Multiple Sclerosis and Human Herpesvirus 6

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Abstract

Background: A possible but as yet unproven relationship has been proposed between the onset or persistence of multiple sclerosis (MS) symptoms and herpesviruses, including, most recently, human herpesvirus 6 (HHV-6). A study was conducted to investigate the presence of HHV-6 DNA and the synthesis of antibodies against HHV-6, cytomegalovirus (CMV) and Epstein-Barr virus (EBV) in serum and cerebrospinal fluid (CSF) of patients with MS.
Materials and Methods: PCR and ELISA were used to detect HHV-6 DNA and specific antibodies against HHV-6, CMV and EBV in 211 samples (139 sera and 72 CSF). There were three groups of samples: group I, paired samples of serum and CSF from 41 MS patients; group II, paired samples of serum and CSF from 31 patients with neurological diseases other than MS (OND); group III, 67 serum samples from 27 different MS patients undergoing serologic follow-up.
Results: No HHV-6 DNA was found in any sample. Group I sera showed elevated anti-HHV-6 IgG and IgA levels. In group II, anti-CMV IgG was detected in one CSF sample and anti-HHV-6 IgM in one serum sample. Group III sera showed high concentrations of anti-HHV-6 IgG, IgA and IgM.
Conclusion: Given the clinical implications of the presence of antibodies against HHV-6 in MS patients, a viral reactivation cannot be excluded as an environmental factor.

Key Words

Herpesvirus · Multiple sclerosis · HHV-6 · CMV · EBV

Infection 2002; 30: 145–149
DOI 10.1007/s15010-002-2056-7

Introduction

Humans are the primary host for the eight known species of human herpesvirus (HHV). The implication of these species in multiple sclerosis (MS) has yet to be definitively ruled out and remains under investigation [1, 2]. HHV species, including HHV-6, can be latent, can periodically reactivate and most are neurotropic and induce demyelination in animal models [3, 4]. Among other sites, HHV-6 has been detected in oligodendrocytes of plaques with demyelination, [5–7].

This study investigated the presence of HHV-6 and the synthesis of antibodies against HHV-6, cytomegalovirus (CMV) and Epstein-Barr virus (EBV) in serum and CSF of MS patients. Sparse studies have been published on the joint application of direct and indirect methods to detect herpesvirus in MS patients. This dual approach reveals the viral status at the moment of the study as well as at earlier stages of the disease. The study of antibodies against the three viruses was designed to reveal whether there was a nonspecific response to all three or a specific response to each.

Materials and Methods

211 samples (139 sera, 72 CSF) were extracted from patients recruited at two regional referral centers in southern Spain (University Hospital Carlos Haya, Malaga: group I and II patients; University Hospital San Cecilio, Granada: group III patients).

Group I

Paired serum and CSF samples were collected from 41 patients (23 females, 18 males) with MS (32 with relapsing-remitting MS (RRMS), five secondary progressive MS (SPMS) and four primary progressive MS (PPMS). Mean patient age and mean disease evolution time were 39.2 ± 12.7 years and 8.9 ± 8.1 years, respectively. The neurological dysfunction of these patients was assessed by means of the Expanded Disability Status Scale (EDSS) [8].

Group II

Paired serum and CSF samples were collected from 31 patients (18 females, 13 males) with other neurological diseases (OND) (six with idiopathic intracranial hypertension, three amyotrophic lat-

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Received: April 5, 2001 • Revision accepted: January 22, 2002

