

Active Human Herpesvirus 6 Infection in Patients With Multiple Sclerosis

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Context: Human herpesvirus 6 (HHV-6) has been linked with multiple sclerosis (MS).

Objectives: To determine HHV-6 viral load in patients with MS, and to analyze separately its 2 variants, HHV-6A and HHV-6B.

Patients and Methods: We analyzed 149 blood and serum samples; 103 were from patients with relapsing-remitting MS (33 during an MS relapse and 70 during remission), and 46 were from healthy blood donors. To determine whether the HHV-6 genome and its variants were present, we analyzed viral DNA using quantitative real-time polymerase chain reaction, which has a sensitivity of 1 copy.

Results: We found HHV-6 DNA in the peripheral blood mononuclear cells of 53.4% of patients and 30.4% of healthy blood donors; HHV-6A was found in 20.4% of patients and

4.4% of controls, and HHV-6B was found in 33.0% vs 26.1%, respectively. Mean viral load in both groups was 7.4 copies of HHV-6 per microgram of DNA (range, 1-15 copies). Analysis of serum samples showed that none of the healthy blood donors were positive for HHV-6, although 14.6% of patients were positive for the virus, specifically the HHV-6A variant. There was no difference between patients during remission or relapse. Mean viral load was 26.3 copies/ μ g microgram of DNA (range, 1-86 copies).

Conclusions: Despite the low viral load and the lack of clinical correlation, and given the biological characteristics of the virus, our results suggest that there was active HHV-6A infection in 14.6% of patients with MS. Further quantitative real-time polymerase chain reaction studies will help us understand the clinical significance of such a low viral load.

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MULTIPLE SCLEROSIS (MS) is characterized clinically by a variable course and pathologically by a progressive accumulation of demyelinating plaques in the central nervous system white matter. Numerous epidemiologic studies support the hypothesis that MS appears in a genetically predisposed population that may also be affected by an environmental factor, which is probably infectious.^{1,2} During the last few decades, numerous viruses have been studied, but so far, none have been definitely associated with MS.

Since the first papers linking human herpesvirus 6 (HHV-6) to MS appeared in 1993,^{3,4} many others have presented contradictory results on its possible role in the disease. Some authors defended the association,⁵⁻¹⁴ whereas others denied it.¹⁵⁻²⁴ The methods used in these studies were heterogeneous, although most authors applied some variant of the polymerase chain reaction (PCR). Using these techniques, several authors have shown higher than

expected HHV-6 DNA presence in patients with MS.⁹⁻¹⁴ Nevertheless, the role of the virus in the pathogenesis of MS is still unclear.

The detection of HHV-6 genomes by quantitative real-time PCR has not yet been applied to the study of MS. Quantitative real-time PCR is a new and highly sensitive assay that can detect a single copy of DNA; it also provides quantitative results, allowing us to measure the viral load in samples of blood and serum. For these reasons, and given the previous results obtained by our group,²⁵ we designed a new study with 2 goals.

1. To use quantitative real-time PCR to determine HHV-6 viral load in whole blood and serum from patients with MS and a control group of healthy blood donors. The results will establish the role of an active viral infection in determining the course of the disease.

2. To study HHV-6 variants A and B separately to determine if there is a difference in their degree of involvement in MS.

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PATIENTS AND METHODS

PATIENTS

We analyzed 149 whole-blood and serum samples; 103 were from patients with relapsing-remitting MS who were treated at the Department of Neurology, San Carlos Hospital, Madrid, Spain, and 46 were from healthy controls who had donated blood at the same institution. In the patient group, 33 were having an MS relapse when blood was drawn (48 hours after onset of symptoms), and 70 were in remission. There were 28 men (mean age, 36.8 years; age range, 22-63 years) and 75 women (mean age, 35.3 years; age range, 21-57 years). In the control group of healthy blood donors, there were 25 men (mean age, 28.6 years; age range, 18-42 years) and 21 women (mean age, 26.9 years; age range, 18-39 years). Sixty-one patients were receiving interferon β treatment, but none received steroid treatment prior to blood sampling. This study was approved by the San Carlos Hospital Ethical Committee of Clinical Investigation.

