SHORT REPORT

Human herpesvirus 6 is latent in peripheral blood of patients with relapsing-remitting multiple sclerosis

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Abstract

Studies of the association between HHV-6 and multiple sclerosis are hindered by the difficulty in discriminating between latent and active infection. A follow up study was undertaken of patients with multiple sclerosis, searching peripheral blood mononuclear cells for molecular markers associated with HHV-6 latency and lytic replication. The results show that HHV-6 is latent and did not support systemic infection in patients with multiple sclerosis. Likewise, patients with multiple sclerosis did not show any evidence of active infection with other human herpesviruses HHV-7 and HHV-8.

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Human herpesvirus 6 (HHV-6) is a lymphotropic virus, with a preferential tropism for CD4+ T cells. Infection is common, as shown by the rate of seropositivity values higher than 90% in the healthy adult population. The presence of viral DNA sequences in peripheral blood mononuclear cells (PBMCs) of healthy people supports the view that HHV-6 establishes persistent infections. The pathogenic associations of HHV-6 range from exanthem subitum and benign febrile diseases in childhood to pneumonitis and other life threatening conditions in immunodepressed subjects.¹

It has been suggested that HHV-6 might be involved in the pathogenesis of multiple sclerosis. HHV-6 infects cells of neural origin and tissues of the CNS¹ where it can be associated with acute diseases.²⁻⁴ The distribution of HHV-6 in the brain of patients with multiple sclerosis is different from controls, and it infects oligodendrocytes associated with the multiple sclerosis plaques.⁵ Patients with multiple sclerosis have antibody titres against HHV-6 higher than controls,⁶ HHV-6 DNA was found by polymerase chain reaction (PCR) in the CSF of three of 21 patients with multiple sclerosis, but not in controls⁷ and, more recently, increased IgM serum antibody responses to HHV-6 early antigen were described in patients with relapsing-remitting multiple sclerosis.⁸ However, other reports did not show

any correlation between clinical features and HHV-6 PCR findings or HHV-6 antibodies.⁹⁻¹³

It is not easy to find a clear solution to this controversy. In general, all studies of association between HHV-6 and pathological states need to take into account the fact that viral infection is very common in the healthy population,¹⁴ and there is the need to discriminate between latent and infectious virus. Determination of viral load does not address the question, as even low concentrations of HHV-6 replication might cause disease.¹⁵

We have recently reported that HHV-6 establishes latent infection in PBMCs of healthy donors.¹⁶ We also described that viral latency is characterised by the presence of U94 mRNA in the absence of transcripts from other immediate-early (IE) genes, as disclosed by rtPCR. Thus, an rt-PCR assay might allow for relatively simple differentiation between latent and active viral infection in patients with multiple sclerosis.

A 1 year follow up study was undertaken analysing PBMCs of patients with multiple sclerosis. Therefore, the intent of this study was to:

- (1) Evaluate whether HHV-6 establishes an active infection during multiple sclerosis.
- (2) Determine the state of viral replication, searching the molecular markers of latent and productive infection.
- (3) Verify whether therapy with β -interferon affects HHV-6 infection.
- (4) Investigate the frequency and a possible role of the other novel lymphotropic herpesviruses, HHV-7 and HHV-8, in patients with multiple sclerosis.

Materials and methods

Seventeen relapsing-remitting patients with multiple sclerosis, diagnosed on the basis of Poser's criteria¹⁷ and subjected to β -interferon 1B therapy, were enrolled in the study. According to the Italian Ministry of Health standards of 1996, β -interferon prescription criteria include an expanded disability status scale (EDSS) score of 1 to 3.5, at least two exacerbations in the previous 2 years, and age range 18-50 years. PBMCs were obtained from each patient before the β -interferon treatment and at 4 month intervals for 1 year. The