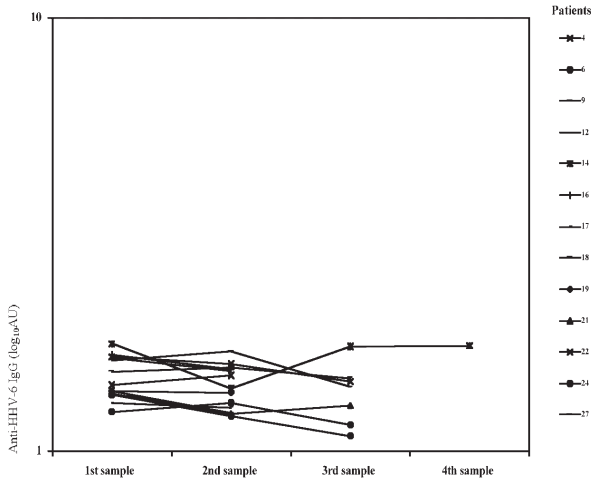

Figure 1. Anti-HHV-6 IgG results in followed-up MS patients (group III).

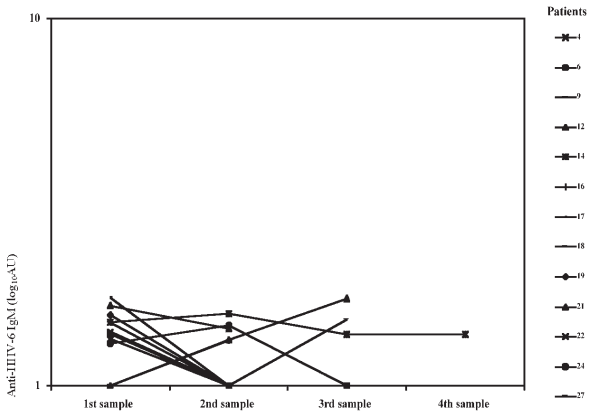

Figure 2. Anti-HHV-6 IgM results in followed-up MS patients (group III).

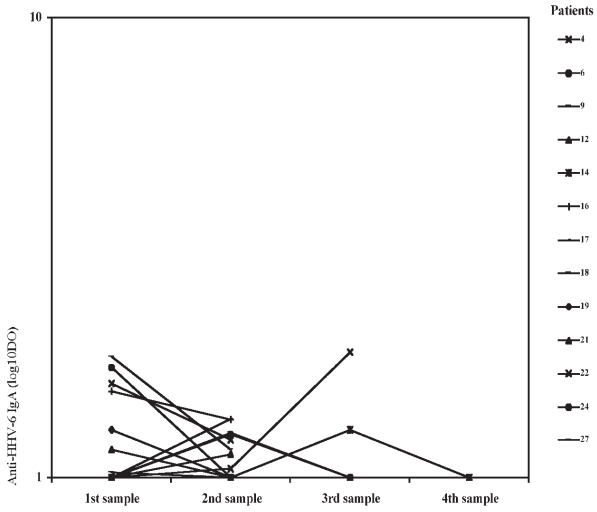

Figure 3. Anti-HHV-6 IgA results in followed-up MS patients (group III).

eral sclerosis, seven cerebral vascular disease, three Guillain-Barré syndrome, seven epilepsy, two chronic inflammatory demyelinating polyneuropathy, two dementia and one neurosyphilis). Mean patient age was 48.2 ± 16.2 years. This group was used as control.

Group III

67 serum samples were collected from 27 patients (15 men, 12 women) with MS (17 RRMS and 10 SPMS) undergoing clinical and serologic follow-up. Two to four serum samples were extracted from each patient during the course of the disease (Figures 1, 2, 3). The evolution of antibody titers during the follow-up was recorded. Mean patient age and mean disease evolution time were 35 ± 10 years and 9 ± 8.3 years, respectively.

Group I and II samples were investigated for the presence of specific anti-HHV-6 IgG, IgM and IgA, specific anti-CMV and EBV IgG and IgA and for HHV-6 DNA. When antibodies were detected in CSF samples, their protein and cell concentrations were also determined in order to rule out rupture of the blood-brain barrier (normal: ≤ 0.45 mg proteins/dl and ≤ 5 cells/µl). Group III sera were investigated for the presence of IgG, IgM and IgA antibodies against HHV-6. There was no statistical difference in patient age between the three groups under study ($p = 0.085$, Student's t-test).