SAMPLES

We obtained 10 mL of whole blood with EDTA and 2 mL of centrifuged serum from each patient and control. Both samples were processed immediately after the extraction. The protocol used to extract DNA from peripheral blood mononuclear cells involved lysing erythrocytes with a sucrose solution. After consecutive washes, we added proteinase K and sodium dodecyl sulfate to the clean lymphocyte pellet. Finally, the DNA was precipitated with 1 volume of 2-propanol and dissolved with Tris-EDTA buffer (10mM Tris, pH 7.5; 1mM EDTA). The DNA was extracted from serum samples using Qiagen columns (QIAamp Blood Kits; Qiagen, Venlo, the Netherlands), according to the manufacturer's protocol. After being extracted and dissolved in the

buffer solution, whole-blood and serum DNA were quantified by spectrophotometry (Eppendorf, Hamburg, Germany), with a reading of absorbancy along the 260-nm wavelength. Aliquots of the samples were measured to the same concentration (50 ng/ μ L for whole-blood DNA; 5 ng/ μ L for serum DNA). The samples were frozen at -80°C .

QUANTITATIVE REAL-TIME PCR

Quantitative real-time PCR, a sensitive and reproducible method for detecting viral DNA,²⁶ was used to detect HHV-6, HHV-6A, and HHV-6B DNA in a real-time cyclor (Rotor-Gene 2000; Corbett Research, Sydney, Australia). Real-time PCR uses the 5' \rightarrow 3' exonuclease activity of *Taq* polymerase to digest an internal probe labeled with 2 fluorescent dyes, the reporter and the quencher. Primers and *TaqMan* probes (Genotek Transloc SBD, Barcelona, Spain) to detect the common strain of HHV-6 and variants A and B were located in the putative immediate-early 1 region of the HHV-6 genome.²⁷⁻²⁹ These primers and probes have been found to be specific when tested with HHV-1, HHV-2, cytomegalovirus, Epstein-Barr virus, varicella-zoster virus, HHV-7, and HHV-8 target DNA. To control amplification, a set of primers and an exonuclease probe located in the human β -globine gene were used³⁰ (**Table 1**). The optimal reaction conditions for the HHV-6 quantitative real-time PCR were 10mM Tris hydrochloride, pH 8.3; 50mM potassium chloride; 1.5mM magnesium chloride; 400 μ M each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate; 0.5 μ mol/L of HHV-6 primers; 0.25 μ mol/L of HHV-6 *TaqMan* probe; 1 U of *Taq* DNA polymerase (*AmpliTaq* Gold; PE Applied Biosystems, Foster City, Calif); 200 ng of whole blood-purified DNA or 50 ng of serum-purified DNA; and water to achieve a final reaction volume of 25 μ M. Each sample was analyzed 4 times:

RESULTS

PURIFIED WHOLE-BLOOD DNA

We found that 53.4% of patients with MS had HHV-6 DNA in their peripheral blood mononuclear cells, compared with 30.4% of healthy blood donors (OR, 1.75; $P < .001$). When we tested for HHV-6A, we also found statistically significant differences: 20.4% of patients with MS vs 4.4% of controls (OR, 4.69; $P < .001$). However, although we found a higher prevalence of HHV-6B DNA in both groups, 33.0% for patients with MS and 26.1% for healthy blood donors, the difference was not statistically significant (OR, 1.3; $P = .06$). When samples were positive for HHV-6 or either of its variants, the viral load was only between 1 and 15 copies of HHV-6 per microgram of DNA (mean, 7.4 copies) in patients and controls.

PURIFIED SERUM

No healthy blood donors had serum samples that contained HHV-6 DNA; 14.6% of serum samples from patients with MS contained HHV-6 DNA, all of which was variant A ($P < .001$). We did not find any significant differences between patients experiencing an MS relapse

(12.1%) and patients in remission (15.7%) (**Table 2**). Among the HHV-6A-positive samples, the viral load was between 1 and 86 copies of HHV-6A per microgram of DNA (mean, 26.3 copies).

