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Evidence for human herpesvirus 6 variant A antibodies in multiple sclerosis: diagnostic and therapeutic implications

JO Virtanen,[1,2] M Färkkilä,[3] J Multanen,[3] L Uotila,[4] AJ Jääskeläinen,[1,2] A Vaheri,[1,2] and M Koskiniemi[1]

[1]*Department of Virology, Haartman Institute, University of Helsinki, Helsinki, Finland*
[2]*Department of Virology, HUSLAB Laboratory Services, Helsinki, Finland*
[3]*Department of Neurology, Helsinki University Central Hospital, Helsinki, Finland*
[4]*Department of Clinical Chemistry, HUSLAB Laboratory Services, Helsinki, Finland*

Human herpesvirus 6 (HHV-6) has been linked to the pathogenesis of multiple sclerosis (MS). HHV-6 antibodies in serum and cerebrospinal fluid (CSF) of 27 patients with clinically definite MS (CDMS) were compared with age- and sex-matched controls, including various other neurological diseases and symptoms (OND). In addition, we studied a series of 19 patients with clinically or laboratory supported possible MS (CPMS). Seroprevalence to HHV-6A was 100% in patients with MS, both in CDMS and CPMS, compared to 69.2% in patients with OND ($P = .001$ and .007). The mean immunoglobulin G (IgG) titers were significantly higher in patients with CDMS and CPMS than in controls ($P = .005$ and .00002). The proportion of acute primary infections without CSF involvement was similar in all groups; however, primary infections with intrathecal HHV-6 antibody production were more frequent in MS. In CSF, HHV-6A–specific antibodies were present in three (11.5%) and four (21.1%) patients with CDMS and CPMS, compared to none with OND ($P = .06$ and .01, respectively). Serological suggestions to HHV-6A infection occurred more often in both CDMS and CPMS than in OND (14.8% versus 21.1% versus 3.8%). We conclude that a subpopulation of MS patients, and even a greater proportion of possible MS subjects, has serological evidence of HHV-6A infection, which might provide new markers for diagnosis and therapy. *Journal of NeuroVirology* (2007) **13,** 347–352.

Keywords: cerebrospinal fluid; human herpesvirus 6; HHV-6A; intrathecal antibody production; multiple sclerosis

Introduction

Viral studies in multiple sclerosis (MS) have been the focus of interest for years (Panitch, 1994). Several microbes have been associated with the disease, including *Chlamydia pneumoniae*, and lately Epstein-Barr virus (DeLorenze *et al*, 2006). Human herpesvirus 6 (HHV-6) has been one of the main candidates (Challoner *et al*, 1995; Friedman *et al*, 1999; Chapenko *et al*, 2003). HHV-6 was discovered 20 years ago (Salahuddin *et al*, 1986) and its impact has been increasing, especially in MS and other neurological disorders (Campadelli-Fiume *et al*, 1999; Moore and Wolfson, 2002).

HHV-6 has the intriguing property to modulate the immune respone because it infects immune cells (Ablashi *et al*, 1987). Two variants of HHV-6 are known, HHV-6A and HHV-6B (Braun *et al*, 1997). Variant B has been identified as the causative agent of exanthema subitum (Yamanishi *et al*, 1988). The

Address correspondence to Jussi Oskari Virtanen, Department of Virology, Haartman Institute, P.O.B. 21 FIN-00014, University of Helsinki, Finland. E-mail: oskari.virtanen@helsinki.fi

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disease associations of variant A have remained elusive, although the originally isolated HHV-6 strain was A (GS) (Salahuddin *et al*, 1986).

Both variants HHV-6A and HHV-6B are widely distributed in brain (Chan *et al*, 2001; Cuomo *et al*, 2001); however, variant A may be more predominant (Hall *et al*, 1998), and in the cerebrospinal fluid (CSF) HHV-6A has been identified more frequently than HHV-6B. The reports have been mainly based on nucleic acid detection by polymerase chain reaction (PCR). Results on variant-specific serological studies are lacking.

HHV-6A seems to be associated more closely with MS than with any other neurological disease (Akhyani *et al*, 2000; Moore and Wolfson, 2002). The reports on the association between HHV-6 and MS are based on antigen or nucleic acid detection and antibody findings (Friedman *et al*, 1999; Knox *et al*, 2000; Alvarez-Lafuente *et al*, 2004, 2006; Rotola *et al*, 2004; Virtanen *et al*, 2005). The timing of infection, although it has been suggested an active infection in early disease (Knox *et al*, 2000; Rotola *et al*, 2004), as well as the variant specificity have remained uncertain. Anyhow, using mRNA as a marker of active HHV-6 infection, variant A has been detected in the blood of patients with relapsing and remitting MS (RRMS) (Alvarez-Lafuente *et al*, 2004, 2006) and in CSF cells (Rotola *et al*, 2004).