HHV-6 DNA Detection

A specific HHV-6 DNA polymerase gene sequence fragment was investigated with a commercial kit (Herplex®, Pharmagen, Spain) [9] from serum or total CSF. Primers: 5'-GAGGTAATTTATGGTGATACGGA-3', 5'-TGTCTACCAATGTATCTTTTTTT-3', probe for amplicon detection: 5'-CTCTGCGAAGGATTGCGCCGA-3'. The test was able to detect the presence of both HHV-6 variants (A and B). The computation was performed at the Swiss Institute of Bioinformatics using the BLAST network service. The reported sensitivity is 100 genome copies [10]. In order to further improve the sensitivity of the assay, the reliability of negative results was verified by purifying and concentrating the DNA by ultrafiltration (DNA Purification kit, Pharmagen, Spain) and repeating the assay. To avoid false positive amplifications, procedures recommended to prevent contamination were meticulously observed [11].

Detection of Specific Antibodies Against HHV-6, CMV and EBV

The tests were performed automatically using a sample pipettor (Tecan, Megaflex, Switzerland) and an ELISA plate processor (Dade Behring ELISA Processor III, Behringwerke, Germany). The reproducibility of the results was previously tested by repeating the procedure in the first 25% of samples, accepting variations of ≤ 5% in the absorbance results.

HHV-6 IgM ELISA Test (PanBio, Australia). Samples were prediluted 1 : 10 in serum diluent (Tris-buffered saline) and diluted 1 : 10 in specimen absorbent (goat anti-human IgG precipitating antibody preparation in Tris-buffered saline) (RF-Absorbent, Behringwerke, Germany). The samples (1 : 100 diluted), controls and calibrators were dispensed into HHV-6 purified antigen-coated polystyrene wells. A horseradish peroxidase conjugated sheep anti-human IgM was used as secondary antibody. Tetramethylbenzidine (TMB) was added as substrate. The stop solution was 1M phosphoric acid and the absorbance was read at

450 nm/600-650 nm. The results were interpreted semi-quantitatively by the calculation of arbitrary units (AU = [sample absorbance/mean calibrator absorbance] × 10). AU < 20 was regarded as a negative result and AU ≥ 20 as positive.

In-House IgA Indirect ELISA Test. The IgA assays were carried out using a homemade indirect ELISA [12]. The samples were diluted (1 : 40) with a mixture (1/1) of phosphate buffer saline (PBS) and anti-IgG antibody solution (RF-Absorbent). 200 ml of each diluted specimen were incubated in a microtiter plate containing specific HHV-6 antigens (PanBio, Australia). Antihuman IgA conjugate (Anti-human IgA-POD, Behringwerke, Germany) was diluted 1 : 50 with PBS buffer and added to each well. The substrate was a mixture (1/10) of TMB (5 g/l) and hydrogen peroxide (0.1 g/l) (Supplementary Reagents for Enzygnost/TMB, Behringwerke, Germany). The results were positive when sample absorbance (A_{450}s) was equal to or greater than the mean cutoff calibrator absorbance (A_{450}c) and negative when it was lower.

HHV-6 IgG ELISA Test. We followed a procedure previously published by *Sloots* et al. [13].

Specific Anti-CMV and EBV IgG and IgA Tests. The antigens and procedures used in these tests were previously published by our group [14, 15].

Statistics

The relationships between each quantitative variable and disease were analyzed with the Student's t-test. Fisher's exact χ^2 test was used to associate percentages of positivity with the qualitative variables. The Wilcoxon test was employed to study the correlation between antibody titers and the sequential order of followed-up samples. Data were processed with SPSS for Windows, version 6.01 (SPSS Inc, Chicago, Illinois, USA) [16]. The prevalence of antibodies is expressed at 95% CIs.

