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# Human herpes virus 6 and human herpes virus 8 DNA sequences in brains of multiple sclerosis patients, normal adults and children

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**Abstract** In order to determine whether the newly discovered human herpesviruses (HHVs) are involved in multiple sclerosis (MS), we investigated by polymerase chain reaction the presence of specific deoxyribonucleic acid (DNA) sequences belonging to human herpesvirus 6 (HHV-6) and to human herpesvirus 8 (HHV-8), in the peripheral blood mononuclear cells (PBMCs), and in the brain and spinal cord plaques from MS patients. Normal adult and stillborn children's brains were investigated as controls. PBMCs from 56 MS patients contained HHV-6 DNA in only 3 cases and in none were there HHV-8 sequences. The cerebral DNA from 5 MS patients was positive for HHV-8 and not for HHV-6 sequences, while the nervous tissue of one patient who died with neuromyelitis optica was positive for HHV-6 and negative for HHV-8. The brains of 4/8 adult controls were positive for HHV-6, as were 3/8 for HHV-8; none of the 7 stillborn children's cerebral tissue contained HHV-6 sequences, while 2 contained HHV-8 DNA. Although these data do not support a hypothesis that there is a role for these two HHVs in the pathogenesis of MS, nevertheless it may be suggested that (1) the two viruses possess strong neurotropism and the central nervous system seems to be a reservoir for them (2) HHV-6 infection is probably not transmitted maternally, but is acquired later in infancy.

**Key words** Multiple sclerosis · Human herpesvirus 6 · Human herpesvirus 8 · Polymerase chain reaction · Brain DNA

## Introduction

Human herpesvirus 6 (HHV-6) infection has recently been shown to be associated with some cases of multiple sclerosis (MS) [4]. The presence of HHV-6 has also been demonstrated frequently in normal brains [15], but since some MS brains harbors HHV-6 sequences divergent from the prototype virus, the possibility that a variant strain may play a role in MS pathogenesis has been suggested. These variant HHV-6 sequences have been found in the nuclei of oligodendrocytes, the target cells of the demyelinization process. Moreover, HHV-6 can persist in the central nervous system (CNS) in latent form and is reactivated in immune-compromised patients, such as infants with roseola [2], AIDS patients [11], bone-marrow transplant patients [7], and MS patients [19], and may induce meningitis and occasionally fatal encephalitis.

DNA sequences of a putative new human herpesvirus named Kaposi sarcoma herpes virus (KSHV) or human herpesvirus 8 (HHV-8) have been identified in Kaposi sarcoma (KS) tissues from patients with AIDS [5]. The DNA-specific sequences from KS showed partial similarity to two capsid-protein coding genes of known γ-herpesviruses, namely, Epstein-Barr (EBV) and herpesvirus Saimiri (HVS) [5].

KSHV/HHV-8, like HHV-6, can be found in the tissues of immunocompromised subjects, as in skin lesions of

transplant patients [20], in the lymphoid tissues of AIDS-related lymphomas [3]. It has been speculated that HHV-8 may play a role in lymphoproliferative disorders [3] in addition to KS. Because CD4 T+ cells [17] and CD19+ B cells [1] are thought to be the natural targets of HHV-6 and HHV-8, and because lymphocyte activation is considered crucial for the development of MS, we investigated whether HHV-8 is associated with MS. For this we used polymerase chain reaction (PCR) to identify HHV-6 and HHV-8 sequences in the DNA of peripheral blood mononuclear cells (PBMCs), brain and spinal cord from MS patients and from adults and children who died of non-neurological causes as controls.

## Materials and methods

### DNA samples

HHV-6 and HHV-8-specific sequences were investigated in the DNA extracted from PBMCs from 56 MS patients (17 males, 39 females, mean age 42 years). All the patients were diagnosed as definite MS, according to the criteria of Poser (1993); and PBMCs from 20 healthy subjects (12 males, 8 females, mean age 37 years), as a control group. DNA was also extracted from autopsy from the cerebral tissue from 5 MS patients (2 males, 3 females, mean age 52 years), including gray matter and plaques of demyelinization from different areas of the brains; from the spinal cord and cerebral tissue from a patient who died with fulminant neuromyelitis optica (NMO); from the cerebral tissue from 8 adults who died after traumatic accidents; and from the cerebral tissue from 7 stillborn children.

