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IgM Serum Antibodies to Epstein-Barr Virus are Uniquely Present in a Subset of Patients with the Chronic Fatigue Syndrome

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Abstract. *Background: A unique subset of patients with chronic fatigue syndrome (CFS) and IgM serum antibodies to cytomegalovirus (HCMV) non-structural gene products p52 and CM_2 (UL 44 and UL 57) has been described. Patients and Methods: Fifty-eight CFS patients and 68 non-CFS matched controls were studied. Serum antibodies to EBV viral capsid antigen (VCA) IgM and EBV Early Antigen, diffuse (EA, D) as well HVCMV(V), IgM and IgG; VP (sucrose, density purified V); p52 and CM_2 IgM serum antibodies were assayed. Results: Mean age of CFS patients was 44 years (75% women). Control patients were 9 years older (73% women). Serum EBV VCA IgM positive antibody titers were identified in 33 CFS patients (Group A subset EBV VCA IgM 62.3±8.3, neg.<20), but were not present in other CFS patients, (Group B subset EBV VCA IgM 6.8±0.7) controls ($p<0.0001$). EBV VCA IgM titers remained positive in CFS patients from Group A for 24-42 months. Conclusion: Serum antibody to EBV VCA IgM may be a specific diagnostic test for a second subset of CFS patients.*

Chronic Fatigue Syndrome (CFS) is a progressive illness of young adults (2-4 women to one man) with peak time of onset of 25-45 years old (1,2). Symptoms of CFS are similar to infectious mononucleosis. A virus etiology for CFS has been suspected, but none has been found. Infectious mononucleosis may be caused by Epstein-Barr virus (EBV) (3) or cytomegalovirus (HCMV) (4). Controlled clinical trials of therapy have been performed. No intervention has benefitted the CFS patient (5). We have reported a subset of 16 CFS patients with unique IgM serum antibodies to human cytomegalovirus (HCMV) non-structural gene products of the virus tegument, p52 and CM_2 (UL44 and UL57). The HCMV IgM p52 and CM_2 serum antibodies were not present in 77 various control patients ($p<0.05$) (6). A subset of CFS may be a prolonged infectious mononucleosis-like syndrome caused by HCMV(7-9).

The EBV IgM serum antibody to viral capsid antigen (VCA) tested by ELISA methods is diagnostic for EBV infectious mononucleosis (10-12). Studies to find a single virus etiology for CFS have been unsuccessful (13,14) and subset analysis may be necessary. Here, we assay several EBV and HCMV serum antibodies in 58 CFS patients and 68 control subjects. We report a subset of 33 CFS with IgM serum antibodies to EBV VCA. The IgM EBV VCA serum antibodies were not present in controls.

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Patients and Methods

CFS patients and controls. From January 1998 to June 2001, CFS and control patients from middle-to-upper-middle socioeconomic classes were seen at a single clinic. CFS patients met US Centers for Disease Control and Prevention criteria for diagnosis of CFS (1,2). At initial clinic visits blood for assays of serum antibodies were taken. Criteria for patient selection, study setting and clinical variables are similar to earlier reports (15,16). After serological testing, patients in CFS groups A and B were separated into subsets. Every 6-12 weeks CFS patients were re-tested. There were 33 CFS patients in subset Group A and 25 CFS patients in Group B, and 68 non-CFS controls. Control patients were matched in time, place and, as far as possible, sex and age. For inclusion in this study, at initial clinic visits, CFS patients in both Groups A and B had, additionally, oscillating abnormal T waves at Holter monitoring (15,16). This additional criterion for CFS excluded no patient who met the original CDC criteria.

CFS patients in subset Group A had positive tests for EBV VCA IgM serum antibody, but CFS patients in subset Group B had negative serum EBV VCA IgM antibody titers. Group B CFS patients had positive serum antibody titers to EBV, Early Antigen,

Diffuse (EA, D). Only after initial serological testing did CFS patients in subset Groups A and B become distinguishable. Serum assays for EBV and HCMV antibody titers were performed by one of us (SHB) blindly, with no clinical knowledge of the patient or subject.