Results

No HHV-6 DNA was detected in any sample studied, either in serum or CSF. Table 1 lists antibodies against each virus detected with ELISA in each group.

In group I, elevated anti-HHV-6 IgA was detected in five samples, four from patients with RRMS and one from a patient with SPMS. Anti-HHV-6 IgG was detected in one CSF sample (2.4%) from a woman with RRMS who also showed IgG in serum. However, her CSF sample contained 2 mg proteins/dl and 57 cells/µl and the IgG may have derived from the serum. No anti-HHV-6 IgM was detected, although sera from two women with RRMS were close to the cutoff point (mean value = 19.15 AU). In group I samples, the antibodies detected were not related to age, evolution time, degree of disability ($p > 0.05$; Student's t-test), gender, or clinical form of the disease ($p > 0.05$; Fisher's χ^2 test).

In group II, anti-CMV IgG was detected in one CSF sample (3.4%) from a 53-year-old woman with chronic inflammatory demyelinating polyneuropathy who also showed anti-CMV IgG in serum. However, the CSF sample contained 1mg proteins/dl and 34.4 cells/µl and the IgG may have been serum derived. One group II serum sample showed anti-HHV-6 IgM with a value around the cutoff (21 AU), corresponding to a 53-year-old woman with idiopathic intracranial hypertension who also had anti-HHV-6 IgG in serum.

No IgA or IgM was detected for any of the three viruses in CSF samples from groups I and II.

Group III results for patients with at least one IgM-positive sample (13/27 patients) are shown in figures 1–3. Elevated concentrations of anti-HHV-6 IgG, IgA and IgM antibodies were detected in group III sera, although they revealed no specific behavior pattern over the course of the disease (Wilcoxon test).

Discussion

The prevalence of anti-HHV-6 IgG in sera from our MS patients is similar to that reported by *Braun* et al. [1]. The significant differences between MS patients and controls are

Table 1
Results obtained in serum samples of MS patients (groups I and III) and controls (group II).

	Geometric mean (CI)	% positive	Student's t-test (p) Group I	Group II
Anti-HHV-6 IgG (AU)				
MS (group I)	16.9 (14.3, 19.9)	53.8		
OND (group II)	13.4 (11.0, 16.3)	40	< 0.05	
MS (group III)	16.1 (13.1, 19.7)	100		< 0.005
Anti-CMV IgG(titer)				
MS (group I)	7,793 (5,468.9, 11,104.7)	78.4	0.0869	
OND (group II)	7,209.2 (49,006.3, 10,592.9)	85.2		
Anti-EBV IgG (IU/ ml)				
MS (group I)	219.6 (162.1, 297.4)	91.9	0.955	
OND (group II)	157.8 (116.6, 213.4)	96.3		
Anti-HHV-6 IgA (AU)				
MS (group I)	4.79 (3.45, 6.64)	15.6		
OND (group II)		0	< 0.005	
MS (group III)	5.8 (2.94, 11.41)	49.3		< 0.001
Anti-CMV IgA (OD)				
MS (group I)	1.87 (1.40, 2.49)	26.5	0.237	
OND (group II)	1.70 (1.50, 1.93)	24.1		
Anti-EBV IgA (OD)				
MS (group I)	1.43 (1.28, 1.59)	14.7	0.474	
OND (group II)	1.24 (1.07, 1.43)	13.8		

HHV-6: human herpesvirus 6, CMV: cytomegalovirus; EBV: Epstein-Barr virus (EBV); AU: arbitrary units; OD: optical density; group I: MS samples not followed-up; group II: control samples; group III: MS samples followed-up. MS: multiple sclerosis; OND: other neurological diseases, CI: confidence interval.

in line with findings of *Sola* et al. [17] but not with those reported by *Nielsen* et al. [18].

