy.

It is suggested that the increase in phospholipids associated with CL and all may be associated with defective mitochondrial metabolism and thus apoptosis in regulation of program cell death, especially involved in the immune system, hence immune dysfunction status in CFS disease (27,28).

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