Clinical groups.
1) Group A subset consisted of 33 CFS patients with positive tests for serum EBV VCA IgM antibodies.
2) Group B subset consisted of 25 CFS patients clinically indistinguishable from Group A CFS patients who had positive serum EBV, EA antibody titers, but negative tests for serum EBV VCA IgM antibodies.
3) Group C control consisted of 50 random time matched random patients from a commercial clinical laboratory (Biotech Inc., Farmington Hills, Michigan, USA). The clinical status of these patients was not known to us.
4) Group D control consisted of 18 non-fatigued patients who were cytomegalovirus (HCMV(V) serum antibody-positive patients to conventional structural HCMV(V) antigen. These patients were miscellaneous patients from this clinic.

Serological testing. Antibodies to EBV Viral Capsid Antigen IgM (17,18). Serum antibodies to the EBV and HCMV CFS subsets were tested in patients and controls. Antibodies to EBV-IgM evaluate VCA p18 peptide, a defined VCA-specific marker protein, which is utilized in the ETI-EBV-M reverse assay (DiaSorin, Inc., Stillwater, MN, USA). This peptide consists of 56 amino acids of the BFRF, encoded VCA and contains immunodominant epitopes for VCA. This EBV antigen detects VCA antibodies with sensitivity and specificity. The ETI-EBV-M reverse kit utilizes the enzyme-linked immunosorbent assay (ELISA) based on the antibody capture technique. Diluted patient serum is incubated with mouse monoclonal antibody against human IgM (μ chain specific) bound to solid surface microtiter well. Patient IgM is captured by the surface-bound antibody. The patient anti-VCA-IgM antibodies are then detected and bound by viral capsid antigen p18 peptide antigen which is linked to an anti-p18 monoclonal antibody conjugated to horseradish peroxidase. Bound horseradish peroxidase is reacted with chromogen/substrate solutions resulting in development of color. The absorbance of the solution measured at 450nm is related to the concentration of IgM to Epstein-Barr viral capsid antigen presented in the reaction solution.

Antibodies to EBV-IgG early antigen (diffuse) (17,18). We have used ETI-EA-G Kit (DiaSorin) ELISA testing for quantitative detection of IgG antibodies to EBV Early Antigen Diffuse (EBV-EA-D). Diluted serum was incubated with recombinant EA (D) peptide bound to the solid surface of a microtiter well. The ETI-EA-G assay uses an EA (D) 47 KD, a recombinant polypeptide. The recombinant EA (D) proteins were expressed in *E. coli* and purified. If EA (D) antibodies are present in the patient's serum, antigen-antibody complexes are formed. The complexes bind with horseradish peroxidase-labeled antihuman IgG which react with the addition of chromogen, resulting in color development. The absorbance of the solution, measured at 450nm, is proportional to the concentration of IgG antibodies to EBV, EA, (D) present in the reaction solution.

Rheumatoid factor (RF). The rapid latex agglutination test (Immunostics, Inc., New Jersey, NJ, USA) was used. Polystyrene latex particles were coated with a layer of absorbed human gamma globulin (and RF). The RF in the test serum reacts with the coating material causing agglutination of latex particles. Distinct agglutination indicates a RF content of more than 20 IU/ml in the undiluted sample.

HCMV antigens (6). Four HCMV antigens were used. The HCMV antigen V used for ELISA tests is a lysate of HCMV strain AD69 grown in human fibroblasts in tissue culture. The HCMV virus particle VP antigen is HCMV (V) purified by sucrose density gradient centrifugation. The p52 antigen is a recombinant protein containing the full UL44 gene product. UL44 is a HCMV polymerase-processing factor. CM_2 is a recombinant protein chimeric antigen fused to N and C termini, containing part UL44 and part UL57 gene products. UL57 is a SS DNA-binding protein (19-25). UL44 encodes ICP36 protein family and UL57 encodes the major DNA-binding protein. Antigens (EBV and HCMV) were obtained from DiaSorin, Inc. (1990 Industrial Building, PO Box 285, Stillwater, Minnesota 55082, USA).

