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**IgM Serum Antibodies to Human Cytomegalovirus
Nonstructural Gene Products p52 and CM₂ (UL44 and UL57)
Are Uniquely Present in a Subset of Patients
with Chronic Fatigue Syndrome**

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Abstract

Human cytomegalovirus (HCMV) IgM serum antibodies to two nonstructural gene products UL44 and UL57 (p52 and CM₂) were assayed in patients with the diagnosis of the chronic fatigue syndrome (CFS) according to criteria established by the US Centers for Disease Control and Prevention. A subset of 16 CFS patients demonstrated HCMV IgG, but no HCMV IgM serum antibodies to conformational structural HCMV antigens (designated, V). By convention, these findings are interpreted to indicate only a remote HCMV infection. However, HCMV IgM

p52 and CM₂ antibodies were uniquely present in these 16 CFS patients. Other CFS patients with similar HCMV (V) IgG antibodies (18 patients), non-fatigued HCMV (V) IgG-positive control patients (18 patients), random HCMV (V) IgG-positive control patients from a clinical laboratory (26 patients), and non-fatigued HCMV (V) IgG-negative control patients (15 patients) did not have HCMV, IgM p52 or CM₂ serum antibodies ($p < 0.05$). Control HCMV (V) IgG-positive patients had no serum IgM HCMV (V) antibodies to conventional structural HCMV (V) antigen. Thus, 77 various control patients did not contain IgM p52 or CM₂ serum antibodies. The presence of IgM p52 and/or CM₂ HCMV serum antibodies in this subset of CSF-specific patients may detect incomplete HCMV multiplication in which a part of the HCMV protein-coding content of the HCMV genome is processed, but remains unassembled. These findings suggest that the presence of HCMV IgM p52 and CM₂ serum antibodies may be a specific diagnostic test for the diagnosis of a subset of CFS patients. Further, these data suggest an etiologic relationship for HCMV infection in this group of CFS patients.

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Chronic fatigue syndrome (CFS) is a disorder of unknown cause characterized by life-altering debilitating fatigue, with a marked restriction of exercise and nonexertional activities of daily living. The Centers for Disease Control (CDC) and Prevention has established a case definition for CFS (1, 2). CFS is a major public health problem affecting all socioeconomic groups in the US. CFS is prevalent among young adult women in their prime productive years of age, 40-49. There are approximately 522 CFS women and 249 CFS men per 100,000 US population (836,000 people, based on the US population count of 198,107,000 adults age 18 years and older) (3). Patients with CFS have characteristic life-altering symptoms and laboratory findings, including cognitive deficits, immune disturbances (4-9), elevated mean values for bioactive 2-5A and RNase L activity in peripheral blood mononuclear cells (10), brain abnormalities (11), and positive tilt-table tests (12). Cardiac involvement in CFS is detected by abnormal 24-Hr. ECG monitoring, abnormal cardiac wall motion at stress exercise, and pathologic changes of cardiomyopathy at right ventricular endomyocardial biopsies (13-15). A virus etiology of CFS is suspected and extensive investigations have been reported, but a virus causality has not been proved (16-24). There are no virologic tests to specifically diagnose CFS.

We have studied IgM serum antibodies to specific gene products of the human cytomegalovirus (HCMV). Human cytomegalovirus is a 230 Kbp double-stranded linear DNA genome which is

packaged within a 100 nm diameter icosahedral capsid which in turn is surrounded by a poorly characterized protein structure known as the tegument or matrix (25). Several HCMV-specific capsid proteins and enveloped glycoproteins (GP) have been characterized. Enveloped glycoproteins assayed here are referred to as HCMV (V) include gp UL55 (gB), gp UL75 (gH), gp UL100 (gM), and gp UL4 (gp48). Among the capsid proteins, here called HCMV (VP), are the 155 KDa major capsid protein (MCP), (ORF) UL86 and the 34 KDa minor capsid protein UL46. UL44 and UL57 are processing proteins of the HCMV tegument (26, 27, 28).

We report a subset of 16 CFS patients with specific IgM serum antibodies to recombinant fusion antigens of HCMV to two nonstructural processing gene products, UL44 and UL57 (p52 and CM₂) of the HCMV tegument. These IgM HCMV nonstructural antibodies were not present in control subjects (77 controls).

