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Short Communication

Association of human herpesvirus 6 and human herpesvirus 7 with demyelinating diseases of the nervous system

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Peripheral blood mononuclear cells and plasma of 113 patients with neurological disorders and 150 blood donors were analyzed for HHV-6 and HHV-7 sequences by PCR. The prevalence of HHV-6 was significantly higher in patients with multiple sclerosis ($P < 0.01$) than in cases of nondemyelinating diseases of the central and demyelinating diseases of the peripheral nervous systems and blood donors. HHV-6 viremia was found only in patients with multiple sclerosis, predominantly in the active phase of the disease. A significantly higher frequency of HHV-7 reactivation in patients with demyelinating diseases of the peripheral nervous system suggests also its association with demyelinating processes. *Journal of NeuroVirology* (2001) **7,** 564–569.

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The ubiquitous T-lymphotropic human betaherpesviruses HHV-6 and HHV-7 were first reported in 1986 and 1990, respectively (Salahuddin *et al*, 1986; Frenkel *et al*, 1990). In the adult population, HHV-6 and HHV-7 infections are present in a high proportion: the prevalence of HHV-6 in Western Europe, North America, South Asia ranges between 30% and 90%, and for HHV-7 can reach 98% (Rojo *et al*, 1995; Wilborn *et al*, 1995; Aberle *et al*, 1996; Thawaranantha *et al*, 1999). Primary infection with these viruses occurs in early childhood and remains latent lifelong. Infection is generally asymptomatic in healthy immunocompetent adults, and one of the main reasons of virus reactivation is suppression of the immune system (Carrigan and Knox, 1999; Clark *et al*, 2000).

HHV-6 has been identified as the causative agent of childhood exanthem subitum (Yamanishi *et al*, 1988; Hall *et al*, 1994). The association of HHV-6 with other diseases is under discussion (Braun *et al*, 1997; Campadelli-Fiume *et al*, 1999). The possible association of HHV-6 with multiple sclerosis (MS) is suggested by its neurotropism and probable capacity to induce demyelinization (Caserta *et al*, 1994; Challoner *et al*, 1995; He *et al*, 1996; Soldan *et al*, 1997).

HHV-7 is closely related in sequence and gene arrangement to HHV-6 (Nicholas, 1996). Moreover, primary infection by HHV-7 is also associated with exanthem subitum (Tanaka *et al*, 1994; Asano *et al*, 1995; Caserta *et al*, 1998). Exanthem subitum has been described to be caused by HHV-7 and associated with disturbances of the central nervous system (CNS) (Torigoe *et al*, 1996; van den Berg *et al*, 1999). It can be assumed that this virus also possesses neurotropism, like HHV-6, but the clinical spectrum of HHV-7 infection has not yet been defined.

This work aims to examine the possible association of HHV-6 and HHV-7 infection with demyelinating diseases of the CNS and the peripheral nervous system (PNS). The main clinical models and intensively investigated forms of inflammatory demyelinization in CNS are considered to be MS, in PNS-acute inflammatory demyelinating polyneuropathy as a form of Guillain-Barré syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy.

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A total of 75 randomly selected patients with inflammatory demyelinating diseases of the CNS ($n = 56$) and PNS ($n = 19$), as well as 38 patients with nondemyelinating disorders of the CNS ($n = 21$) and PNS ($n = 17$) were examined. Of these, 40.4% were men and 59.6% were women, aged between 16 and 66 years (mean age 41). All patients with demyelinating disorders of the CNS met Poser's clinical criteria for MS (Poser *et al*, 1983); diagnosis was confirmed by magnetic resonance imaging and laboratory studies. A total of 35 of 56 MS cases were classified as relapsing/remitting type and 21 as chronic progressive (1 primary and 20 secondary progressive). The patient group with demyelinating diseases of the PNS consisted of 15 patients with acute inflammatory demyelinating polyneuropathy, all of whom met the criteria of Asbury and Cornblath (1990) for GBS, and 4 with chronic inflammatory demyelinating polyneuropathy. The type of nerve damage was estimated by nerve conduction studies.

The patient group with nondemyelinating disorders of the CNS included patients with motor neuron diseases, stroke, inherited ataxia, and vascular myelopathy. Peripheral nondemyelinating neuropathies were represented by spondilogenic radiculopathies, entrapment, and vibration-exposed and diabetic peripheral neuropathies.