DNA was extracted from the PBMCs by TurboGen genomic DNA isolation kit (Invitrogen, San Diego, Calif.). Brain specimens were obtained within 18 h of death; crude extracts were prepared from single 5 μm formalin-fixed and paraffin-embedded tissue sections, according to the method described by Luppi et al. [15].

### HHV-6 PCR

Since all the viruses in the herpes family have extensive genomic homology, it was decided to use the ZVH14 sequence of the HHV-6 genome as the target gene for amplification, because the ZVH14 primers did not cross react with non-HHV-6 viral (HHV-7 included) or human sequences in a very large number of assays [10].

PCR analysis was performed in the PBMCs as described by Torelli et al. [23] and in the tissues according to Luppi et al. [15]. The oligonucleotides used as primers and probes were synthesized on an automated solid-phase synthesizer (Applied Biosystems, Mod. 381A), by standard phosphoramidite chemistry. Two sets of primers and probes were used. One set, representing a portion of the gene for the major capsid protein [13], has the following sequence:

1. 5′ATTAGGACCGATCGGCTCTA3′ as sense primer
2. 5′GTGTAGGTGGTCGAATGCGA3′ as antisense primer
3. 5′TCCCGGCGTTCACGGTGAATTGAGTA3′ as internal probe

The second set, representing a portion of the ZVH14 segment [10], has the following sequence:

1. 5′CCCATTTACGATTTCCTGCACCACCTCTCTGC3′ as sense primer
2. 5′TTCAGGGACCGTTATGTCATTGAGCATGTCG3′ as antisense primer
3. 5′CCGTAAAAAATTTACACCTCCATTTCATCTT3′ as internal probe

Thirty cycles of amplification were performed with an automatic thermal cycler (MJ Research, Cambridge, Mass.). The amplification products, a 300 nucleotide segment for the first set and a 186 nucleotide segment for the second set, were then subjected to electrophoresis, transferred to a nylon membrane by vacuum blotting, and hybridized with a oligonucleotide probe end-labelled with γ32P-ATP. Autoradiography was then performed for 2 and 7 days at –80°C. A positive control was represented by HHV-6 infected HSB-2 DNA. Two negative controls (a human placental DNA and a reaction mixture without DNA) were used for each group of ten samples examined.

### HHV-8 PCR

PCR for HHV-8 sequences in the DNA samples was performed using specific primers amplifying a fragment of 233 base pairs (bp) as described [1, 5].

The primers set for KS330 233 sequences are the following:

1. 5′AGCCGAAAGGATTCCACCAT3′ as sense primer
2. 5′CTGGACGTAGACAACACGGA3′ as antisense primer
3. 5′TGCAGCAGCTGTTGGTGTACCACAT3′ as internal probe

Aliquots of 1 γ of PBMC DNA and 10 μl of tissue DNA were submitted to PCR. Each 100 μl reaction contained 30 pmol of each primer, 2.5 U of Taq polymerase, 200 μmol/l each of deoxynucleotide triphosphate, 10 mmol/l Tris-HCl, 1.5 mmol/l MgCl2, and 50 mmol/l KCl (pH 8.3). PCR conditions were as follows: 94°C for 1 min, followed by 45 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 90 s. Reactions were terminated by 7-min extension at 72°C. For each PCR test the positive controls were represented by the crude extracts obtained from HHV-8 positive AIDS-KS biopsy specimens. All standard recommended procedures were performed in order to avoid false-positive results [12]. PCR analysis was invariably performed on tissue sections derived from different paraffin blocks for each patient. Negative controls, consisting of all reagents except sample DNA, were also present during the crude extract preparation and equalled or exceeded the number of the assayed samples. Primers for the DQα gene were used as positive amplification control.