COMMENT

The β -herpesvirus HHV-6 is highly prevalent among healthy adults worldwide; so far, we know of 2 variants, A and B, that have different antigenic, genomic, and biological features to the point of behaving as 2 different viruses. Although HHV-6B has been identified as the causal agent of exanthema subitum, the role of the A variant has not yet been determined.³¹

Many studies support the hypothesis that HHV-6 could be involved in the pathogenesis of MS,⁵⁻¹⁴ but there is still controversy about this theory. A MEDLINE review on the topic yielded more than 60 articles about MS and HHV-6 since 1993,^{3,4} many of which were written in the last 2 years.^{6-10,23-25,32-42} Most of these articles describe molecular biological studies using various PCR assays in different specimens (blood, serum, saliva, urine, brain tissue, etc); other articles describe detailed serologic, antigenic, and immunologic studies as well as cultures; and finally, several re-

2 consecutive PCRs with duplicated samples to detect HHV-6 and variants A and B. The PCR reaction mixture for β -globine was identical to that for HHV-6, except that we used 0.25 $\mu\text{mol/L}$ of β -globine primers instead of HHV-6 primers and 0.125 $\mu\text{mol/L}$ of a specific β -globine TaqMan probe; each sample was analyzed only once. The samples were placed in a real-time cycler and preincubated at 95°C for 15 minutes to activate *AmpliTaq* Gold DNA polymerase. Then, 2-step thermocycling was performed for 45 cycles: denaturation at 95°C for 15 seconds and anneal/extension for 50 seconds at different temperatures (60°C for HHV-6, 64°C for variant A, and 65°C for variant B). Cycling conditions for β -globine were identical to those for the HHV-6 assay. With these primers and conditions, the sensitivity of the real-time PCR assay was only 1 copy for both HHV-6 and β -globine.

When the probe is intact, the reporter and quencher dyes undergo fluorescent resonance energy transfer, thereby suppressing the fluorescence of the reporter dye. When target DNA is present, upon primer elongation, the probe is cleaved by the 5'→3' exonuclease activity of *Taq* polymerase. The reporter dye is no longer physically attached to the quencher dye on the probe, and fluorescent resonance energy transfer is interrupted. This results in an increase of reporter dye fluorescence that is proportional to the amount of PCR product accumulated. The number of DNA copies is determined by calculating the number of PCR cycles necessary for a standard curve of known amounts of DNA to cross a fluorescent threshold and interpolating the unknowns. For the design of the standard curve, we used purified and quantified HHV-6 genome (Advanced Biotechnologies Inc, Columbia, Md). First, we determined the range of copies of HHV-6 in patients with MS; then, we decided that the points of the standard curve would be 1 copy, 5 copies, 10 copies, 50 copies, and 100 copies. Each point was triplicated, and we repeated the standard curve for each of the PCRs.

As the sample tubes spin past the detector modules, a high-powered light-emitting diode strobes the sample, and a photomultiplier collects the increasing fluorescent energy during the amplification. The data are sent to a personal computer, which averages the energy of each sample during several revolutions. These data are displayed on the monitor in real time as fluorescence vs cycle number or temperature plot. For the analysis of the quantitative results, the software plotted the log of the signal normalized to an internal reference (ΔRn) and set the threshold cycle, C_T (the PCR cycle number required for fluorescence intensity to exceed an arbitrary threshold in the exponential phase of the amplification), when ΔRn becomes equal to 10 SDs of the baseline (**Figure 1**). With this method, we obtained a C_T value for each sample. Next, a standard curve was generated by plotting C_T vs the amount of total DNA added to the reaction and was used to compare the amount of HHV-6 DNA in the samples from controls and patients (**Figure 2**). Calculations of C_T and ΔRn , standard curve preparation, and quantification of DNA in the samples were performed by the software provided with the Rotor-Gene 2000.

STATISTICAL ANALYSIS

Quantitative variables were presented by their frequency distribution. The associations among qualitative variables were analyzed with a χ^2 test or the Fisher exact test. The median and mean values of quantitative variables were determined, and the associations among these variables were calculated with a *t* test. Correlations were established with the Spearman rank correlation. The validity of our tests was analyzed through sensitivity, specificity, positive predictive value, negative predictive value, and global and observed concordance. Risks were quantified with odds ratios (ORs) and 95% confidence intervals. We considered differences to be statistically significant at $P < .05$.