The absence of anti-HHV-6 IgM in the MS patients of group I is not consistent with reports by *Soldan* et al. [19] and *Friedman* et al. [20], although our findings in sera from the followed-up MS patients (group III) were in line with their studies. It is possible that the IgM detected in their studies reflected a non-specific clonal stimulation of B cells and may not have been true antiviral IgM; in our followed-up patients, the status of the IgM was confirmed by subsequent IgG elevation. Anti-HHV-6 IgA was present in many of the group I and III sera, which may perhaps reflect a previous reactivation of the virus, with or without clinical relevance. No comparable studies on IgA have been published. Anti-HHV-6 IgG was only detected in one CSF sample, well below the prevalence reported by *Ongradi* et al. [21] and *Ablashi* et al. [22].

Regarding the detection of HHV-6 DNA, no genome sequences were detected in group I samples from MS patients, similar to findings of *Martín* et al. [23] and of *Álvarez* et al. [24]. *Goldberg* et al. [25] detected HHV-6 DNA in only one out of 15 serum samples from a patient with SPMS. These findings may suggest that the presence of HHV-6 DNA is not a common event in sera or CSF from MS patients. *Enbom* et al. [26] reported a low prevalence of HHV-6 DNA in CSF from MS patients. In an earlier study [27] on CSF, 14% of MS patients were found to be positive for HHV-6 DNA. The present findings cannot be attributed to PCR inhibition because the Aujezsky virus gene was co-amplified for each sample as internal control. However, the degree of replication of the virus was probably influenced by the type of sample. HHV-6 DNA detection in peripheral blood mononuclear cells (PBMC) has no clinical relevance because the virus can be latent in PBMC or salivary glands [28] and its presence does not discriminate between active infection and latent stages. The same is true for brain tissue, because of the marked neurotropism of HHV-6. It is also possible that an HHV-6 reactivation could produce encephalitis with no presence of viral DNA in the CSF [29]. Thus, serum or plasma samples seem more appropriate to discriminate the status of the infection, although it remains an open question whether positive samples could be detected by a more sensitive method than that used in the present study. On the other hand, it has been shown that there is no relationship between HHV-6 DNA in serum and HHV-6 serological status in individuals over 2 years of age [30].

Our findings for anti-CMV IgG in sera of MS patients are consistent with those obtained by *Myhr* et al. [31] and our results for anti-EBV IgG are in line with those published by *Munch* et al. [32], *Myhr* et al. [31] and *Wandinger* [33]. However, unlike us, *Wandinger* [33] observed a significant presence of IgM and IgA in serum. We detected anti-CMV IgG in only one CSF sample, in contrast to reports by *Izquierdo* et al. [34]. Unlike *Rand* et al. [35], we found no anti-EBV IgA or IgG in CSF samples.

Our failure to detect HHV-6 DNA was probably due to an inadequate replication of the viral particle at the time of the detection. The significant presence of anti-HHV-6 IgM and IgA in the followed-up patients (group III) could have been due to a replication of the viral particle at an earlier time, as shown by the IgG detection in the followed-up samples. The higher concentrations of anti-HHV-6 versus anti-CMV or anti-EBV antibodies in sera from MS patients may indicate that the increased humoral response to HHV 6 is not simply a side effect of disorders associated with MS but is rather a potential cofactor of these disorders. Otherwise, there would have been a generalized increase in the response to each antibody. Given the serologic difference in anti-HHV-6 antibodies from the MS patients, more manifest in the serologic follow-up, an HHV-6 reactivation may be an environmental factor that could, alongside individual genetic predisposition, alter the normal functioning of the immune system. This alteration may induce an autoimmune response that could produce the characteristic inflammation and demyelination of MS. Finally, we cannot rule out the participation of CMV and EBV in MS, because they were not investigated in the longitudinal study.

Acknowledgments

This study was supported by the Health Council of Andalusian Regional Goverments, project 141/2001 and the Spanish Federation of Multiple Sclerosis. Ms. Maria José Soto performed the documentation scientific work.

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