The PCR products were analyzed on 1.5% ethidium bromide-stained agarose gel, which allowed direct visualization of the predicted 233 bp band. In order to confirm the viral origin of the amplified DNA template, one-fifth of the PCR product was subjected to electrophoresis on 1.5% agarose gel blotted on nylon membrane, positively charged (Boehringer Mannheim, Germany) and hybridized with an internal oligonucleotide probe specific for HHV-8 [5], end labelled with γ32P-ATP (Amersham-USB, Cleveland, Ohio, USA).

## Results

### PBMCs

We found HHV-6 genomic sequences in 3 of 56 DNA samples from PBMCs of MS patients and in none of the 20 positive controls. In contrast, in the same pathological and normal samples PCR analysis for HHV-8 was negative (Table 1).

**Table 1** Results obtained by PCR performed to detect HHV-6 and HHV-8 specific sequences, (/ = not done, *PBMCs* peripheral blood mononuclear cells)

| Patients | No. | Tissue sample | | | HHV-6 | | HHV-8 | |
|---|---|---|---|---|---|---|---|---|
| Multiple sclerosis | 5 | Brain: | White matter | Gray matter | White matter | Gray matter | White matter | Gray matter |
| Case 1 | | | 2 plaques | frontal | – | – | – | + |
| Case 2 | | | 3 plaques | parietal, brain stem | – | – | + | + |
| Case 3 | | | 3 plaques | / | – | / | + | / |
| Case 4 | | | 4 plaques | / | – | | + | / |
| Case 5 | | | 5 plaques | temporal | – | – | + | – |
| Neuromyelitis optica | 1 | Brain: | | 2 plaques | + | / | – | / |
| | | Spinal cord: | | 2 plaques | + | / | – | / |
| Controls | | Brain | | | Positive cases | | Positive cases | |
| Normal adults | 8 | | | | 4 | | 3 | |
| Stillborn children | 7 | | | | 0 | | 2 | |
| Multiple sclerosis | 56 | PBMCs | | | 3 | | 0 | |
| Blood donors | 20 | PBMCs | | | 0 | | 0 | |

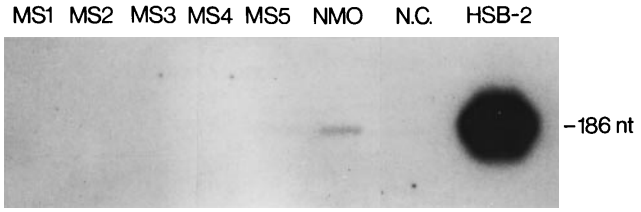


**Fig. 1** HHV-6 polymerase chain reaction (PCR) in brain tissue from multiple sclerosis (MS) and neuromyelitis optica (NMO) spinal cord. Autoradiography of HHV-6 negative PCR in brain of five MS cases *(lanes MS1-MS5)* and one positive NMO patient spinal cord specimen. The positive and the negative controls are represented by the HHV-6 infected *(HSB-2)* and uninfected (NC) cells DNA, respectively. The probe used was the ZVH14 plasmid

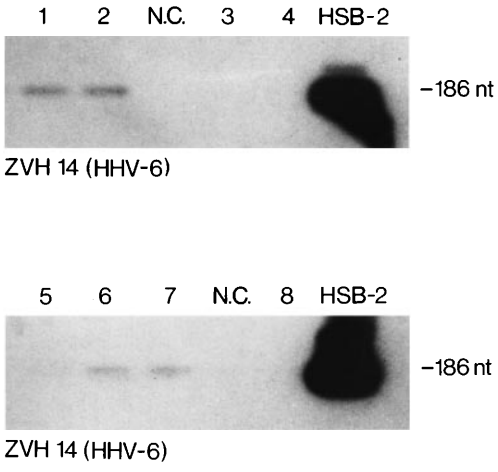


**Fig. 2** HHV-6 PCR in normal adult brains. Autoradiography of HHV-6 positive PCR *(lanes 1,2,6,7)* and negative PCR *(lanes 3,4,5,8)* in normal brains obtained from immunocompetent adults. The positive and negative controls are represented by the HHV-6 infected *(HSB-2)* and uninfected (NC) cells DNA, respectively. The probe used was the ZVH14 plasmid