Patients and Methods

CFS Patients and Controls. There are 16 patients in the study group and 77 controls. CFS patients met CDC criteria for CFS (1, 2). For inclusion in this study, CFS patients had the added criteria of abnormal Holter monitoring (13, 15) and the presence of serum IgG HCMV (V) at their initial clinic visits. CFS patients in Groups A and B are indistinguishable clinically. Approximately 50% of the adult US population contain serum HCMV (V) IgG antibodies (29). Only after tests described here do Group A and Group B become distinguishable. From November 1999 to September 2000, CFS and control patients were seen in a clinic setting from the middle to upper-middle socioeconomic classes (13, 15). Bloods for assays of serum antibodies were taken at initial visits. Assays for serum HCMV antibodies were performed by one of us (SHB) blindly with no clinical knowledge of the patient or subject. Criteria for patient selection, study setting, and clinical variables have been described (13, 15). The results of these serum assays reported here were repeatedly reproducible at blinded testing of repeat specimens of bloods from the patients in this study.

Clinical Groups

- (1) Group A subset consisted of 16 CFS patients with serum HCMV (V) IgG antibodies.
- (2) Group B subset consisted of 18 CFS patients clinically similar to Group A CFS patients who also had serum HCMV (V) IgG antibodies.
- (3) Group C control consisted of 18 HCMV (V) IgG-positive non-fatigued miscellaneous patients from this clinical practice.
- (4) Group D control consisted of 26 HCMV (V) IgG-positive patients from a commercial clinical laboratory (Biotec, Inc., Southfield, Michigan). The clinical status of these patients was not known.
- (5) Group E control consisted of 15 HCMV (V) IgG-negative *non-fatigued* patients from this clinical practice.

HCMV Antigens

Four HCMV antigens were used. The HCMV antigen V used for ELISA tests is a lysate of HCMV strain AD69 grown in human fibroblast 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 processivity factor. CM₂ 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 (30-35). The UL44 and UL57 antigens are pieces of the replicatory machinery of HCMV. UL44 encodes ICP36 protein family, and UL57 encodes the major DNA-binding protein (36). Antigens were obtained from DiaSorin, 1990 Industrial Building, PO Box 285, Stillwater, Minnesota 55082.

Serology Testing

ELISA Testing (35-39)

In this study, HCMV antigens were evaluated with human sera from different sources (see clinical groups) by using ELISA testing (DiaSorin, Stillwater, MN) and scattered light technology Copalis Multiplex assay (DiaSorin). ELISA testing was done using DiaSorin kits for HCMV IgG and HCMV IgM. HCMV IgG kit contains purified HCMV strain AD-169 antigen-coated wells. HCMV IgM ELISA was Φ -capture assay with wells coated with anti-human IgM antibody to same strain AD-169. Sera were diluted 1:101 with sample diluent and incubated for 1 h at 37EC. After washing, HRP-conjugated monoclonal to human IgG (for IgG) or mouse monoclonal anti-HCMV HRP-conjugated antibodies (for IgM) were added for 1 h at 37EC. 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 Inc.).

Copalis HCMV Multiplex Assay (35-38)

The Copalis HCMV multiplex assay (DiaSorin Inc.) is used for detection of both acute infection and convalescent immune status to HCMV. This is a light scattering automated and computerized methodology that quantitatively measures the antibodies to three HCMV antigens p52, CM₂, and VP. Both the CM₂ and the p52 antigens detect early phase IgM antibodies. Reactivity to HCMV VP is characteristic of prior infection.

Statistical Methods

Gender composition among the five patient groups was examined by chi-square analysis. One-way analyses of variance (F ratio $p < .05$) and Tukey's HSD (global alpha < 0.05) were used to examine differences among the patient groups for age, HCMV IgG (V), HCMV (V) IgM, HCMV (VP), HCMV CM₂ and HCMV p52. The Tukey's HSD is a post hoc comparison to determine which group means differ significantly.

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Results

Demographics. The age and sex of the two CFS subsets (A, B) and the non-CFS controls (C, D, E) are shown (Table I). The ages of the CFS patients and controls were similar, except that Group C non-fatigued controls were approximately 10 years older ($p < 0.05$). The mean age of CFS patients was 49 years. Group A and Group B CFS patients were 87.5% and 88.9% women, respectively. Female preponderance in the sex distribution of chronic fatigue syndrome patients is usual (13). Subjects in control Groups C and D were also mostly women (87.5% and 69.2%, respectively ($p > 0.05$)). Non-fatigued persons from Group E were 66.4% men ($p < 0.05$).