Table 1. Primer and Probe Sequences for Quantitative Real-Time Polymerase Chain Reaction (PCR)*

Gene	Primer/Probe	Sequence (5' → 3')	Amplification Size
HHV-6	F-primer	AGTCTAGAACTAGAACTAAA	103 bp
	R-primer	ATCGTAGTTCATAAAAACCTC	
	TQ-probe	JOE-TACCGCATCCTTGACATATTACGC-TAMRA	
HHV-6A	F-primer	CATGAAGATGATGACAATAAAATG	278 bp
	R-primer	TGGAACCATCTTGTCTGTCC	
	TQ-probe	JOE-CCGCCAGATCTGTCACTGAGGCTG-TAMRA	
HHV-6B	F-primer	GAGACCGGTCTGGACAACA	145 bp
	R-primer	GAGTTGCTGAGTTGGTAAAGG	
	TQ-probe	JOE-CTCCAAGTGACCGAAACGCTTCTGG-TAMRA	
β -Globine	F-primer	CAGAGCCATCTATTGCTTAC	71 bp
	R-primer	CATGGTGTCTGTTGAGGTT	
	TQ-probe	FAM-ACACAACCTGTGTTCACTAGC-TAMRA	

*HHV-6 indicates human herpesvirus 6; HHV-6A, HHV-6 variant A; HHV-6B, HHV-6 variant B; F-primer, forward PCR primer; R-primer, reverse PCR primer; and TQ-probe, TaqMan probe.

view papers attempt to analyze the accumulated evidence on the subject. In the end, the question of whether HHV-6 is associated with MS pathogenesis or is just a consequence of the activation of the inflammatory-immune response is still unanswered. If it is associated with MS pathogenesis, at what point does it intervene? Is it mediated by

an active infection? The answers to these questions are heterogeneous and, at times, contradictory.

For the last 3 years, our group has been investigating the possible association between HHV-6 and MS. A first approach using nested qualitative PCR in whole blood²⁵ led us to conclude that there was a statistically significant

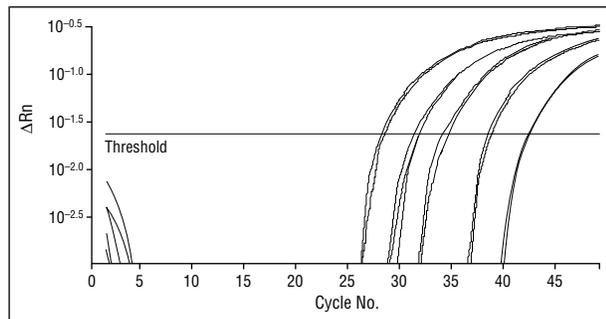


Figure 1. Total DNA input ranging among 100, 50, 10, 5, and 1 copies of human herpesvirus 6 (HHV-6) DNA was plotted by the log of the change in normalized reporter signal (ΔR_n) vs the cycle number. The minimal deviation among triplicate samples indicates the high reproducibility of this approach. The threshold cycle difference among triplicate samples was 0 to 0.36 for HHV-6.

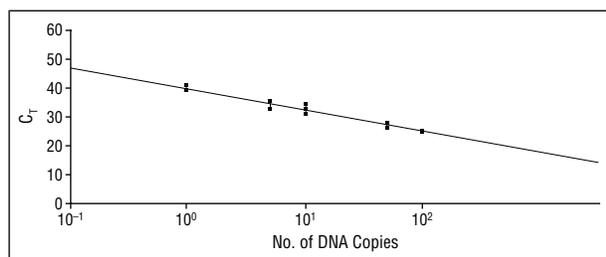


Figure 2. Polymerase chain reaction standard curve and plots of the threshold cycle (C_T) against the input target copy number, or the number of copies of DNA. The standard curve showed a close correlation between copy number and C_T ($R=0.99$).

difference between DNA prevalence of HHV-6 in patients with MS vs healthy blood donors (OR, 2.26; $P < .001$); this difference was not found for the rest of the viruses we investigated: herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus, HHV-7, and HHV-8. Therefore, we redesigned our study and introduced new variables: serum HHV-6 DNA prevalence, the peripheral blood mononuclear cell and serum viral loads, and the differentiation between HHV-6 variants A and B.