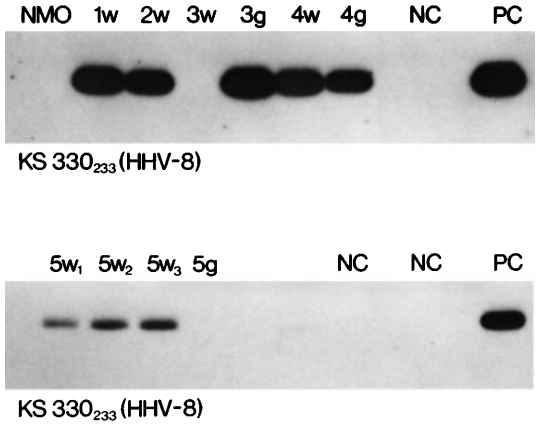


**Fig. 3** HHV-8 PCR in brain tissues from MS and NMO spinal cord. Autoradiography of the PCR for HHV-8 of 1 NMO and 5 MS (lanes 1–5) cases. *Lanes 1w, 2w, 3g, 4w, 4g, and* $5w_{1-3}$ show PCR positive for HHV-8 specific sequences *(KS* $330_{233}$*)*. The positive controls *(PC)* are represented by the crude extracts obtained from KSHV-positive AIDS-KS biopsy specimens. Negative controls (NC) consisted of all reagents except a DNA sample (*w, g* DNA extracted from white and gray matter, respectively)

## Nervous tissue

Since the reported sequences of the KS-specific DNA segments share partial similarity to two EBV capsid-protein coding genes [5], we submitted all the MS and NMO biopsy specimens to PCR for EBV specific sequences in order to avoid false-positive results. All the lesional and perilesional brain specimens from MS as well as from the NMO brain and spinal cord were negative for the presence of EBV genomic DNA.

We examined for HHV-6 and HHV-8 17 plaques and gray matter from 5 MS brains, 2 plaques from brain tissue and 2 from spinal cord tissue of 1 NMO case. As a control

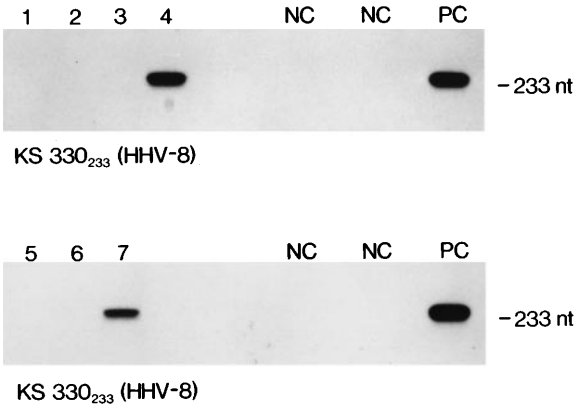


**Fig. 4** HHV-8 PCR in brain tissue from stillborn children. Autoradiography showing the positive PCR for HHV-8 specific sequences *(KS $330_{233}$)* in two stillborn children's brains *(lanes 4 and 7)* and negative PCR in the other five cases *(lanes 1, 2, 3, 5, 6).* The positive controls *(PC)* are represented by the crude extracts obtained from KSHV positive AIDS-KS biopsy. Negative controls *(NC)* consisted of all reagents except a DNA sample

some specimens for each of the 8 normal adult brains and of the 7 stillborn children's brains were examined.

The plaques as well as the gray matter of MS brains were negative for HHV-6 in all the specimens, whereas the plaques from brain and spinal cord tissue of the NMO case contained HHV-6 specific sequences (Fig. 1). In the controls 4 of 8 adult brains (Fig. 2) and none of the 7 stillborn children's brains were positive for HHV-6 (Table 1).

All 5 MS brains contained sequences of HHV-8 in the plaques or in the gray matter or in both, while NMO brain and spinal cord tissues were negative (Fig. 3). In the controls the virus was present in 2/7 infant brains (Fig. 4) and in 3/8 normal adult brains (Table 1).