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PBMCs were purified on Ficoll gradients and DNA was extracted by conventional procedures. When possible, specimens obtained from the third and fourth withdrawal were stored at -80°C in two separate aliquots, to be used, respectively, for DNA and RNA extraction. The presence of HHV-6 DNA was searched by nested PCR for the U31 gene, analysing 1 μg DNA, corresponding to 150 000 cells. Particular care was taken to avoid contamination of the samples and blank reactions, consisting of the extraction mixture alone, were interspersed with every third experimental sample (so as to control for possible cross contamination of DNA samples). The concentration of DNA was determined by reading the optical density at 260 nm. Transcription of HHV-6 was analysed in seven samples positive for HHV-6 DNA. The RNA was extracted from PBMCs with RNAzol B (Biotecx) and was stored at -70°C in ethanol/sodium acetate until utilised for reverse transcription (RT) and PCR amplification. Immediately before reverse transcription, the mRNA pellet was rinsed with 75% ethanol and resuspended in water treated with diethyl pyrocarbonate. First strand cDNA synthesis was carried out with cDNA Cycle Kit (Invitrogen) following the manufacturer's recommendations, with random examer primers. Nested PCR on cDNAs was performed for U16/17, U39, U42, U91, and U94 as recently described.¹⁶ Efficiency of retrotranscription was assessed by analysis of dilutions of cDNA with PCR specific for β -actin. To ensure that viral DNA was not contaminating the RNA samples, positive specimens were analysed by nested PCR without retrotranscription.

The presence of HHV-7 was searched by single step PCR, followed by hybridisation with a specific internal oligonucleotide labelled with P^{32} .¹⁸ To estimate the viral load, the autoradiographic signals were measured by densitometry. HHV-8 DNA was searched by nested PCR, according to the conditions already described.¹⁹ Sensitivities of all PCR reactions were determined by amplification of known amounts of cloned target sequences. All necessary steps to achieve lack of contamination and reproducibility were taken. PBMCs, obtained from healthy blood donors, were included as controls in the study.

Results

The prevalence of viral DNA in PBMCs of patients with multiple sclerosis during the 1 year follow up are shown in the table.

At enrollment, 41% (seven of 17) patients were positive for HHV-6, and the prevalence

Table 1 Presence of herpesvirus DNA in PBMCs

Virus	MS patients: blood sample No				Healthy donors
	1	2	3	4	
HHV-6	7/17 (41%)	5/17 (29%)	7/17 (41%)	6/17 (35%)	7/24 (29%)
HHV-7	16/17 (94%)	16/17 (94%)	14/15 (93%)	14/14 (100%)	21/24 (87%)
HHV-8	2/17 (12%)	1/17 (6%)	0/17	0/17	5/56 (9%)*

Samples 1–4 were obtained from patients with multiple sclerosis (MS), at enrollment and 4, 8, and 12 months after the beginning of the study, respectively.

*Already published (Bigoni et al¹⁹).

(A) Analysis of PBMCs from six patients with multiple sclerosis, negative for HHV-6 DNA (samples 1–3) or harbouring viral sequences (samples 4–6). The DNA was amplified by nested PCR with primers specific for U31 and analysed by agarose electrophoresis after staining with ethidium bromide. A positive control of DNA extracted from HHV-6 infected cells is shown (D). The size of the amplicon is expressed in base pairs (bp). (B) From the same patients PBMCs were analysed by rtPCR for U94 transcripts. RNA was extracted from PBMCs, retrotranscribed in cDNA (RT+) and analysed by nested PCR using primers specific for the U94. The same mRNAs, positive for U94 transcript, were analysed without retrotranscription (RT-) to ensure that viral DNA was not contaminating the samples. A positive control of mRNA from HHV-6 infected cells, retrotranscribed and amplified is shown (R). The size of the amplicon is expressed in base pairs (bp). Control reactions, to test for possible contamination, are shown in lanes B1 (extraction blank) and B2 (blank reaction).

remained constant throughout the study (table). The sensitivity of PCR allowed the detection of 1000 target molecules of HHV-6 DNA with single step PCR and 10 molecules with nested PCR (data not shown). Positive signals were detected only after nested PCR (figure A), indicating that low amounts of virus were present in the positive samples. None of the patients were positive to HHV-6 in all four specimens collected, with HHV-6 DNA being detected only in some blood samples but not in others.