HCMV ELISA testing (25-28). Cytomegalovirus antigens were evaluated with human sera from different sources (see clinical groups) by ELISA testing (DiaSorin) and scattered light technology Copalis Multiplex assay (DiaSorin). ELISA testing was done using DiaSorin kits for HCMV IgG and HCMV IgM. The HCMV IgG kit contains purified HCMV strain AD-169 antigen-coated wells. The HCMV IgM ELISA was a μ-capture assay with wells coated with anti-human IgM antibody to same strain AD-169. Sera were diluted 1:10 with sample diluent and incubated for one hour at 37°C. The wells were washed three times in washing buffer and bound HRP label was detected with 3.3', 5. 5'-tetramethylbenzidine as substrate for 30 minutes in the dark, after which the color reaction was stopped by the addition of stop solution as recommended by the manufacturer's manual. The absorbance was measured at 450/650 nm using Biotech reader (Biotech Clinical Laboratories, Inc., Farmington Hills, MI, USA).

Copalis HCMV multiplex assay (6). The Copalis HCMV multiplex assay (DiaSorin) is a light scattering automated computerized methodology that quantitatively measures the antibodies to three HCMV antigens: p52, CM_2 and VP. Both CM_2 and p52 antigens detect IgM antibodies.

Statistical analyses. Among the four patient groups demographic differences were determined by analysis of variance for age and Chi-square analysis was used for gender distribution. Difference in duration of CFS for the two CFS patient groups was determined by a *t*-test. Differences in EBV VCA IgM; EBV EA(D); HCMV (V) IgG; HCMV (VP); HCMV p52 IgM; and HCMV CM_2 IgM levels among the four patient groups were determined by analysis of variance; when appropriate pair wise comparisons were performed using Tuckey-Kramer HSD (global alpha=0.05). The percent of patients who were negative for EBV VCA IgM and EBV EA were examined using Chi-square analysis.

Results

Demographic characteristics. The age and sex of the two CFS subsets (Groups A, 45.2+13.9* yrs., 69% ♀ and B, 42.0±10.1 yrs., 80% ♀) and the non-CFS controls (Group C, 47±19.0 yrs., 66% ♀ and Group D, 68±13.6 yrs., 83% ♀) are shown (Table I). The ages of the CFS patients (Groups A, B) and control Group C were not statistically different. Control

Table I. *Demographic characteristics of CFS patients with elevated serum antibody titers to Epstein-Barr virus (EBV) viral capsid antigen (VCA) IgM and/or Early Antigen (diffuse, EA) and controls.*

Group	Number of Patients	Age (years)	Sex (% ♀)	Duration of CFS (years)
A) CFS patients EBV, VCA IgM-positive subset	33	45.2±13.9*	69%	3.7±4.5*
B) CFS patients EBV VCA, IgM-negative, EBV, EA-positive subset	25	42.0±10.1	80%	2.3±1.8
C) Random control patients from a clinical laboratory	50	47±19.0	66%	–
D) Random non-fatigued control patients from a physician's practice	18	68±13.6†	83%	–
Test (*p*-value)	–	ANOVA (<0.001)	Chi-square (0.39)	T-test (0.17)

* mean±SD
†Group D is significantly different from Groups A, B and C

Table II. *Epstein-Barr virus serum antibody titers in two subsets of CFS patients and controls.*

Group	Number of Patients	Serum antibody titers: EBV VCA, IgM (neg <20)	EBV EA (D) (neg<20)
A) CFS patient subset	33	62.3±8.3 * (100%)†	55.4±9.6 (79%)
B) CFS patient subset	25	6.8±0.7 (0%)	49.5±6.3 (100%)
C) Random control patients from clinical laboratory	50	10.4±2.4 (8%)	23.7±6.0 (30%)
D) Random non-fatigued control patients from physician's practice	18	14.1±9.4 (6%)	39.5±8.8 (50%)
Test (*p*-value)		Chi-square+ (< 0.0001) ANOVA (< 0.0001)	Chi-square++ (<0.0001) ANOVA (0.008)

* Mean±SEM
† Number of patients in group with positive tests
\+ Group A is significantly different from Groups B, C and D
++ Group A is significantly different from Group C

Group D (random non-fatigued patients from a physician's practice) was older ($p<0.001$). Female preponderance of CFS patients is usual (5,7, 15). Subjects in control Groups C and D were mostly women (66% and 83%, respectively). When first seen, Group A CFS patients had already been ill with CFS for a mean ±SD of 3.7±4.5 years, while Group B CFS patients had been ill (2.3±1.8 years).