## Discussion

HHV-6 uses as a receptor mainly the CD4+ molecule [22], which is also thought to be very important in the pathogenesis and development of MS. Although a previous study [21] found a significative increase of HHV-6 antibody titres in MS sera compared with normal subjects, our present data show that HHV-6 was found in the PBMCs of only 3/56 MS samples examined. This finding may signify that HHV-6 antibody-high titre represents an unspecific activation of the immune system in MS, in the absence of detectable viral sequences, more than an etiological phenomenon. Interestingly, all three positive patients suffered from a very aggressive form of the disease; two of them died of infectious complications, while the third underwent an intercurrent encephalitic episode [19]. It could be speculated that in these cases HHV-6 was reactivated because of the immunological derangement, which as a rule takes place in the more severe forms of MS.

The autopsy brain tissues, the plaques, and the perilesional gray matter of the five MS patients did not score positive for HHV-6, like the seven brains from stillborn children.

In contrast, 4/8 normal adult brains contained HHV-6 genomic DNA in agreement with previous reports [4, 15], which showed 6/9 and 40/54 normal brain specimens positive for HHV-6 specific sequences, respectively. Altogether these findings strongly suggest that HHV-6 is probably a common commensal virus for brain cells; in addition, our data indicate for the first time that HHV-6 is probably not transmitted to the fetus in pregnancy but is acquired later in life, like EBV [9].

All the MS brain DNA samples examined were negative for HHV-6 sequences; only the spinal cord and brain specimens from the NMO case were positive for HHV-6. Like the three HHV-6 positive MS patients in the PBMCs, the NMO patient also suffered a very acute form of the disease, with extensive demyelinization of the spinal cord and death within 4 months from the onset.

In the NMO case, we may also argue that the dramatic impairment of the immunocompetent system may have reactivated a latent infection of the virus in the CNS with the well-known effect, in common with all the other human herpesviruses [8]. Moreover, very recently HHV-6 has been recognized as being implicated in the etiology of myelopathy, still now considered to be of unknown origin [18].

HHV-8 was not detected in all the PBMC DNA examined, whether from MS or from normal subjects, although this novel herpesvirus has been found with very high frequency in CD19+ B cells from the PBMCs of KS patients with and without AIDS [1].

In this view, the large number of HHV-8 positive samples in MS, normal and stillborn children's brains, argues in favor of a strong neurotropism for this novel herpesvirus. The fact that all five MS cases examined scored positive for HHV-8 sequence in the plaques, the gray matter or both (Table 1) is tantalizing; however, because 3/8 normal adult brains and 2/7 infant brains also contained specific viral sequences it is difficult to ascertain whether HHV-8 may play a role in MS. In contrast, brain and spinal cord specimens from the NMO case were devoid of viral sequences belonging to HHV-8.

The discrepancy between the absence in the PBMCs of all the MS cases of HHV-8 and the presence of the same herpesvirus in most of the brain and spinal cord specimens examined may reflect the fact that, as for HHV-6, the virus is present mainly at the site of pathological lesions, or that HHV-8 resides latent in many regions of the brain.

The two herpesviruses do not seem to co-infect the nervous tissues of the same patient; in fact, samples positive for HHV-6 are negative for HHV-8 and vice versa. If this finding is confirmed by other analyses, it could be speculated that viral interference plays a role in the occupation of the same nerve cell, possibly the oligodendro-

cyte, which has been shown to be the host cell for HHV-6 in MS brains [4].

Moreover, the integration of HHV-6 in the PBMCs has been reported in vivo in the chromosome 17p13 [24], and this seems of crucial importance in understanding the cell–virus relations and the clinical consequences of the infection, because the integration may alter the expression of host-cell protein directly or through the transactivation of cellular genes. To our knowledge, it is not known whether HHV-8 integrates in the host genome like EBV [9] and Marek's disease virus [6]; certainly it would be of great interest to establish whether HHV-8 also integrates in the host genome, and to investigate the influence of a pre-existing infection by HHV-6 on HHV-8 infectivity in oligodendrocytes in vitro.

The understanding of these events and of the mechanism of reactivation may answer the numerous questions regarding the role of these novel herpesviruses in the normal and pathological CNS.

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