HCMV IgG Serum Antibodies, V, Antigen. (Table II, Figure 1) The mean HCMV (V) ELISA IgG titers were highest in the CFS subsets (A, $122 \nabla 9.5$; B, $115 \nabla 8.9$), followed by the control subjects in Group D ($99 \nabla 7.5$) whose clinical status was unknown. Non-fatigued patients (Group C) had lower HCMV IgG titers by ELISA tests assayed with the V antigen ($79 \nabla 8.9$). Non-fatigued control patients Group E had negative HCMV (V) ELISA IgG serum titers (e.g., $8 \nabla 9.8$, negative < 18) (Table II, Figure 1). In these HCMV assays a serum antibody titer of >18 is positive. For HCMV (V) IgG, Group A and Group B is different from Groups C and D ($p < 0.05$). Groups A and B are not statistically different from Group D ($p > 0.05$).

HCMV IgG Serum Antibodies, VP, Antigen. (Table II, Figure 1) Assays for HCMV, VP antigen, yielded the HCMV serum antibody titers in CFS Group A ($14.87 \nabla 1.89$), CFS Group B ($8.97 \nabla 1.79$), Control Group C ($5.5 \nabla 1.79$), Control Group D ($6.3 \nabla 1.49$) and Control Group E ($0.12 \nabla 1.96$). For these HCMV assays, a serum antibody titer of >1 is positive. For HCMV (VP), Groups A and B are similar ($p > 0.05$). However, Group A is different from Groups C, D, and E ($p < 0.05$).

HCMV IgM Serum Antibodies, V Antigen. (Table II, Figure 2) CFS subsets

(A, B, $8.64 \nabla 0.7 : 6.4 \nabla 0.6$, respectively) and Control Groups (C, D, E, $5.1 \nabla 0.6 : (6.4 \nabla 0.5)$ and ($4.9 \nabla 0.7$), respectively) were all negative (< 18) for HCMV IgM antibodies to HCMV (V) antigen. These results “suggest” that the HCMV IgG antibodies to both V and VP antigens represent “past experience,” but offer no evidence of current HCMV multiplication. The single exception to this statement was a 39-year-old woman in CFS subset A with a positive HCMV IgM serum titer to HCMV, V which was 24 (< 18 , negative), (Table II, Figure 2).

HCMV IgM Serum Antibodies, p52 and CM₂ Antigens. In these assays, a value >1 is positive. HCMV p52 and CM₂ IgM antibodies were strikingly positive in CFS patients in subset Group A (p52, $2.05 \nabla 0.15$: CM₂, $3.13 \nabla 0.48$). All CFS patients who were positive for p52 and CM₂ IgM antibodies were similarly positive for IgG antibodies to VP antigen (Table II, Figures 1 and 2). HCMV IgM serum antibody titers to p52 and CM₂ were, to the contrary, negative in CFS subset B (p52, $0.23 \nabla 0.14$: CM₂, $0.21 \nabla 0.06$) and Control Groups C (p52, $0.22 \nabla 0.14$, CM₂, $0.27 \nabla 0.06$), D (p52, $0.27 \nabla 0.11$: CM₂, $0.43 \nabla 0.06$) and E (p52, 0.09∇

0.15 : CM₂, 0.12 ∇ 0.02). There were, however, two subjects from a total of 26 subjects from Control Group D random, HCMV (V) IgG-positive persons of “unknown” clinical status from Biotec Clinical Laboratory who were HCMV, IgG (V) and (VP) positive, with positive IgM p52 (1.25 : 1.25) and IgM CM₂ (1.12 : 1.33) serum titers. The clinical status of these two patients is unknown. The HCMV IgM p52 and CM₂ serum antibodies appear to be unique to CFS subset A (p < 0.05).

