Human Herpesviruses 6 and 7 in Chronic Fatigue Syndrome: A Case-Control Study

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We conducted this study to determine whether infection with human herpesvirus (HHV) 6A, HHV-6B, or HHV-7 differed between patients with chronic fatigue syndrome and control subjects. We recruited 26 patients and 52 nonfatigued matched control subjects from Atlanta. Serum samples were tested by enzyme immunoassay for seroreactivity to HHV-6, and all were seropositive. Lymphocyte specimens were cocultivated with cord blood lymphocytes and assayed for HHV-6 and HHV-7; neither virus was isolated. Finally, lymphocytes were tested by use of 3 polymerase chain reaction methods for HHV-6A, HHV-6B, and HHV-7 DNA. HHV-6A or HHV-6B DNA was detected in 17 (22.4%) of 76 samples, and there were no significant differences (by matched analyses) between patients (3 [11.5%] of 26) and control subjects (14 [28%] of 50). HHV-7 DNA was detected in 14 subjects, and although control subjects (12 [24%]) were more likely than patients (2 [7.7%]) to be positive, the difference was not statistically significant. We found no evidence that active or latent infection with HHV-6A, HHV-6B, HHV-7, or any combination these 3 HHVs is associated with chronic fatigue syndrome.

Chronic fatigue syndrome (CFS) is distinguished by debilitating fatigue that lasts for ≥6 months and is accompanied by symptoms that include neurocognitive problems, musculoskeletal pain, sore throat, and lymphadenitis [1]. No physical signs or diagnostic test can confirm CFS, and its etiology remains unknown. Many patients with CFS report having a flulike illness just before the onset of CFS, and it is possible that CFS or a subset of cases of CFS is associated with viral infection [2]. Human herpesvirus (HHV) 6 and HHV-7 have been suggested as possible causes of CFS.

HHV-6 was first described in 1986 [3], and the existence of 2 variants (HHV-6A and HHV-6B) was documented in 1993 [4]. HHV-7 was described in 1990 [5]. HHV-6A, HHV-6B, and HHV-7 are members of the Roseolovirus genus in the subfamily Betaherpesvirinae. The viruses share similar cell tropism and can infect CD4+ T lymphocytes and salivary glands in vivo [6, 7].

The results of seroepidemiological studies have consistently shown that as many as 83% of children are infected with HHV-6 by age 13 months and that ≥95% of the population aged ≥2 years is seropositive [6]. Approximately 70% of children become infected with HHV-7 in the first 5 years of life, usually between the ages of 6 months and 2 years. Of the adult population, ~90% is seropositive for HHV-7 [7]. HHV-6B causes exanthema subitum (roseola) and other acute febrile illnesses in young children [8, 9]. The clinical spectrum of HHV-6A and HHV-7 infections in children is not well described, and causal associations with disease in adults remain speculative.

Because most people have been infected with HHV-6 and HHV-7 in early childhood, it is unlikely that CFS is precipitated by primary infection. Rather, reactivation of latent infections may precipitate CFS or (more likely) could reflect an underlying pathophysiology. These viruses are activated in immunocompromised hosts (HHV-infected individuals, renal-transplant recipients, and bone marrow–transplant recipients) and in patients with various lymphoproliferative diseases [6, 7].

Studies of HHV-6 and CFS have produced conflicting results. In part, this inconsistency reflects varying degrees of rigor in defining patients and selecting appropriate control subjects, a lack of unified standards for defining and subcategorizing patients with CFS (in terms of the type of onset, duration of illness, or current clinical status), and an inability to differentiate HHV-6A, HHV-6B, and HHV-7 either serologically or by virus detection. The objective of this study was to use sensitive assays to examine lymphocyte specimens to determine whether infection with HHV-6A, HHV-6B, or HHV-7 differed between patients with CFS and control subjects.