The sensitivity of PCR specific for HHV-7 was 1000 molecules by visualisation with ethidium bromide and 10 molecules, after hybridisation with a specific probe (data not shown). HHV-7 DNA was present in most samples at enrollment (94%), and the prevalence remained high throughout the study. The viral loads of the four samples from each patient were compared, and no significant trend was detected: the amount of viral DNA decreased in eight patients, increased in three, and was constant in six.

HHV-8 specific PCR detected 400 target molecules after single step PCR and as low as four targets after nested PCR (data not shown). Two patients (12%) were positive for HHV-8 in the first sample, and were negative in the follow up specimens, with the exception of one case (table). Positive signals were detected only after nested PCR, showing that low amounts of viral sequences were harboured in positive samples.

Eleven patients had relapses during the study, but there was no relation with presence of virus, neither with an increased viral load.

Samples for RNA analysis were available for seven patients positive for HHV-6 DNA, and corresponded to either one of the last two time

points. Transcripts of five different IE genes (U94, U16/17, U39, U42, U91) were analysed by nested rtPCR: all reactions had the same sensitivity. Only U94 was detected after nested PCR (figure B). Furthermore, no residual DNA contamination of RNA samples was detected by analysing the same amount of RNA, without the initial reverse transcription reaction (figure B).

Discussion

The rationale of our research is based on the finding that HHV-6 and HHV-7 have a preferential tropism for cells involved in demyelination, such as T lymphocytes and macrophages. Multiple sclerosis is a disease restricted to the CNS, but HHV-6 activation in the blood of patients with multiple sclerosis has been suggested on the basis of increased concentrations of IgM and the presence of viral DNA in serum of patients.⁸ Therefore, analysis of viral transcription in peripheral blood could give important indications on viral replication and could represent an important marker for at least a subset of patients.

The prevalence of HHV-7 is similar to that reported in the healthy population (table) and no significant variation in viral load was found during the follow up period. HHV-8 was detected in up to 12% of patients with multiple sclerosis, confirming that it can be harboured in PBMCs of patients not affected by Kaposi's sarcoma.¹⁹ The low prevalence excludes a role for this virus in multiple sclerosis, as already suggested by a previous study.²⁰

HHV-6 showed similar distributions in patients with multiple sclerosis and in healthy people. The finding that the virus is detected only in some samples, but not in others of the same patient, probably reflects the low amount of viral sequences.

Mayne *et al.*¹³ recently reported the infrequent detection of HHV-6 in PBMCs of patients with multiple sclerosis and at a first analysis the results dismiss the hypothesis of a systemic involvement in the course of multiple sclerosis. However, it is well established that even low levels of productive infection by HHV-6 may cause clinical disease.¹⁵ The low viral load estimated for the three herpesviruses included in the study would argue for a lack of an active involvement in the course of multiple sclerosis, but PCR is not suitable to distinguish between latent and chronic infection. We recently described that latency of HHV-6 is associated with the presence of U94 mRNA in the absence of other mRNAs transcribed during the IE phase of infection.¹⁶ In this report, we unequivocally show that HHV-6 is indeed latent in PBMCs of patients with multiple sclerosis, because U94 is the only transcript found, and all other IE genes, transcribed with high levels during productive and restricted infection,²¹ were not detected.

Finally, no differences in virus load or frequency were found in the follow up period,

suggesting that β -interferon therapy has no effect on virus persistence.

The association of HHV-6 and multiple sclerosis is controversial and far from being established. Our results clearly show that active infection (or reactivation) of HHV-6 does not take place in the context of multiple sclerosis. However, before conclusively dismissing the possibility that HHV-6 might be involved in the pathogenesis of the disease, the state of viral persistence in the CNS should be clearly defined by further studies.