Using an immunofluorescence test for both HHV-6A and HHV-6B variants, we show here a significantly higher seroprevalence to HHV-6A in patients with MS than with other neurological diseases, and frequent presence of intrathecal antibody production (ITAP) to HHV-6A.

Results

HHV-6A and HHV-6B antibodies in serum

The prevalence of HHV-6A immunoglobulin G (IgG) antibodies in serum was 100% in patients with MS, both with clinically definite MS (CDMS) and clinically or laboratory supported possible MS (CPMS), compared to 69.2% in patients with OND ($P = .001$ and .007, respectively) (Table 1). The mean IgG titers were significantly higher in patients with CDMS and CPMS than in the other neurological diseases and symptoms (OND) group ($P = .005$ and .00002) (Figure 1). High levels of antibodies (>100 reciprocal titer) appeared in patients with MS but in no case in the reference group. For HHV-6B IgG antibodies, no significant differences appeared (Table 1). HHV-6 antibodies appeared variant-specific in immunofluorescence (Figure 2).

Table 1 Antibody findings to HHV-6A and HHV-6B from serum and CSF of patients with confirmed or possible MS (CDMS or CPMS) and reference group of other neurological diseases (OND)

	CDMS (n = 27)	*CPMS (n = 19)*	*OND (n = 27)*
S-HHV-6A IgG + (%)	27 (100)*	19 (100)†	18 (69.2)
Primary infection (%)	2 (7.4)	3 (15.8)	1 (3.8)
CSF-HHV-6A IgG (%)	3 (11.1)‡	4 (21.1)§	0 (0)
S-HHV-6B IgG + (%)	10 (37.0)	4 (21.1)	10 (38.5)
Primary infection (%)	0 (0)	0 (0)	1 (3.8)
CSF-HHV-6B IgG (%)	0 (0)	0 (0)	0 (0)

P values CDMS or CPMS versus OND: * $P = .001$; † $P = .007$; ‡ $P = .06$; § $P = .01$; chi-square test.

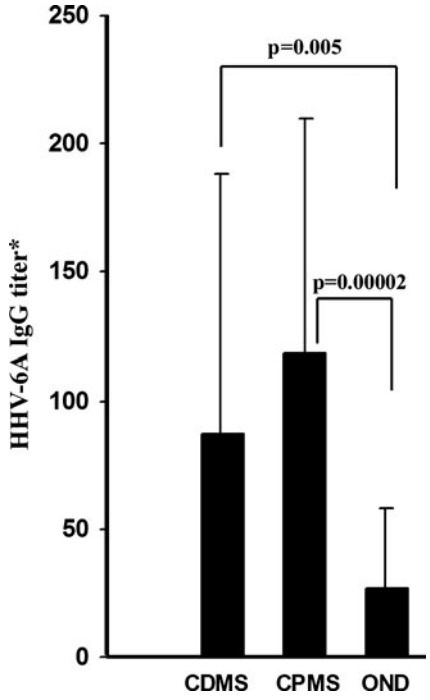

Figure 1 The level of specific HHV-6A IgG antibodies was significantly higher (two-tailed Student's *t* test) in both clinically defined and possible MS (CDMS and CPMS) compared to patients with other neurological diseases (OND). *Reciprocal of titer.

Acute primary infections

Low avidity of HHV-6A IgG antibodies was observed in two patients with CDMS and three with CPMS, suggestive of acute primary infection, compared to one patient with OND (Table 1). One acute primary infection to HHV-6B appeared in the group with OND in a patient with polyneuropathy but not in the MS groups.

HHV-6A antibodies in CSF and ITAP

HHV-6A IgG antibodies in the CSF were detected in three patients with CDMS. One of them had serum/CSF antibody ratio of 20, and antibody index (AbI) was 7.56, both suggestive of specific ITAP. In two patients the serum/CSF ratio was unremarkable, but one of them had an abnormal AbI 2.023. HHV-6A IgG antibodies were present in four patients with CPMS compared to none in OND ($P = .01$) (Table 1, Figure 3). In three the AbI was abnormal and two of them also had an acute primary HHV-6 infection. HHV-6B IgG antibodies were not detected in the CSF.