Discussion

These data indicate that IgM HCMV antibodies to UL44 and UL57 gene products are present in a subset of CFS patients with prior infection with HCMV, as indicated by persisting HCMV (V) IgG serum antibodies, (e.g., Group A). However, other CFS patients, who, too, have HCMV (V) IgG serum antibodies are not positive for CM₂ and p52. This presence of HCMV (V) IgG serum antibodies to structural HCMV antigens is evidence of past HCMV infection. CFS patients (Group A, Group B) do not have active HCMV infection by attempts at virus isolation from buffy coats of blood, urines, or cardiac biopsies. These studies are negative (40, 41). In these latter CFS patients, two of 11 cardiac biopsies, along with similar positive results in several control patients with known cardiac disease, yielded HCMV DNA by polymerase chain reaction (41). The IgM p52 and CM₂ HCMV antibodies were not present in control non-fatigued patients.

The IgM p52 and CM₂ HCMV assays are highly specific, sensitive assays of HCMV multiplication (31). Cytomegalovirus IgM CM₂ and p52 IgM antibodies are rarely seen in patients with human immunodeficiency virus infections or other immunocompromised patients where HCMV complete virion (V antigen) virus titers are high, and HCMV is easily detected in blood (33, 34). Unlike the immunocompromised patient, the CFS patient who is *not* immunocompromised appears able to prevent the careful, orderly complex assembly of the 230 proteins encoded in the HCMV DNA genome of approximately 240,000 base pairs (25).

HCMV incomplete virus multiplication may be present in Group A CFS patients with positive IgM serum antibody titers to HCMV p52 and/or HCMV CM₂. Serum assays for HCMV p52 and CM₂ IgM antibodies may recognize these unassembled specific HCMV gene products in this CFS subset Group A. UL44 and UL57 proteins are unassembled replication proteins vital to the HCMV virion. The data suggest that in the CFS patient, the unassembled UL44 and UL57 gene products may contribute to an intracellular disorganization intrinsic to the pathology of CFS. Further, in human cardiac tissues, this may be a non-inflammatory pathologic process (40).

To be sure, it is possible, and we believe probable, that the CFS syndrome is caused by one or several herpesviruses (HCMV, Epstein-Barr virus, human herpesvirus 6 singly, or, in co-infections in the same affected patient (28). This variable herpesvirus causality may account for the differences between the findings in CFS subsets, Groups A and B, reported here. Moreover, the IgM serum antibody response to UL44 and UL57 (p52, CM₂) suggests that these specific HCMV processing proteins are transcribed, but are not assembled into the complete HCMV virion in CFS subset, Group A, reported here. When HCMV virions are completely assembled, UL44 and UL57 internal tegument proteins are hidden within the virus and do not induce serum antibodies (28). Martin and co-workers have previously recognized in bloods of several CFS

patients naked HCMV processing genes with partial homology to HCMV UL34 and UL35 (42, 43). These authors have further suggested that by deleting HCMV genes UL55 and UL83 which are the major antigenic targets for T-cell immune responses, intracellular survival of the dispersed HCMV proteins may be facilitated (44). Martin has named such a non-immunologically recognized virus a “stealth virus” (43). No prior association of IgM antibodies to UL44 and UL57 and CFS patients has been made, to our knowledge (41).

Primary HCMV infection in an immunologically intact human is followed by an explosive synthesis of fully assembled and partially unassembled HCMV gene products (28, 31). Following initial primary infection with HCMV, IgM anti-p52 and anti-CM₂ antibodies are transiently present along with IgM and later, IgG antibodies to HCMV complete virions here called V. As reported here and elsewhere, IgM HCMV (V) antibodies, and rises in HCMV, IgG (V) antibodies do not commonly occur in the CFS patient with prior HCMV infection (28). In the bloods and cardiac tissues of the CFS subset Group A patient with positive HCMV IgM p52, CM₂ antibody titers (27), we could not isolate HCMV in tissue cultures. We found no rises in IgM or IgG antibodies to HCMV (V) (40). In other HCMV CFS patients, IgM antibodies to still other HCMV processing proteins of the HCMV tegument may also be present. Such patients may be represented by CFS patients reported here in Group B with equally high HCMV IgG (V) antibody titers as CFS patients in Group A, but negative IgM antibody titers to UL44 and UL57 (CM₂, p52). For the time, however, HCMV p52 and CM₂ IgM antibodies may be highly useful in virologically identifying a subset of CFS patients. This test clearly separated this CFS subset patient from controls. A specific virologic etiologic role for HCMV in the CFS patient in subset Group A is suggested.*