During February 1997–April 1998, blood samples of 113 patients were collected at the Department of Neurology of the Latvian Medical Academy. The control group consisted of 150 randomly selected blood donors. Out of these, 48.7% were men (mean age 34.5) and 51.3% were women (mean age 40.2). In March 1998, donor blood samples were taken at the State Blood Donors Centre.

Cohorts were established with the approval of the local Ethics Committee and, prior to their examination, all participants provided informed consent. The diagnosis of virus infection in our study was based on the detection of viral DNA in the peripheral blood mononuclear cells (PBMC) by PCR. The detection of viral DNA in blood plasma was used to assess the active stage of the infection.

DNA was extracted from cultured PBMC and blood plasma. PBMC were separated from EDTA anticoagulated blood samples by Ficoll-Hypaque gradient centrifugation and maintained for 72 h in RPMI-1640 culture medium supplemented with 20% FCS, 0.1 U/ml recombinant human interleukin-2, gentamicin (40 μg/ml) at 37°C in a CO_2 incubator. After cultivation, cells were washed three times in phosphate buffered saline and lysed in 80 μl of 5× proteinase K buffer (0.375 M NaCl, 0.12 M EDTA pH 8.0), 20 μl of 20% SDS, 30 μl of proteinase K (10 mg/ml), and 240 μl of deionized water (to the final volume 400 μl) followed by incubation at 55°C for 1 h. DNA was extracted by phenol-chloroform treatment, precipitated by ethanol and dissolved in 50–150 μl deionized water. The QIAamp Blood Kit (Qiagen, Germany) was used for extraction of DNA from plasma. A globin PCR was performed to assure the quality of the extracted DNAs (Vandamme *et al*, 1995).

Nested PCR assays were used for the detection of HHV-6 and HHV-7 infection. PCR was performed in the presence of DNA from PBMC of individuals and their blood plasma. An amount of 1 μg of DNA was amplified in the final volume of 50 μl in the presence of PCR buffer (Fermentas, Lithuania), 0.2 mM of each deoxyribonucleoside triphosphate, 1.5 mM $MgCl_2$, 1 U Taq DNA polymerase (Fermentas, Lithuania), and 0.2 μM of each specific primer. DNAs isolated from HHV-6B (Z-29) and HHV-7 virions (donated by Dr. S. Dewhurst, University of Rochester Medical Centre, NY, USA) were used as positive controls for the corresponding viruses. To avoid possible contamination during PCR, HHV-6- and HHV-7-negative DNAs, as well as water controls, were processed side by side in each experiment.

The detection of HHV-6 DNA was carried out according to Bandobashi *et al* (1997) with a nested primer set, complementary to the gene coding the major capsid protein, which recognizes both variants (A and B) of the virus. The nucleotide sequences of the primers were 5′-GCGTTTTCAGTGTGTAGTTCGGCAG-3′ and 5′-TGGCCGCATTCGTACAGATACGGAGG-3′ (outer primers), and- 5′-GCTAGAACGTATTTGCTGCAGAACG-3′ and 5′-ATCCGAAACAACTGTCTGACTGGCA-3′ (inner primers) for the first and second round of PCR, respectively, allowing us to amplify the 258-bp fragment of HHV-6. The first round of PCR was performed at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. Terminal extension of 72°C for 7 min was performed after completion of the 30 cycles. A volume of 10 μl of the PCR product was amplified with inner primers following the same conditions described for the first round of PCR. According to Secchiero *et al* (1995), the sensitivity of these primers allows the detection of <10 molecules of the template.

To assess the sensitivity of HHV-6- and HHV-7-specific primers in our hands, 10-fold dilutions of the first round amplification products of positive HHV-6 or HHV-7 controls purified by using the QIAquick PCR Purification Kit (Qiagen, Germany) were used as a template for PCR amplification. In our experiments, the sensitivity of HHV-6-specific primers was 1×10^{-18} g per reaction, which allowed the detection of 3 copies of HHV-6 genome.

The primer set targeted at the U10 gene of HHV-7 (Pfeiffer *et al*, 1995) and reaction conditions described by Berneman *et al* (1992) were used to detect HHV-7 DNA in the examined samples. Thirty cycles of PCR amplification with outer primers (5′-TATCCC AGCTGTTTTCATATAGTAAC-3′ and 5′-GCCTTGCGGTAGCACTAGATTTTTTG-3′) were carried out: at 94°C for 1 min, at 60°C—2 min, at 72°C—2 min with an increase of 2 sec per cycle, and an additional final extension at 72°C for 7 min. PCR amplification with inner primers

(5′-CAGAAATGATAGACAGATGTTGG-3′ and 5′-TAGATTTTTTGAAAAAGATTTAATAAC-3′) was performed by using the same protocol as described for the outer primers, with annealing at 55°C instead of 60°C. The expected size of the amplified fragment was 124 bp. According to Ablashi *et al* (1998a), the sensitivity of outer primers allowed the detection of less than 482 genome copies. In our experiments, the sensitivity of HHV-7-specific primers after the second round corresponded to 5 copies of the HHV-7 genome.