Subjects and Methods

Subjects. Subjects and study design have been presented in detail elsewhere [10, 11]. In brief, 26 patients with CFS (23 women and 3 men) were recruited from the Atlanta arm of the 4-city CFS
surveillance system of the Centers for Disease Control and Prevention (CDC) [10]. Patients met the 1988 research case definition for CFS [12] and had been ill for more than 10 years. Two age- (± 5 years), race-, and sex-matched nonfatigued control subjects were contacted by means of random-digit dialing of telephone numbers of individuals in the 5-county Atlanta area and were selected to be matched with each case patient. We interviewed the patients and the matched control subjects, and we obtained specimens from all individuals on the same day. No subject was taking immunosuppressive drugs.

Specimens. Specimen collection has been described in detail elsewhere [11]. In brief, blood specimens were collected in vacu- tainers containing either heparin or EDTA. Lymphocytes were iso- lated on lymphocyte separation medium (Organon Teknika, Dur- ham, NC), were washed, and were either cultured for herpesviruses (see below) or frozen at −70°C until testing was done for DNA.

Serological testing for HHV-6. Titers of antibody to HHV-6 were determined with the use of an ELISA using 96-well plates [13]; results have been reported elsewhere [14].

Procedures for isolation of HHV-6 and HHV-7. Each sample of peripheral blood lymphocytes was cocultivated with 3 independent phytohemagglutinin-stimulated cord blood lymphocyte cultures [15] and was passaged into independent fresh cord blood lymphocytes at 1-week intervals for 3 weeks. Cultures were monitored weekly for cytopathic effect and for the presence of HHV-6 and HHV-7 antigens with use of specific monoclonal antibodies [16]. (Antibodies to HHV-7 were kindly provided by Dr. Koichi Yamamishi, Osaka University, Osaka, Japan.) After 3 weeks, cultures were also tested for HHV-6A, HHV-6B [17], and HHV-7 [18] DNA by means of PCR amplification.

PCR amplification. Frozen lymphocytes were available from all 26 patients and from 50 of 52 matched control subjects and were screened by PCR assays for HHV-6A, HHV-6B, and HHV-7 DNA. For each subject, 1 × 10⁷ cells were suspended in 300 μL of lysis buffer (1% 10-lauryl ether, 10 mM Tris [pH 8.5], and 200 μg of proteinase K/mL in water), heated at 65°C for 2 h, and transferred to a 98°C heat block for 10 min. In general, 10-μL (1.5 × 10⁷ cells) aliquots of the cell lysate were used for PCR amplification of HHV-6A, HHV-6B, and HHV-7; primer sets specific for each virus were used. Reactions consisted of 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM MgCl₂; 0.01% (wt/vol) gelatin (GeneAmp PCR Buffer, PE Biosystems, Norwalk, CT); 200 μM each of dATP, dCTP, dGTP, and dTTP (Roche Molecular Biochemicals, Indianapolis, IN); and 2.5 U Taq DNA polymerase (AmpliTaq Gold, PE Biosystems). Primers were used at a final concentration of 1.0 μM each.

Nested primer PCR assay for HHV-6. This method used a nested PCR assay based on primer sequences located in the teg-ument gene. Common outer primers with variant-specific inner primers were used for nested PCR analysis [19]. Cycling conditions for the outer primers, SIE1 and SIE2, consisted of initial denaturation at 95°C for 10 min, followed by denaturation at 95°C for 3 min and by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min. Variant common amplification with primers SIE1 and SIE2 resulted in a 249-bp product. Nested PCR assay was then done with the use of 5 μL of the aforementioned product with the following parameters: HHV-6A DNA was amplified using primers LTP-A1 and LTP-A2, resulting in a 146-bp product, and HHV-6B DNA was amplified using primers LTP-B1 and LTP-B2, resulting in a 147-bp product. Variant A cycling parameters consisted of initial denaturation at 95°C for 10 min, followed by denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 47°C for 1 min, extension at 62°C for 1 min, and final extension at 62°C for 10 min. Variant B cycling conditions were the same as variant A cycling conditions, with the exception of annealing at 51°C and extension at 68°C.