Table I Age and Sex of CFS Patients and Controls

Group +	Number of Patients	Age (Yrs) ++ (mean)	Sex
A) CFS-HCMV (V) p52, CM ₂ positive Subset	(16)	48.8 ∇ 3.9	87.5% &
B) CFS-HCMV (V) positive p52, CM ₂ negative	(18)	49.8 ∇ 3.7	88.9% &

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Group +	Number of Patients	Age (Yrs) ++ (mean)	Sex
Subset			
C) Non-fatigued HCMV (V) IgG positive Control patients	(18)	67.9 ∓ 3.7	87.5% &
D) Random HCMV (V) IgG Control patients from commercial clinical laboratory	(26)	54.4 ∓ 3.1	69.2% &
E) Non-fatigued HCMV (V) IgG negative Control patients	(15)	57.5 ∓ 4.1	33.6% &

- Patients in Groups A-D had HCMV (V) IgG, but no HCMV (V) IgM serum antibodies to structural HCMV antigens (V). Group E control patients had no HCMV (V) IgG or IgM serum antibodies to HCMV (V) structural antigens.

++ The ages of patients in Groups A and B are different from Group C ($p < 0.05$). The gender of Group E is different from that of the other Groups (A-D), $p < 0.05$. The standard errors were calculated within a pooled estimate of error variance.

Values are means and standard error.

Table II Cytomegalovirus Serum Antibodies in CFS Patients and Controls

CFS Subset Groups			Control Groups		
Serum Antibody Titers	A) CFS B HCMV (V) p52, CM ₂ positive Subset (16) ⁺	B) CFS B HCMV (V) positive p52, CM ₂ negative Subset (18)	C) Non-fatigued HCMV (V) positive Control (18)	D) Random HCMV (V) IgG positive Control from commercial clinical laboratory (26)	E) Non-fatigued HCMV (V) IgG negative Control (15)
HCMV (V) IgG (neg. <18)	122 ∇ 9.5 ⁺⁺	115 ∇ 8.9	79 ∇ 8.9	99 ∇ 7.5	8 ∇ 9.8
HCMV IgG (VP) (neg. <1)	14.87 ∇ 1.89	8.97 ∇ 1.79	5.50 ∇ 1.79	6.3 ∇ 1.49	0.12 ∇ 0.03
HCMV (V) IgM (neg. < 18)	8.64 ∇ 0.7	6.4 ∇ 0.6	5.1 ∇ 0.6	6.4 ∇ 0.5	4.9 ∇ 0.7
HCMV (p52) IgM (neg. < 1)	2.05 ∇ 0.15	0.23 ∇ 0.14	0.22 ∇ 0.14	0.27 ∇ 0.11	0.09 ∇ 0.15
HCMV (CM ₂) IgM (neg. < 1)	3.13 ∇ 0.48	0.21 ∇ 0.06	0.27 ∇ 0.06	0.43 ∇ 0.06	0.12 ∇ 0.02

⁺ Number in parenthesis is number of patients in CFS Subset Group or Control Group.

⁺⁺ Mean ∇ standard error of the mean. The standard error of the mean was calculated using a pooled estimate of error variance.

Statistical evaluations:

- a) HCMV (V) IgG B Groups A and B are different from Groups C and E (p < 0.05). Groups A and B are not statistically different from Group D.
- b) HCMV (VP) B Groups A and B are similar. Group A is different from Groups C, D, and E (p < 0.05).
- c) HCMV (p52) IgM B Group A is different from all other groups (p < 0.05).
- d) HCMV (CM₂) IgM B Group A is different from all other Groups (p < 0.05).

Legends to Figures

Figure 1. Serum HCMV IgG (V) antibody titers in the study groups are shown. There are no striking differences between Groups A-D with positive HCMV (V) IgG titers. Group E is the HCMV (V) IgG negative control.

Similarly, HCMV IgG (VP) antibody titers in the study Groups A-D are positive, but in Group E HCMV (VP) IgG titers are negative (see text for details).

Figure 2. Serum HCMV IgM antibody titers in the study groups are shown. Essentially, all groups have no HCMV IgM antibody titers to V (conformational structural epitopes). However, subset Group A CFS patients strikingly differs from all others groups by the presence of IgM CM₂ and p52 antibody titers. IgM CM₂ and p52 antibody titers are negative in Groups B-E ($p < 0.05$), (see text for details).

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