Relapsing-Remitting Multiple Sclerosis and Human Herpesvirus 6 Active Infection

Roberto Álvarez-Lafuente, MD; Virginia De las Heras, PhD; Manuel Bartolomé, PhD; Juan José Picazo, MD; Rafael Arroyo, MD

Background: Recent studies have focused on the relationship between human herpesvirus 6 (HHV-6) and multiple sclerosis (MS).

Objective: To analyze HHV-6 messenger RNA expression in patients with relapsing-remitting (RR) MS vs healthy blood donors (HBDs).

Design: One hundred fifty-four subjects were enrolled in the study: 105 patients with RRMS (32 in relapse) and 49 HBDs. Total DNA and messenger RNA were extracted from serum and blood samples, respectively, and analyzed by quantitative real-time reverse transcription-polymerase chain reaction for the detection of 3 HHV-6 immediate-early genes (U16/U17, U89/U90, and U94) and both HHV-6 variants (HHV-6A and HHV-6B).

Results: Active HHV-6 infection was detected in 16% of patients with RRMS vs 0% of HBDs (P = .003). Seven patients with RRMS with exacerbation had HHV-6 active replication, and the virus remained latent in only 1 of them. We did not find any statistically significant difference between HHV-6 active or latent infection for patients in remission (P = .12). Among patients with RRMS with HHV-6 active replication, viral load was higher when they experienced an acute attack than when in remission (P = .04). In those patients with RRMS who had an active infection only, HHV-6A was found. Cell-free HHV-6 DNA detected in serum samples confirmed the results.

Conclusions: The results show that a subset of patients with RRMS experience HHV-6 active infection, and there likely is an association between the viral active replication and relapses; therefore, HHV-6 active infection may imply a greater risk of exacerbations in a subgroup of patients with RRMS.

Arch Neurol. 2004;61:1523-1527

Multiple Sclerosis (MS) is a chronic, inflammatory, demyelinating disease of the white matter in the human central nervous system. It is characterized as a T-cell–mediated, autoimmune, pathogenic process in genetically predisposed individuals. The origin of MS is still not clear and eludes our comprehension despite many epidemiological, immunological, and neurological studies. The hypothesis that viral infections would be involved in disease genesis has been repeatedly suggested; the virus might play a critical role in the pathogenesis of MS, both at the onset of the disease or during it, and may, for example, trigger the exacerbations in the relapsing-remitting (RR) form. Although an etiological correlation is still uncertain, during the past few years, many studies have focused on the relationship between human herpesvirus 6 (HHV-6) and MS. Human herpesvirus 6, a newly described β-herpesvirus, was first isolated in 1986 from the peripheral blood of patients with AIDS and lymphoproliferative disorders, and in 1993 2 variants were described: HHV-6A and HHV-6B. It is widely spread in the human population, and primary infection usually takes place in the first years of life. As with other herpesviruses, it is highly neurotropic, infects cells of both the immune and nervous systems, and establishes latency. In addition, HHV-6 genomic sequences can be found in T cells of healthy individuals. However, HHV-6 reactivates under certain conditions, causing an active infection that has been associated with RRMS and, specifically, with exacerbations.

Because viral messenger RNA (mRNA) expression constitutes an unambiguous marker of active viral infection, the aim of this study was to analyze HHV-6 mRNA to determine if there is an association be-
between the presence of an acute attack in patients with RRMS and HHV-6 active infection. In the present study, we report the measurements of 3 HHV-6 immediately-early (IE) genes by quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR): U94 (which is the only viral gene expressed during latency), U16/U17, and U89/U90; Epstein-Barr virus (EBV) mRNA was studied as the control. In parallel, to confirm the active infection, we analyzed the presence of HHV-6 and EBV in serum samples from the same patients. Finally, we established which specific HHV-6 variant was present among HHV-6–positive samples.

METHODS

PATIENTS AND SAMPLES

The case population of this case-control study was composed of 105 patients with RRMS (26 men between 22 and 64 years of age and 79 women between 21 and 63 years of age) followed up at the Multiple Sclerosis Unit of Hospital Clínico San Carlos of Madrid, Spain. The study period lasted from November 1, 2001, to February 28, 2003. To be eligible for the study, patients had to have a diagnosis of definite MS according to the criteria described by Poser et al.14 Cases were classified by the presence or absence of acute relapse at the time of sampling; thus, 32 blood and serum samples were obtained from 32 patients with RRMS within 2 days from the onset of a clinical acute attack. Patients were invited to participate in the study in consecutive visits.