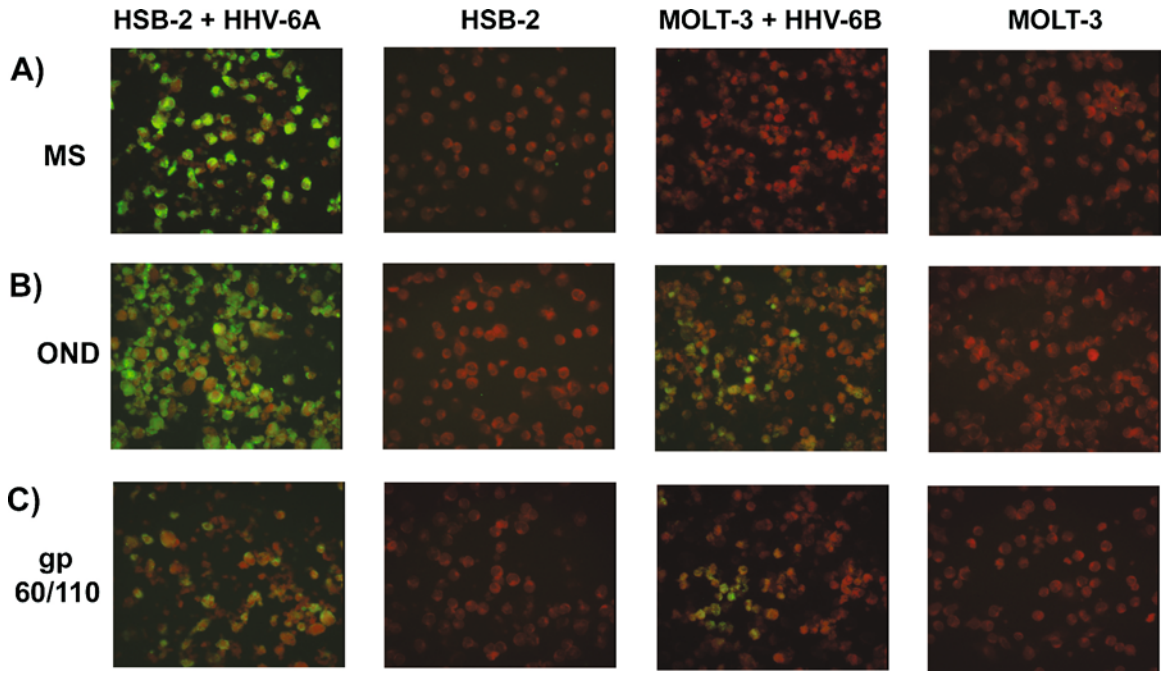

Figure 2 Representative image of immunofluorescence analysis of one patient with MS (**A**) and one with OND (**B**). Both sera reacted with HHV-6A–infected HSB-2 cells. Serum of the patient with MS did not react with HHV-6B–infected cells, but serum of the patient with OND did, suggesting the specificity of the HHV-6 antibodies in sera. Uninfected control cells did not give any signal. Monoclonal antibody to HHV-6 gp60/110 reacted with both variants, but not with uninfected control cells (**C**).

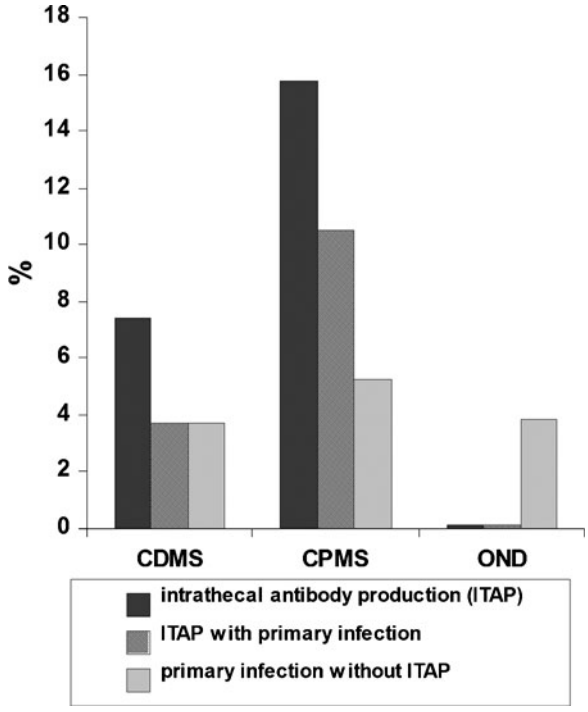

Figure 3 Intrathecal HHV-6 variant A–specific antibody production in patients with MS was significant both in CDMS and CPMS. No patient with other neurological diseases (OND) had specific antibodies in CSF. The number of primary infections without intrathecal antibody production was similar in all groups.