The products of DNA amplification were analysed by electrophoresis on 1.7% agarose gel and visualized by ethidium bromide staining. Statistical differences between tested groups were judged by the Fisher's Exact Test (2-Tail P-value). As shown in Table 1, the HHV-6 sequence was identified in PBMC DNA of 67.9% (38/56) of patients with MS and only in 28.6% (6/21) of patients with other diseases of the CNS ($P = 0.004$) and 28.7% (43/150) of control blood donors ($P = 0.001$). There was no statistically significant difference in the prevalence of HHV-6 latent infection between patients with relapsing/remitting MS (26/35, 74.3%) and chronic progressive cases (12/21, 57.1%). HHV-7 DNA was found in 73.2% (41/56) of patients with MS, in 57.1% (12/21) of patients with nondemyelinating diseases of the CNS, and in 75.3% (113/150) of examined control blood donors. There was no statistically significant difference in the prevalence of latent HHV-7 infection between these examined patient groups and between each group of patients with neurological disorders and the control group (Table 1).

Co-infection of HHV-6 and HHV-7 was recorded in 48.2% (27/56) of MS patients (Table 1), which was 5 times more frequent than in patients with other diseases of the CNS (2/21; 9.5%; $P = 0.0016$), and 2.8 times more frequent than in the control blood donors (26/150; 17.3%; $P = 0.00002$).

In the group of inflammatory demyelinating diseases of the PNS, HHV-6 and HHV-7 sequences in PBMC were found in 6 (31.6%) and 15 (78.9%) of 19 individuals, respectively. In PBMC of patients with other peripheral neuropathies, HHV-6 DNA was detected in 5 (29.4%), and HHV-7 in 14 (82.4%), of 17 tested samples (Table 1). Dual viral infection was observed both in patient groups with demyelinating and nondemyelinating PNS pathologies −26.3% and 23.5%, respectively. The differences in prevalence of latent HHV-6, as well as HHV-7 and dual viral infection, between the examined groups of patients with disorders of the PNS and compared with the control blood donors were not statistically significant.

To estimate the possible reactivation of the viruses in latently infected persons, their blood plasma was tested for the presence of HHV-6 and HHV-7 DNA. HHV-6 viremia was indicated in 36.8% (14/38) of patients with MS (Table 2); eight with relapsing/remitting MS and six with the chronic progressive type of the disease. In the group of patients with relapsing/remitting MS, seven were in relapse and one was in the remission phase. Of six patients with chronic progressive MS, one was in the primary progressive and five in the secondary progressive course of the disease, all in the exacerbation phase.

Plasma samples of all control blood donors and patients with other neurological disorders were HHV-6 negative. However, the HHV-7 sequence was found in the blood plasma of individuals in all examined groups (Table 2). The highest prevalence of HHV-7 viremia (53.3%; 8/15) was observed in the group of patients with acute and chronic inflammatory polyneuropathies, whereas the prevalence of HHV-7 infection in the patients with nondemyelinating disorders of the PNS and the control blood donors was only 14.3% (2/14) and 10.6% (12/113), respectively.

Table 1 Prevalence of latent HHV-6 and HHV-7 infection in patients with neurological disorders and blood donors

Groups examined	*HHV-6*	*HHV-7*	*HHV-6 + HHV-7*
Patients with CNS diseases:			
Demyelinating (MS)	38/56 (67.9%)	41/56 (73.2%)	27/56 (48.2%)
Nondemyelinating	6/21 (28.6%)	12/21 (57.1%)	2/21 (9.5%)
Patients with PNS diseases:			
Demyelinating (AIDP + CIDP)	6/19 (31.6%)	15/19 (78.9%)	5/19 (26.3%)
Nondemyelinating	5/17 (29.4%)	14/17 (82.4%)	4/17 (23.5%)
Blood donors	43/150 (28.7%)	113/150 (75.3%)	26/150 (17.3%)

Number positive/number examined.
CNS, central nervous system; PNS, peripheral nervous system; PBMC, peripheral blood mononuclear cells; MS, multiple sclerosis; AIDP, acute inflammatory demyelinating polyneuropathy; CIDP, chronic inflammatory demyelinating polyneuropathy.