For the control group, 49 subjects were randomly selected among healthy blood donors (HBDs). None of them had clinical symptoms suggestive of a viral infection. This study was approved by the local ethics committee, and all the participants gave written informed consent before the enrollment.

EXTRACTION AND RT

Total mRNA was extracted from peripheral blood mononuclear cells (PBMCs) from 400 µL of whole blood using Qiagen spin columns (QIAGEN Blood RNA Mini Kit; Qiagen Inc, IZASA, Barcelona, Spain), according to the protocols supplied by the manufacturer. Single-stranded complementary DNA (cDNA) was synthesized by rTth (recombinant Thermus thermophilus) DNA polymerase (Roche Diagnostics, Barcelona, Spain) using random hexamer primers (Roche Diagnostics). Finally, DNA was extracted from serum samples using Qiaquant columns (QIAGEN Blood Kits), according to the manufacturer’s protocol, and then was quantified by spectrophotometry (Eppendorf, Hamburg, Germany) and put into 5-ng/µL aliquots before freezing at –80°C.

DATA ANALYSIS

Univariate odds ratios and exact 95% confidence intervals were calculated with standard microcomputer software (Epi-Info version 2000; Centers for Disease Control and Prevention, Atlanta, Ga, and SPSS version 11.0; SPSS Inc, Chicago, Ill). The χ² or 2-tailed Fisher exact test was used to test differences in categorical variables. Kruskal-Wallis analysis or the Wilcoxon rank sum test was used to test differences in continuous variables. P<.05 was considered statistically significant.

RESULTS

The prevalences of total mRNA belonging to EBV and HHV-6 considered in the PBMCs collected from patients with RRMS and from HBDs are given in Table 1. A total of 17 patients with RRMS (16%) had an early HHV-6 active replication (only those patients with transcripts of the 3 IE genes), whereas none of the HBDs had detectable HHV-6 (P=.003). As given in Table 1, we did not find any statistically significant difference (P=.75) when we analyzed HHV-6 latency in patients with RRMS and HBDs (detected as the presence of U94 transcripts)

### Table 1. Messenger RNA Prevalences of HHV-6 and EBV in PBMCs of Patients With RRMS and HBDs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients With RRMS, No. (%)</th>
<th>HBDs, No. (%)</th>
<th>OR (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHV-6 gene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U89/U90 + U16/U17 + U94</td>
<td>17 (16)</td>
<td>0</td>
<td>1.4 (0.4-5.6)</td>
<td>.75</td>
</tr>
<tr>
<td>U94*</td>
<td>9 (9)</td>
<td>3 (6)</td>
<td>2.4 (0.3-21.1)</td>
<td>.66</td>
</tr>
<tr>
<td>EBV</td>
<td>5 (5)</td>
<td>1 (2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations: CI, confidence interval; EBV, Epstein-Barr virus; HBDs, healthy blood donors; HHV-6, human herpesvirus 6; OR, odds ratio; PBMCs, peripheral blood mononuclear cells; RRMS, relapsing-remitting multiple sclerosis.

*Latent infection: samples with transcripts of U94 in the absence of transcripts of the other HHV-6 genes.

QUANTITATIVE REAL-TIME PCR

The cDNAs were analyzed by quantitative real-time PCR for the presence of HHV-6 genomes, human β-globin gene as internal control (to ensure that cDNA strands were suitable for DNA amplification), and EBV sequences to compare with HHV-6 results. To discriminate mRNA from genomic DNA, we focused on the detection of spliced gene expression. Primers and TaqMan probes were located in 3 IE genes (U16/U17, U89/U90, and U94) of the HHV-6 genome and in the BamHI-K gene of the EBV sequence.15-17 To detect the presence of HHV-6 genomes and EBV sequences in serum samples, we used primers and TaqMan probes as described elsewhere.16 Because it has been previously described,11 the presence of U94 gene transcripts in the absence of expression of the other HHV-6 genes was considered HHV-6 latent infection.