HHV-6A serological results

Serological evidence for HHV-6A infection was observed in 4 of 27 (14.8%) patients with CDMS and in 4 of 19 (21.1%) patients with CPMS, compared to 1 of 27 (3.8%) patients with OND, when combining the results of acute primary HHV-6A infection and/or intrathecal HHV-6A–specific antibody production.

Herpesviridae DNA

Specific DNA was not detected from CSF or serum samples when using multiplex-PCR and oligonucleotide microarray, except one CSF of the reference patients with OND contained HSV-1 DNA.

Discussion

HHV-6A–specific IgG antibodies were found in serum of all patients with MS compared to 2/3 of controls, and the levels were significantly higher in MS. The level was most prominent in patients with CPMS. This group might represent patients in transition to CDMS. In addition, two patients with CDMS, with a relapsing disease course, and three patients with CPMS, had intrathecal HHV-6A antibody production. This raises a question of antiviral therapy for patients with menacing MS, although it might be possible that HHV-6 activation in MS is a consequence, not a cause. Even in this scenario, HHV-6 might contribute to the course of the disease. Carefully designed clinical

trials are urgently needed to clarify the role of HHV-6 in MS.

Previously Derfuss *et al* (2005) reported intrathecal HHV-6 antibody production in 21% of MS patients when using an antibody index. In our series the frequency of intrathecal antibody production to HHV-6A was nearly the same, 11% in CDMS and 21% in CPMS. As criteria, we used the serum (S)/CSF antibody ratio (Levine *et al*, 1978) and HHV-6–specific AbI (Deisenhammer *et al*, 2006). The report by Derfuss *et al* (2005) did not distinguish between the HHV-6A and -6B variants. We emphasize the presence of intrathecal HHV-6A antibody production.

Acute primary HHV-6A infection associated with CSF antibody finding occurred more often in patients with CDMS and in CPMS than in patients with OND. An active HHV-6 infection in patients with multiple sclerosis has been reported (Knox *et al*, 2000; Alvarez-Lafuente *et al*, 2004, 2006; Rotola *et al*, 2004). In three papers only HHV-6A was found among patients with active infection (Alvarez-Lafuente *et al*, 2004, 2006; Rotola *et al*, 2004). The active HHV-6 infection has been suggested to play a greater part in early disease (Knox *et al*, 2000; Rotola *et al*, 2004). Soldan *et al* (2000) showed a lymphoproliferative response to HHV-6A in 67% of the patients with MS and 78% to HHV-6B. In contrast, in their study only 33% of the healthy controls responded to HHV-6A lysate. Our findings on acute primary infections and ITAP to HHV-6A in 14.8% and 21.1% of the patients agree with their results, suggestive of the possible role of HHV-6A in early MS and in relapses, although cell-free samples were negative for specific DNA. Our serological findings are in line with reported nucleic acid and virus culture findings (Knox *et al*, 2000; Alvarez-Lafuente *et al*, 2004, 2006; Rotola *et al*, 2004), and together suggest that there might be a subpopulation of MS with HHV-6A infection, which might benefit from antiviral medication.

MS is predominantly regarded as an autoimmune disease (Grigoriadis and Hadjigeorgiou, 2006). Infections could present as triggers in genetically susceptible individuals. Previously others and we have detected HHV-6 antigen by immunohistochemistry in brain tissue of MS patients (Challoner *et al*, 1995; Friedman *et al*, 1999; Knox *et al*, 2000; Virtanen *et al*, 2005). In those studies the variant specificity was not defined. According to recent data and publications (Alvarez-Lafuente *et al*, 2004, 2006; Rotola *et al*, 2004), HHV-6A might be associated with MS.

In our survey, HHV-6A antibody titers were high and higher in MS than in controls. In contrast, titers to HHV-6B were low or undetectable, possibly reflecting the fact that HHV-6B infection, exanthema subitum, occurs early in life, and titers may decline with years (Yamanishi *et al*, 1988). With respect to HHV-6A the results were consistent and the patients with MS differed significantly from the reference group. A conspicuous finding was the specific intrathecal HHV-6A IgG production, in many cases already during the acute primary infection, but the highest antibody production appeared in patients with nonprimary infection.