EBV, VCA IgM and EBV, IgG EA (D) serum antibody titers (Table II). The mean serum EBV VCA IgM antibody titer in CFS subset Group A was (62.3±8.3, Mean±SEM). This value is different from EBV VCA IgM serum antibody titers in CFS subset Group B (6.8±0.7) and control Groups C and D (10.4±2.4 and 14.1±9.4), $p<0.0001$). On the other hand, serum EBV EA (D) IgG antibody titers were not different in CFS Groups A (55.4±9.6, Mean±SEM); CFS Group B (49.5±6.3) and control Group D (39.5±8.8) ($p>0.05$). Control Group C (random patients from a clinical laboratory) had a lower mean EBV EA serum antibody titer (23.7±6.0). Tests for rheumatoid factor were performed in 25 patients in subset CFS Group A. Each test was negative.

Longitudinal measures of serum antibody titers to EBV VCA IgM in subset group A. We repeated serum antibody titers to EBV VCA IgM from Group A subset every 3 months in 12 CFS patients. In the 12 CFS patients, serum IgM EBV VCA mean antibody titers were: initial visit: 90±15 (neg.<20); three months, 98.4±13.6; six months, 96.2±16.4; nine months, 112±24.7; 12 months 97±25.7; 15 months, 86.7±20; 18 months, 119±14.9; and 24-42 months, 110±31 (negative<20).

HCMV serum antibody titers (Table III). Serum HCMV antibodies in CFS subsets (Groups A and B) and control Groups C and D were not different. In patients in Groups A-D there were uniformly negative serum HCMV IgM (V) antibody titers. Serum HCMV IgG (V) antibody titers were Group A (57.5±9.7, negative<18); Group B (40.8±10); Group C (56.5±7.4) and Group D (79.0±8.9, negative<18. Serum HCMV IgG (VP) antibody titers were Group A (4.2±1.0); Group B (3.3±1.1); Group C (4.3±1.2); and Group D (5.5±1.8), negative<1. Serum HCMV p52 IgM antibody titers were Group A (0.24±0.12; Group B (0.17±0.08); Group C (0.16±0.04), and Group D (0.22±0.14). Serum HCMV CM_2 IgM antibody titers were Group A (0.22±0.05); Group B (0.19±0.05); Group C (0.32±0.04); and Group D (0.27±0.06), negative<1.

Discussion

These data indicate that IgM EBV VCA serum antibodies are present and may persist for several years in a significant subset of CFS patients (here, 33 CFS patients). Other CFS patients with EBV infections do not have IgM EBV VCA serum antibodies. Serum antibody titers to EBV EA (D) do not differentiate CFS patients from controls (14). The EBV EA (D) antibody is an "early" polymeric nonstructural antibody to at least 12 early EBV polypeptides 17-85 KD_a, BMLF1, BM-RF1, BAMH1-0 and BMRF1 which are transactivators for the lytic cycle (29). The EBV VCA is a "late" gene product of the EBV genome.

Table III. *Cytomegalovirus (HCMV) serum antibodies in subsets of CFS patients and controls.*

	CFS patients		Control patients		
Group Serum Antibody Titers†	A (33)* EBV, VCA, IgM-positive	B (25) EBV, VCA, IgM-neg., EA-positive	C (50) Random controls clinical laboratory	D (18) Non-fatigued control patients	Test (*p*-value)
HCMV (V) IgG (neg. <18)	57.5±9.7** (31)	40.8±10 (25)	56.5±7.4 (50)	79.0±8.9 (18)	ANOVA 0.12
HCMV (VP) (neg. <1)	4.2±1.0 (32)	3.3±1.1 (25)	4.3±1.2 (50)	5.5±1.8 (18)	ANOVA 0.80
HCMV p52 IgM (neg.<1)	0.24±0.12 (32)	0.17±0.08 (25)	0.16±0.04 (50)	0.22±0.14 (18)	ANOVA 0.88
HCMV (CM_2) IgM (neg. <1)	0.22±0.05 (32)	0.19±0.05 (25)	0.32±0.04 (50)	0.27±0.06 (18)	ANOVA 0.19