Table 2 Prevalence of active HHV-6 and HHV-7 infection in patients with neurological disorders* and blood donors*

Groups examined	*HHV-6*	*HHV-7*
Patients with CNS diseases:		
Demyelinating (MS)	14/38 (36.8%)	9/41 (22.0%)
Nondemyelinating	0/6	2/12 (16.7%)
Patients with PNS diseases:		
Demyelinating (AIDP + CIDP)	0/6	8/15 (53.3%)
Nondemyelinating	0/5	2/14 (14.3%)
Blood donors	0/43	12/113 (10.6%)

*All tested individuals had HHV-6 and/or HHV-7 latent infection.
Number positive/number examined with latent viral infection.
CNS, central nervous system; PNS, peripheral nervous system; MS, multiple sclerosis; AIDP, acute inflammatory demyelinating polyneuropathy; CIDP, chronic inflammatory demyelinating polyneuropathy.

The frequency of HHV-7 in plasma of patients with demyelinating diseases of the PNS significantly differed from that in the patient group with nondemyelinating disease of the PNS and in the control blood donors ($P = 0.05$ and 0.0003, respectively).

In MS patients, active HHV-7 infection was found 2 times more frequently than in the blood control donors, but this difference was not significant. In our study, the HHV-6 and HHV-7 sequences in PBMC DNA of randomly selected control blood donors was detected in 28.7% and 75.3% of cases, respectively (Table 1). This indicates that, in the healthy Latvian population, latent HHV-7 infection is 2.6 times more widespread than HHV-6 infection ($P = 0.001$).

Latent HHV-6 infection in patients with MS occurred 2.4 times more frequently than in patients with nondemyelinating diseases of the CNS ($P = 0.004$) and in control blood donors ($P = 0.001$), and two times more frequently than in the patients with demyelinating diseases of the PNS ($P = 0.007$) (Table 1). Moreover, HHV-6 DNA in plasma, i.e., virus reactivation, was detected in MS patients only (Table 2). The lack of viremia cannot exclude the presence of early stages of virus replication, and RT-PCR should be used to prove the absence of HHV-6 reactivation.

Despite some data not conforming with a correlation between HHV-6 infection and MS development (Hay and Tenser, 2000; Rotola *et al*, 2000; Taus *et al*, 2000), our findings do indicate a higher prevalence of HHV-6 infection among MS patients than in the other examined groups, and confirm the association between HHV-6 and MS reported earlier by other authors (Soldan *et al*, 1997; Ablashi *et al*, 1998b, 2000; Akhyani *et al*, 2000; Knox *et al*, 2000). Moreover, a correlation between HHV-6 reactivation and disease activity was detected. In the group of MS patients with relapsing/remitting phases, seven were in relapse and one in the remission phase. Of five patients with secondary progressive MS and active HHV-6 infection, all were in the exacerbation phase. The results show that active HHV-6 infection is associated with clinical activation of the disease. The difference in frequency of active HHV-6 infection between MS patients in clinically active and inactive phases of the disease (13/27 and 1 /11, respectively) is statistically significant ($P = 0.03$).

Table 1 shows that a high frequency of latent HHV-7 infection was displayed in all groups of patients with neurological disorders, but there was no statistically significant differences in the prevalence of this infection between the groups. Similarly, active HHV-7 infection was also observed in all groups (Table 2). Statistically significant difference in the prevalence of HHV-7 active infection was observed between the groups of patients with demyelinating and nondemyelinating diseases of PNS ($P = 0.05$) as well as between the former group and control blood donors ($P = 0.0003$).

The obtained results indicate that not only HHV-6, but also HHV-7, can be associated with demyelinating diseases. The high frequency of dual latent infection (HHV-6 + HHV-7) in MS patients in comparison with the control blood donors group (2.8 times higher; $P = 0.00002$) and with patients with nondemyelinating diseases of the CNS (5 times higher; $P = 0.0016$) suggests that further studies are required to investigate the dual infection and relationship between these viruses. Moreover, the reactivation of HHV-6 by HHV-7 *in vitro* has been described earlier (Katsafanas *et al*, 1996; Tanaka-Taya *et al*, 2000). Our study further supports the previously described association between HHV-6 infection and MS, and the correlation between the active viral infection and the clinical activity of the disease. The association of HHV-7 reactivation with demyelinating diseases of the peripheral nervous system suggests involvement of this virus in demyelinating processes.

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