PCR assay for the HHV-6 immediate-early gene. We used a nonnested PCR assay based on primer sequences from the HHV-6 immediate-early gene. Primers were used to bracket a variant-specific deletion [20]. After initial denaturation at 95°C for 10 min, cycling conditions were the same as those previously described.

PCR assay for HHV-7. Primers were used to generate a 186bp amplified product as described elsewhere [18]. After initial denaturation at 95°C for 10 min, cycling conditions were the same as those previously described.

Southern blot hybridization. Gels were blotted unidirectionally onto Nytran membranes (Schleicher & Schuell, Keene, NH) and were hybridized with the appropriate probe labeled with ³²P (Random Prime Labeling Kit, Roche Molecular Biochemicals). Hybridization solution consisted of 4 × SSC, 0.2% sodium pyrophosphate, 500 μg of heparin/mL, 5% SDS, and 1% blocking reagent (Roche Molecular Biochemicals). After overnight hybridization at 65°C, the membranes were washed 3 times for 10 min each at room temperature with 2 × SSC and 0.1% SDS and then were washed for 1 h at 65°C with 1 × SSC and 0.1% SDS. Membranes were exposed to Kodak X-Omat AR film (Eastman Kodak Scientific Imaging, New Haven, CT) at −70°C for 24 h with intensifying screens.

Double-stranded templates for probe preparation that lacked the primer sequences used in diagnostic PCR analysis were generated by a PCR assay with the following primers: for the primer set described by Cone et al. [19], TTTATGAACTACGATCACA and GAATCTTTGAAATATGC; for the primer sets described by Yamamoto et al. [20], CAAACAAACCCTAACTGTGTA and GTCTCTACAACTACTGAATCG for an HHV-6A probe (these probes were mixed during hybridization); and for the HHV-7 primer set described by Berneman et al. [18], ACCAAATGCTTTTCTATCC and TAAACAATTTGAA-GAGGGA. Cycling conditions for all analyses were as follows: initial denaturation at 95°C for 10 min, followed by 30 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min.

Statistical analysis. The Cochran-Mantel-Haenzel test was used to account for the matching feature of the design and to allow for an unequal number of control subjects per case patient. We did not compare patients with control subjects when the data were sparse (i.e., the proportion of positive results was <5%). Comparisons of the 2 groups included outcome, total proportion of positive results, and P value. P ≤ .05 was considered significant.

Results

Serology. As described elsewhere [14], all patients and con- trol subjects were seropositive for HHV-6. The median titer of...
antibody to HHV-6 was 1460 for patients and 1715 for control subjects. This difference was not significant.

**Virus isolation.** Neither HHV-6 nor HHV-7 was isolated from peripheral blood lymphocytes from patients or control subjects; no culture developed a cytopathic effect, nor did monoclonal antibody or PCR testing of cultures reveal evidence of HHV-6 infection.

**Detection of HHV-6A, HHV-6B, and HHV-7 by PCR analysis.** Although all analyses were matched, pooled numbers are reported for descriptive purposes. PCR assay detected HHV-6A or HHV-6B DNA in 17 (22.4%) of 76 subjects, and there was no significant difference in the distribution of monotypic infection with HHV-6A or HHV-6B or dual HHV-6A/HHV-B infections between patients and control subjects (table 1). The nested PCR assay detected HHV-6A DNA in 1 (1.3%) of 76 subjects and HHV-6B DNA in 13 (17.1%) of these subjects. HHV-6B DNA was also detected in the subject infected with HHV-6A. The PCR assay for the HHV-6 immediate-early gene detected DNA in 4 additional patients (HHV-6A in 2 patients and HHV-6B in 2).