* Number in parenthesis is a number of patients in CFS Subset Group or Control Group
† HCMV (V) IgM serum antibody titers were uniformly negative in all patients from Groups A-D
** Mean±SEM

The positive IgM EBV VCA serum antibody titers which were present in the 33 CFS patients in the Group A subset of this report were not present in blinded tests of 25 CFS patients in the Group B subset or in 68 controls. Thus, serum antibody to EBV VCA IgM may define a "new" CFS subset. This new CFS subset (Group A) is different from the earlier HCMV CFS patients with IgM serum antibodies to the nonstructural gene products p52 and CM_2 (UL 44 and UL 57). Here, in studies in both CFS and control groups, tests of several HCMV serum antibodies were not statistically different. These present data describing the EBV VCA IgM subset do not address, nor do we yet know, the relative number of CFS patients who have positive EBV VCA IgM serum antibodies.

Epstein-Barr virus VCA IgM serum antibody is diagnostic of infectious mononucleosis (IM) (30-35). In adolescents with IM EBV VCA IgM serum antibody disappears within 3-6 months (11). Apparently, a latent inactive state of Epstein-Barr virus cannot be established in the Group A CFS subset (36). The EBV VCA antigen is not neutralizing. Anti-membrane EBV antibody (MA) is protective (37). When EBV MA is present, EBV VCA IgG antibody is always present. The EBV viral capsid antigen represents an incomplete virion. Viral tegument proteins, viral genome fragments, empty virus capsids, non-enveloped double-stranded DNA-containing capsids and empty virions are other forms of non-assembled EBV (Figure 1) (37). Subset analysis such as that which we have outlined with a HCMV subset and, here, with a further EBV subset with IgM antibodies to VCA, may further differentiate CFS patients into clinically important groups.

Since the work of Robert Koch who revolutionized scientific medicine, proving the singular cause of tuberculosis, evidence-based medicine has sought one infectious aetiology for a single "clinical" disease (38). A herpesvirus etiology of CFS may be tenable, and subset analysis of groups of CFS patients may facilitate our understanding of the CFS conundrum. The present work adds new diagnostic direction for CFS. All CFS patients in this study were seen by the same infectious diseases physician (AML), and EBV, HCMV virus titers were done in this research laboratory by one of us (SHB) with no knowledge of the place of the patient in the study. We hope that previous work done seeking a "single" Epstein-Barr virus etiology for CFS will not prejudice the data of this report. A subset analysis among CFS patients appears essential to a specific diagnosis for the CFS patient (6-7).

This work may be aided by the contribution of the polymath Gunther Stent who defines a hypothesis as "scientific prematurity" if it cannot be connected by a series of simple logical steps to canonical knowledge of the time (39). Hook suggests that investigators with "premature scientific hypothesis" according to Stent's definition attempt to (1) link the premature findings to canonical knowledge and, in fact by linking it, perhaps alter a canon in some respect (40). Alternatively, the "premature" investigator can attempt (2) to harness the alleged findings for some practical use. In the present case, our use of the hypothesis "incomplete herpesvirus multiplication" may fit the Stent definition of a premature scientific claim. This CFS hypothesis is based upon a) the unique presence of HCMV IgM serum antibodies to the nonstructural gene products of p52 and CM_2 (UL44 and UL57) in a unique subset of CFS patients,