HHV-6 VARIANT DETECTION

Human herpesvirus 6 variants (HHV-6A and HHV-6B) were characterized in HHV-6–positive samples by quantitative PCR, as previously described.16

COMPARATIVE STUDY OF HHV-6 mRNA PREVALENCES

The prevalences of total mRNA belonging to EBV and HHV-6 considered in the PBMCs collected from patients with RRMS and from HBDs are given in Table 1. A total of 17 patients with RRMS (16%) had an early HHV-6 active replication (only those patients with transcripts of the 3 IE genes), whereas none of the HBDs had detectable HHV-6 (P=.003). As given in Table 1, we did not find any statistically significant difference (P=.75) when we analyzed HHV-6 latency in patients with RRMS and HBDs (detected as the presence of U94 transcripts).
in the absence of expression\textsuperscript{13} of \textit{U89/U90} and \textit{U16/U17}. These results were confirmed with the parallel study of the cell-free HHV-6 DNA in serum samples: only patients with transcripts of \textit{U89/U90}, \textit{U16/U17}, and \textit{U94} tested positive for the presence of HHV-6 genomes in their serum samples, confirming the occurrence of HHV-6 active infection (Table 2). We did not find any correlation between EBV and MS.

### COMPARATIVE STUDY OF HHV-6 VIRAL LOAD

There was a statistically significant difference (Table 3) in HHV-6 median viral load between patients with RRMS with active and latent infection ($P < .001$). However, when we considered those patients with RRMS and controls with latent infection, a statistically significant difference was not observed ($P = .06$).

Regarding EBV, we found a median viral load of 25.2 (range, 19.9-29.3) genomes per microgram of RNA in patients with RRMS, which was comparable with 1 positive HBD (31.4 genomes per microgram of RNA), providing that the 5 patients with RRMS who were positive for that virus had a latent infection for EBV. These results were confirmed when we analyzed the serum samples of these patients; none of the patients with RRMS had EBV in their serum sample.

### RELATIONSHIP BETWEEN HHV-6 INFECTION AND DISEASE ACTIVITY

The number of copies and prevalences of HHV-6 mRNA in PBMCs of patients with RRMS based on disease activity are reported in Table 4. Of note, we found a statistically significant difference for HHV-6 DNA prevalence and viral load between patients with RRMS in active relapse and patients with RRMS in remission. Among patients with RRMS who had an acute attack, an active HHV-6 infection was observed more frequently (22\%) than in patients with a latent viral infection (3\%). This was not observed for those patients with RRMS in remission.

Human herpesvirus 6 viral load during active HHV-6 infection was significantly greater ($P = .04$) among patients with RRMS in exacerbation than in remission: 229.6 vs 181.2 genomes per microgram of RNA for \textit{U89/90} (26.7\% of increase in viral load during acute attack) and 198.7 vs 134.5 genomes per microgram of RNA for \textit{U16/U17} (28.6\% of increase in viral load during acute attack). For latent infections, we did not find any statistically significant difference (Table 4).

Regarding EBV, there were no differences in DNA prevalence or in viral load between patients with RRMS with active relapse and stable or inactive disease patients (data not shown).
HHV-6 VARIANT STUDY

Among patients with RRMS with HHV-6 active infection (including patients with active relapsing and inactive disease), we only found the HHV-6A variant; this result was confirmed when we analyzed the cell-free samples. In those patients with RRMS with HHV-6 latent infection, we found HHV-6A in 3 of 9 and HHV-6B in 6 of 9. Of interest, in the HBD control group, we only found variant B.