Besides MS, many other chronic neurological diseases, including chronic fatigue syndrome (CFS), myelopathies, encephalopathies, and progressive neurological syndromes, both in children and in adults, may have etiological or associated relationship with HHV-6 (Josephs *et al*, 1991; Salonen *et al*, 2002; Hall *et al*, 2004; Ward *et al*, 2005). Especially in MS, the costs, both social and economic, are high. Besides interferon, antiviral compounds have been administered with the presumption that a virus may be associated. Friedman *et al* (2005) used in their clinical trial valacyclovir to treat MS patients. A positive effect was suggested in clinical picture, but not in magnetic resonance imaging (MRI). HHV-6 testing would be an essential part in such of trials.

We conclude that the prevalence and level of HHV-6A–specific IgG antibodies, primary infections based on the avidity of IgG, and ITAP can be measured from serum and CSF, and these tests may benefit both diagnostics and therapeutic assessment in a considerable subpopulation of MS.

Patients and methods

Patients

We studied 27 patients with clinically definite MS (CDMS), selected according to the criteria described by McDonald *et al* (2001). For comparison we had age- and sex-matched controls including various other neurological diseases and symptoms (OND), 10 male and 17 female patients. In addition, we had a series of 19 patients with clinical symptoms and signs or laboratory findings suggestive of MS but not fulfilling the clinically definite criteria, 5 male and 14 female patients. We designated them as clinically possible MS (CPMS).

Serum and CSF were obtained during a visit at the Neurological Outpatient Department of Helsinki University Central Hospital. The patients were evaluated to have a lumbar puncture for clinical reasons. The sample for the present study was obtained on the same occasion. The Ethics Committee of University of Helsinki and Helsinki University Central Hospital had approved the study. All patients were informed personally about this extra research sample and they gave written informed consent before the enrollment.

Cells and viruses

HSB-2 and MOLT-3 T cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), glutamine, and antibiotics. HSB-2 cells were infected with HHV-6 type A (strain GS) and MOLT-3 cells with HHV-6 type B (strain Z29). The viral antigen was purified by ultracentrifugation through a 40% *w*/*v* sucrose cushion at 24000 rpm

overnight in an SW28 rotor (Beckman). The variant specificity of HHV-6A and HHV-6B antigens was confirmed using immunoblot and microarray tests (Jääskeläinen *et al*, 2006).

Immunofluorescence avidity assays
Six to 10 days post infection, the cells were collected and washed with phosphate-buffered saline (PBS), pH 7.4, applied to diagnostic glass slides and fixed with ice-cold acetone. Monoclonal antibody to gp60/110 (MAB8537; Chemicon, CA, USA) recognizing both variants of HHV-6 was used for the detection of infected cells (Figure 2). Patients' sera and CSF were diluted serially and the reciprocal of the last dilution that gave a bright green fluorescence clearly above of the negative-control serum was determined to be the titer. Fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Monosan, Uden, The Netherlands) was used to detect bound HHV-6 antibodies, and cells were counterstained with Evans blue. For avidity of IgG antibodies the low-avidity antibodies were eluted by urea wash as described previously (Ward *et al*, 1993). A $\geq$12-fold decrease in titer was considered diagnostic for acute primary disease, 8- to 10-fold for equivocal, and $\leq$6 for past infection. To determine ITAP, serum and CSF titers were compared in the same dilution and given the S/CSF antibody ratio determined (Levine *et al*, 1978). Values $\leq$20 were regarded as a marker of ITAP. Antibody index (AbI) was estimated by dividing CSF Ab/S Ab with CSF IgG/S IgG (Deisenhammer *et al*, 2006). Values $\geq$1.5 were regarded as pathologic, to indicate specific HHV-6 antibody production in the central nervous system (CNS).

Herpesvirus multiplex-PCR and oligonucleotide microarray
DNA was extracted from 200 μl of serum ($n = 71$, 26 CDMS, 19 CPMS, and 26 OND) or CSF ($n = 49$, 16 CDMS, 15 CPMS, and 18 OND) samples using High Pure Viral Nucleic Acid Kit (Roche Applied Science, Basel, Switzerland). Multiplex-PCR and microarray for herpes simplex virus (HSV)-1, HSV-2, cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella-zoster virus (VZV), HHV-6A, HHV-6B, and HHV-7 was carried out as published (Jääskeläinen *et al*, 2006) with modifications (Jääskeläinen *et al*, manuscript in preparation). Briefly, part of the DNA polymerase gene was amplified by multiplex-PCR. The amplified product was transcribed to single-stranded RNAs and hybridized to specific oligonucleotides on microarray. Commercial specific viral DNA samples were used as controls in each microarray experiment.

Statistical tests
Chi-square test was used to determine statistically significant differences between the groups. Two-tailed student's *t* test was used to determine the statistically significant differences between the mean titers of each group. P value $<.05$ was considered statistically significant.

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