The prevalence of HHV-6 DNA in whole-blood samples was similar to results obtained in a previous study by our group: 53.4% vs 49.0% positivity, respectively, for the patients with MS and 30.4% vs 22.0% for controls; the differences between groups were statistically significant in both studies. The increase in the prevalence of samples containing HHV-6 DNA in the current study is due to the greater sensitivity of quantitative real-time PCR compared with the nested PCR used in the previous study.

The prevalence of viral DNA in whole blood gives stationary information about the viral implication in the disease. This limitation prompted us to determine the prevalence of HHV-6 DNA in serum, which would be a better indicator of active infection. The current study showed a statistically significant difference in HHV-6 DNA prevalence in serum between patients (14.6%) and controls (0%). However, despite the difference and the accumulated evidence,⁵⁻¹⁴ it is risky to infer that a virus with such a low prevalence has a major role in the pathogenesis of MS.

To solve this dilemma, we hypothesized that subjects whose serum samples were positive for HHV-6 DNA could belong to a subgroup of patients who were expe-

Table 2. HHV-6 DNA Prevalence in Serum*

Subjects	HHV-6	HHV-6A	HHV-6B
Patients with MS	14.6	14.6	0
In relapse	12.1	12.1	0
In remission	15.7	15.7	0
Healthy blood donors	0	0	0

*HHV-6 indicates human herpesvirus 6; HHV-6A, HHV-6 variant A; HHV-6B, HHV-6 variant B; and MS, multiple sclerosis. Data are presented as percentage of subjects.

riencing an attack at the time of the study, whereas the serum from patients in remission would be negative for the virus. Tomson et al¹² found HHV-6 viremia only in patients with MS, predominantly those in the active phase of the disease. In this study, however, this hypothesis did not prove to be true; 12.1% of relapsing patients and 15.7% of patients in remission had positive results. Another possibility was that patients whose samples were positive for HHV-6 were not receiving immunomodulating treatment with interferon β , but there were no differences between treated and untreated patients (data not shown).

With no plausible explanation for our results, we tried a new approach to this question: measuring the viral load with a quantitative real-time PCR, an extremely sensitive method that can detect a single copy of DNA. This approach was based on the assumption that an active infection indicates a high viral load, as has proved true for other diseases.²⁹

The viral load averaged between 1 and 15 copies of HHV-6 per microgram of whole blood-purified DNA, both in patients and controls without any statistically significant difference. However, in the serum of patients with MS, the viral load ranged between 1 and 86 copies of HHV-6 per microgram of DNA. Are these quantities significant enough to infer an active HHV-6 infection in patients with MS? The presence of HHV-6 DNA in serum is used as a marker of active HHV-6 infection; however, because this is the first quantitative TaqMan assay used to study the association between HHV-6 and MS, perhaps this statement should be reconsidered based on the viral load in other diseases for which viral pathogenesis is proven. Nevertheless, there must be a justification for the differences obtained for HHV-6 as opposed to other herpesviruses, not only in our studies but also in others on this subject.⁵⁻¹⁴ Maybe the explanation lies not in the agent itself but in when and how HHV-6 plays its role. To determine the accuracy of this hypothesis, it would be necessary to study patients with MS in the early stages of the disease and conduct prospective follow-up of several years to relate the viral load in blood, serum, and cerebrospinal fluid with the clinical manifestations during the development of the disease.

Finally, we decided to study separately the 2 viral variants, HHV-6A and HHV-6B, in addition to HHV-6 as a whole. The difference in viral positivity in peripheral blood mononuclear cells from patients and controls was mainly due to a difference in HHV-6A prevalence (20.4% and 4.4%, respectively), whereas HHV-6B prevalence was similar in both groups. We obtained congruent results in serum samples: all the positive samples contained the HHV-6A variant. Viral load values for these variants confirmed the

results obtained for HHV-6 because the number of copies was within the ranges previously mentioned. These findings show that, of the 2 variants, HHV-6A would be the one involved in the pathogenesis of MS. Any further studies aiming to better define the role of HHV-6 in MS should separate the variants in their analyses.

In conclusion, our study suggests a possible HHV-6A active infection in patients with MS. At this point, however, we cannot say whether this association has a pathogenic significance in the origin or development of MS. There is a need for further studies to better define the role and association of HHV-6 in this puzzling disease.

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