COMMENT

Numerous studies have implicated HHV-6 in the etiology of MS; however, this association is controversial, and, as is the case, experimental evidence linking this organism to MS is equivocal. In fact, it is difficult to establish a causative role of HHV-6 due to its high prevalence: more than 95% of people older than 2 years are seropositive for HHV-6. Furthermore, the entity known as HHV-6 consists of 2 closely related yet distinct viruses, designated variant A (HHV-6A) and variant B (HHV-6B). Human herpesvirus 6B is the etiologic agent of the common childhood illness exanthema subitum (also known as roseola infantum, or sixth disease) and related febrile illnesses and is frequently active in immunocompromised patients. Human herpesvirus 6A has not been etiologically linked to any human disease; however, a possible association between this variant and MS has recently been described based on the findings of HHV-6 DNA in PBMCs and serum samples of MS patients compared with control groups. The presence of viral genomes in cell-free samples led us to hypothesize about a possible HHV-6 active infection in a subset of MS patients, which ought to be confirmed with mRNA analysis. It recently has been reported that latency of HHV-6 is associated with the presence of U94 mRNA in the absence of other mRNA transcribed during the IE phase of infection, and a role in the maintenance of the latent state through the regulation of viral gene expression has been suggested. In addition, Mirandola et al found that during HHV-6 active infection, all other IE genes are transcribed with high levels during productive and restricted infection. In the past few years, several authors have reached the same conclusions. Nevertheless, controversy exists among the studies that analyzed mRNA in MS patients and control groups. Rotola et al analyzed 3 HHV-6 genes with transcripts that belong to the IE transcriptional class (U94, U16/17, and U91) by nested PCR; they report that HHV-6 is latent in PBMCs of MS patients, because U94 was the only transcript found. Chapenko et al studied PBMC RNA in 5 patients with MS, 3 with RRMS, and 2 with secondary progressive MS; transcription of viral mRNA, which indicates early HHV-6 replication, was found in 2 of 3 PBMC RNA samples from patients with RRMS.

In this study, we analyzed the association between HHV-6 active infection and RRMS with a recently developed quantitative real-time RT-PCR assay through the detection of the transcripts of the following HHV-6 IE genes: U16/17, U89/U90, and U94. Two population groups were enrolled in this assay: patients with RRMS and HBDs.

The results of our study show that a subset of patients with RRMS had HHV-6 active infection. The hypothesis of the molecular mimicry recently has been implicated in the pathogenesis of MS. In addition, Tejada-Simon et al reported that an identical sequence was found in both myelin basic protein and HHV-6; they showed that greater than 50% of T cells recognizing residues 93 to 105 of myelin basic protein cross-reacted with and could be activated by a synthetic peptide corresponding to residues 1 to 13 of HHV-6 U24 in MS patients. This study provides important evidence in the understanding of the potential role of HHV-6 in the activation of autoimmune reactivity.

When we analyzed the possible relationship between HHV-6 active infection and the disease activity, we found that 7 (22%) of 32 patients with RRMS in active relapse had HHV-6 active replication, whereas only 1 (3%) of 32 had viral latency; the median viral load was almost 30% higher during an acute attack than remission. These results led us to think that there is a correlation between HHV-6 active infection and the relapses in RRMS, whereas HHV-6 latent infection is related to the stable phase of the disease. The HHV-6 variant study was conclusive, because only HHV-6A was found among patients with RRMS who had an active infection. Regarding EBV, our results differ from those who described a possible association with MS. The prevalence and viral load results for EBV were similar in patients with RRMS and in the control group, and no difference between both phases of the disease was detected. These results were confirmed with the parallel study of the cell-free HHV-6 DNA in serum samples.

In conclusion, our results suggest that HHV-6A active replication is implicated in a subset of patients with RRMS, and specifically in relapses, showing that HHV-6A active infection increases the risk of an exacerbation in these individuals. We believe cerebrospinal fluid analysis would be particularly suitable in future studies, because this specimen would probably add some valuable information regarding the role of HHV-6 in the pathogenesis of MS.

Accepted for Publication: December 30, 2003.
Correspondence: Roberto Álvarez-Lafuente, MD, Laboratorio de Investigación 7a Norte, Hospital Clínico San Carlos, C/ Profesor Martín Lagos s/n, 28040 Madrid, Spain (labesmul@hcsc.es).

Author Contributions: Study concept and design: Álvarez-Lafuente, De las Heras, and Arroyo. Acquisition of data: Álvarez-Lafuente and Bartolomé. Analysis and interpretation of data: Álvarez-Lafuente, De las Heras, Picazo, and Arroyo. Drafting of the manuscript: Álvarez-Lafuente and Bartolomé. Critical revision of the manuscript for important intellectual content: De las Heras, Picazo, and Arroyo. Statistical expertise: De las Heras. Obtained funding: Arroyo. Study supervision: Picazo and Arroyo.

Funding/Support: This study was supported by Fundación des Esclerosis Múltiple, Barcelona, Spain, and Fundación Alfonso Martín Escudero, Madrid.

Acknowledgment: We thank Ms María Concepción Ramírez and Ms María Jesús Díez, who collected the specimens.
REFERENCES