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- Braun KS, Dominguez G, Pellett PE. Human herpesvirus 6. *Clin Microbiol Rev* 1997;10:521-67.
- Asano Y, Yoshikawa T, Kajita Y, *et al.* Fatal encephalitis/encephalopathy in primary human herpesvirus-6 infection. *Arch Dis Child* 1992;67:1484-5.
- Caserta MT, Hall CB, Schnabel K, *et al.* Neuroinvasion and persistence of human herpesvirus 6 in children. *J Infect Dis* 1994;170:1586-9.
- Suga S, Yoshikawa T, Asano Y, *et al.* Clinical and virological analyses of 21 infants with exanthem subitum (roseola infantum) and central nervous system complications. *Ann Neurol* 1993;33:597-603.
- Challoner PB, Smith KT, Parker JD, *et al.* Plaque-associated expression of human herpesvirus 6 in multiple sclerosis. *Proc Natl Acad Sci USA* 1995;92:7440-4.
- Sola P, Merelli E, Marasca, Human herpesvirus 6 and multiple sclerosis: survey of anti-HHV-6 antibodies by immunofluorescence analysis and of viral sequences by polymerase chain reaction. *J Neurol Neurosurg Psychiatry* 1993;56:917-19.
- Wilborn F, Schmidt CA, Brinkmann V, *et al.* A potential role for human herpesvirus type 6 in nervous system disease. *J Neuroimmunol* 1994;49:213-14.
- Soldan SS, Berti R, Salem N, *et al.* Association of human herpes virus 6 (HHV-6) with multiple sclerosis: increased IgM response to HHV-6 early antigen and detection of serum HHV-6 DNA. *Nat Med* 1997;12:1394-7.
- Liedtke W, Malessa R, Faustmann PM, *et al.* Human herpesvirus 6 polymerase chain reaction findings in human immunodeficiency virus associated neurological disease and multiple sclerosis. *J Neurovirol* 1995;1:253-8.
- Martin C, Enbom M, Soderstrom M, *et al.* Absence of seven human herpesviruses, including HHV-6, by polymerase chain reaction in CSF and blood from patients with multiple sclerosis and optic neuritis. *Acta Neurol Scand* 1997;95:280-3.
- Fillet AM, Lozeron P, Agut H, *et al.* HHV-6 and multiple sclerosis. *Nat Med* 1998;4:537.
- Coates ARM, Bell J. HHV-6 and multiple sclerosis. *Nat Med* 1998;4:537-8.
- Mayne M, Krishnan J, Metz L, *et al.* Infrequent detection of human herpesvirus 6 in peripheral blood mononuclear cells from multiple sclerosis patients. *Ann Neurol* 1998;44:391-4.
- Di Luca D, Mirandola P, Ravaioli T, *et al.* Distribution of HHV-6 variants in human tissues. *Infect Agents Dis* 1996;5:203-14.
- Carrigan DR, Knox KK. Bone marrow suppression by human herpesvirus-6: comparison of the A and B variants of the virus. *Blood* 1995;86:835-6.
- Rotola A, Ravaioli T, Gonelli A, *et al.* U 94 of human herpesvirus 6 is expressed in latently infected peripheral blood mononuclear cells, and blocks viral gene expression in transformed lymphocytes in culture. *Proc Natl Acad Sci USA* 1998;95:13911-16.
- Poser CM, Paty DW, Scheinberg L, *et al.* New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann Neurol* 1983;13:227-31.
- Di Luca D, Mirandola P, Ravaioli T, *et al.* Human herpesvirus 6 and 7 in salivary glands and shedding in saliva of healthy and human immunodeficiency virus positive individuals. *J Med Virol* 1995;45:462-8.
- Bigoni B, Dolcetti R, de Lellis L, *et al.* Human herpesvirus 8 is present in the lymphoid system of healthy persons and can be reactivated in the course of AIDS. *J Infect Dis* 1996;173:542-9.
- Merelli E, Bedin R, Sola P, *et al.* Human herpes virus 6 and human herpes virus 8 DNA sequences in brain of multiple sclerosis patients, normal adults and children. *J Neurol* 1997;244:450-4.
- Mirandola P, Menegazzi P, Merighi S, *et al.* Temporal mapping of transcripts in herpesvirus 6 variants. *J Virol* 1998;72:3837-44.