HHV-7 DNA was detected in 14 (18.4%) subjects. Again, control subjects (12 [24%] of 50) were more likely than patients (2 [7.7%] of 26) to be positive, but the difference was not statistically significant in matched analyses. Three subjects had dual infections with HHV-6B and HHV-7.

Because of the biological similarities of HHV-6 and HHV-7 and because few dual infections were detected, we examined the detection of HHV-6 or HHV-7 DNA by any of the PCR methods. Overall, DNA was detected in 28 (36.8%) of 76 subjects. Significantly more control subjects (23 [46%] of 50) than patients (5 [19.2%] of 26) were positive ($P = .04$). This distribution was similar when data were stratified according to type of CFS onset, wellness score, or age (data not shown).

**Discussion**

The objective of this study was to compare the characteristics of infection with HHV-6A, HHV-6B, and HHV-7 in patients with CFS and nonfatigued control subjects. Patients were recruited from a population-based surveillance program and were rigorously classified according to the 1988 case definition of CFS [12]; matched control subjects were randomly selected from the same population from which the patients were selected [10]. All subjects were seropositive for HHV-6 and thus had been infected at some time in the past. Patients and control subjects had virtually identical antibody levels. Neither HHV-6 nor HHV-7 was isolated from peripheral blood lymphocytes from any of the 76 study participants; therefore, there was no evidence of active infection or viral reactivation. Finally, 28 subjects had various mixtures of HHV-6A, HHV-6B, or HHV-7 DNA detected. In all instances, control subjects were more likely than patients to have HHV DNA detected, but the differences were likely to have occurred by chance. Overall, 46% of the control subjects and 19.2% of the patients had HHV-6 and/or HHV-7 DNAs detected, and this difference would occur 4% of the time by chance in a homogeneous population. Thus, the results of our study suggest that patients were slightly less likely than control subjects to support active, reactivated, or latent infections with HHV-6A, HHV-6B, or HHV-7.

There have been at least 7 other previously reported studies measuring markers of HHV-6 or HHV-7 infection and CFS [21–27]; it is difficult to critically compare these studies with our study because of varying degrees of rigor in the description and classification of patients, inappropriate controls, inadequate statistical methods, and quite different methods for estimation of HHV-6 infection.

The earliest studies measured active HHV-6 infection in peripheral blood mononuclear cell (PBMC) cultures. In 1991, HHV-6 growth was reported in PBMC from 3 of 7 patients with CFS and from neither of 2 control subjects [21]. This report did not include statistical testing, but this difference would occur 42% of the time by chance alone. Patients were said to have met the 1988 case definition of CFS [12]; however, 2 of the 3 patients from whom HHV-6 was isolated had severe cerebral dysfunction and severe polylymphadenopathy, and the third had acute Epstein-Barr virus infection. The 3 patients likely had illnesses other than CFS that could account for the findings.

A more comprehensive study reported isolation of HHV-6 from PBMC from 79 (70%) of 113 patients with chronic fatigue and 8 (20%) of 40 control subjects [22]. Unfortunately, this study did not characterize the fatigue or classify patients by standard criteria, nor did the researchers report evaluation of patients systematically to consider known causes of chronic fatigue. Similarly, the study did not characterize the control subjects; therefore, it is impossible to critically evaluate the findings. Moreover, the investigation did not present statistical analyses for elucidation of observed differences, did not address possible interactions between risk factors, and did not control for possible confounding variables.