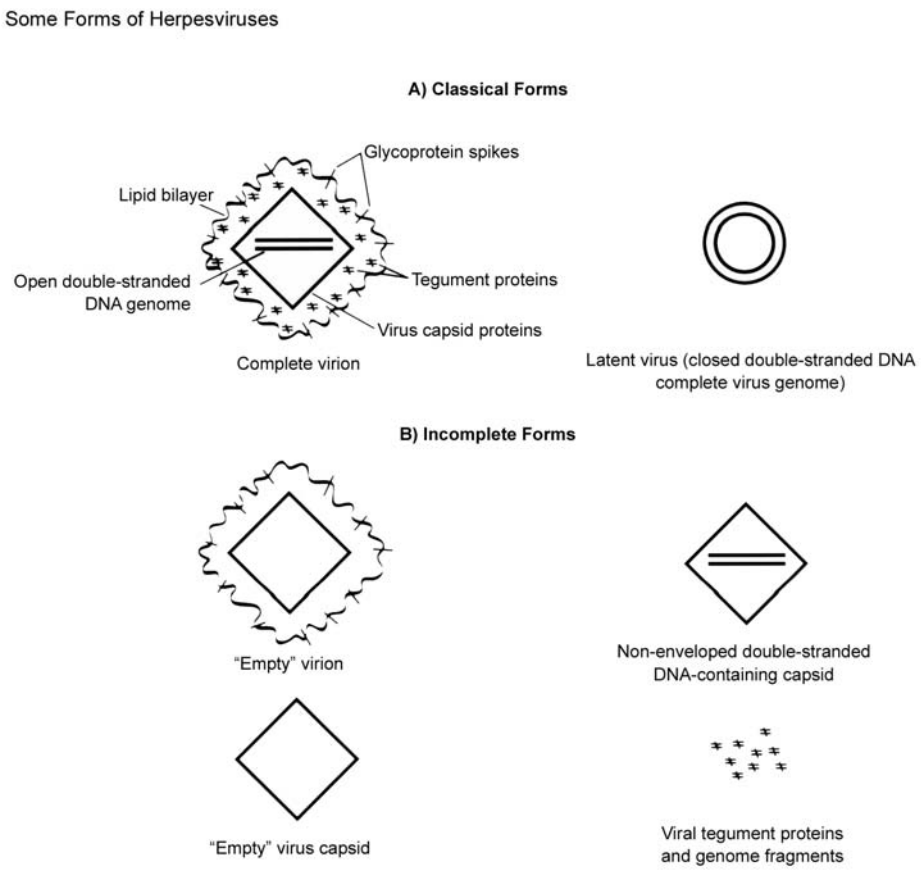

Figure 1. *Possible herpesvirus forms, complete and incomplete are shown. The "complete" herpesvirus virion (EBV, a beta herpesvirus; HCMV, a gamma herpesvirus) is shown with component parts: a) lipid bilayer, b) glycoprotein spikes, c) tegument proteins, d) virus capsid proteins and e) open double-stranded DNA complete virus genome. Latent herpesvirus is the complete genome as a closed non-integrated inactive circular episome situated in the nucleus of an infected cell (e.g. EBV (B-lymphocyte), HCMV (monocyte-macrophage). "Incomplete" unassembled herpesvirus forms are empty virion (no DNA genome); non-enveloped double-stranded DNA-containing capsid; empty virus capsid and viral tegument proteins and genome fragments.*

(6) and b) the unique prolonged presence of EBV IgM serum antibodies in a separate CFS subset (this report). Clearly, (2), if subset directed antiviral therapy in controlled clinical trials is beneficial to the CFS patient for whom there is currently no useful therapy then, (5) attention to this possibly "premature scientific hypothesis" may be warranted. As a corollary to the above argument, incomplete non-assembled herpesviruses are regularly seen at electron micrographic review of herpesvirus multiplication in tissue culture (37). No clinical significance to incomplete virions has yet been ascribed.

Incomplete herpesvirus multiplication indeed "may" be a variable intermediate involving early: early and middle: and early, middle and late genes in an orderly sequential pattern. Incomplete herpesvirus multiplication, latency and complete virus multiplication may all be commonplace as stages of the life cycle between the latent state (no virus gene product) and complete virion. Finally, except as reported in the CFS subset, EBV IgM VCA serum antibodies are not demonstrable in the general population and their "presence" is virtually diagnostic of acute infection (41). The prolonged finding of VCA IgM serum antibodies in a significant subset of CFS patients may be etiologic in these specific patients (42).

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