Three studies used PCR analysis to measure HHV-6 infection [23–25]. The first study enrolled 13 patients with CFS and 13 age-matched healthy control subjects [23]. All 26 subjects were seropositive for HHV-6, and HHV-6 could not be detected in direct culture of PBMC from any subject. However, PCR analysis detected HHV-6 DNA in 7 (54%) patients compared with no control subjects (Fisher’s exact test; $P = .003$). Unfortunately, no information was provided concerning patients, other

<table>
<thead>
<tr>
<th>Subjects</th>
<th>HHV-6A</th>
<th>HHV-6B</th>
<th>HHV-6A and HHV-6B</th>
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</thead>
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<tr>
<td>Patients with CSF</td>
<td>0/26</td>
<td>2/26 (7.7)</td>
<td>1/26 (3.8)</td>
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<tr>
<td>Control subjects</td>
<td>2/50</td>
<td>12/50 (24.0)</td>
<td>0/50</td>
</tr>
<tr>
<td>Total</td>
<td>2/76</td>
<td>14/76 (18.4)</td>
<td>1/76 (1.3)</td>
</tr>
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</table>

* Data in the columns are mutually exclusive.
than that they met the 1988 case definition of CFS [12], and no information was provided concerning control subjects, except that they were healthy and age-matched. It is unclear whether subjects were also matched for sex in addition to age or whether matched analyses were performed. Finally, the study was conducted in Japan, and the results cannot be generalized to other racial/ethnic populations.

A less extensive Italian study of 36 patients with CFS and 24 control subjects found HHV-6 DNA in PBMC from 16 (44%) of the patients and 7 (29%) of the control subjects ($\chi^2$ test; $P = .23$) [24]. HHV-6B was found in 22% of patients and 25% of control subjects, and HHV-6A was found in 22% of patients and 4% of control subjects (Fisher’s exact test, $P = .06$). Thus, the observed differences were not statistically significant. In addition, HHV-7 DNA was detected in 82% of patients and 83% of control subjects. Patients were not described, with the exception that they met the 1994 case definition of CFS [1]. The 24 control subjects were unsellected healthy blood donors who came from the same geographic region as did the patients and who were matched for age and sex. Such matching cannot, of course, be achieved with 36 patients and 24 control subjects.

The most recent study enrolled 74 patients with CFS and 71 control subjects [25]. All subjects were seropositive for HHV-6, and direct culture of PBMC was not done. HHV-6 DNA was detected in 35% of patients and 72% of control subjects, and HHV-7 DNA was detected in 77% of patients and 70% of control subjects. Patients met the 1994 case definition of CFS [1] and were well described. Unfortunately, control subjects were physician- or self-referred and thus represent a convenience sample; therefore, it is impossible to critically evaluate the meaning of their results compared with those for patients.

Finally, 2 studies, which either did not measure active HHV-6 infection or did not test for DNA, reported serological data on HHV-6 for patients with CFS and control subjects [26, 27]. An excellent study blindly measured IgG and IgM antibodies to HHV-6 early antigen in 154 well-characterized patients with CFS from 2 clinical practices and 165 healthy blood donors from the same geographic region (control subjects) [26]. The researchers found that 40% of patients and 8% of control subjects had IgG antibody to HHV-6 early antigen and that 60% of patients and 4% of control subjects had IgM antibody. A second, seriously flawed study measured antibody to 2 strains of HHV-6 in 20 serum samples from patients aged 21–37 years and 26 control subjects, aged 20–48 years, who were recruited from the staff of laboratories at the Japanese National Institutes of Health (Shinjuku-ku, Japan) and the University of Massachusetts Medical School (Worcester, MA) [27]. Although significantly more patients than control subjects were seropositive and had higher geometric mean titer of antibody, the groups are inappropriate for epidemiological comparison.

In summary, we found no association between CFS and infection with HHV-6A, HHV-6B, or HHV-7 in a group of well-characterized, rigorously defined patients with CFS and randomly selected matched control subjects. Primary infection with HHV-6 or HHV-7 is not likely to be causally associated with CFS, because infection is virtually ubiquitous by the age of 2–5 years. Moreover, it is unlikely that reactivation of latent HHV-6 or HHV-7 is causally associated with a substantial proportion of cases of CFS because of the lack of consensus among studies and because reactivation of most latent herpesviruses is common, occurs sporadically, and is usually asymptomatic.

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References


