



account the fact that viral infection is very common in the healthy population (reviewed by Di Luca *et al*, 1996) and there is the need to discriminate between latent and infectious virus. The presence of viral DNA is often analysed by polymerase chain reaction (PCR), but the extreme sensitivity of this technique is a serious limit to determine the pathogenic significance of a positive finding. Positive amplification of a genomic region is not indicative of viral replication nor does it prove that the identified agent is playing an etiologic role in the disease. This is particularly important in the case of herpesviruses, that establish lifelong latent infections in the host and persist in a non replicative state.

We undertook a study to determine whether HHV-6 establish a systemic active infection in the course of MS, and to investigate possible roles of HHV-7, a member of the herpesvirus family closely related to HHV-6.

## Results

The PCR reactions for HHV-6 and HHV-7 DNA had similar sensitivities and both allowed to detect 1000 target molecules with the first round of PCR and ten molecules after nested PCR. A reconstruction experiment showing the sensitivity of HHV-6 is shown in Figure 1.

PCR for detecting human  $\beta$ -actin gene was performed on 10 ng of all samples, to ensure that they were suitable for DNA amplification. The results on the presence of HHV-6 and HHV-7 DNA are shown in Table 1. HHV-6 sequences were

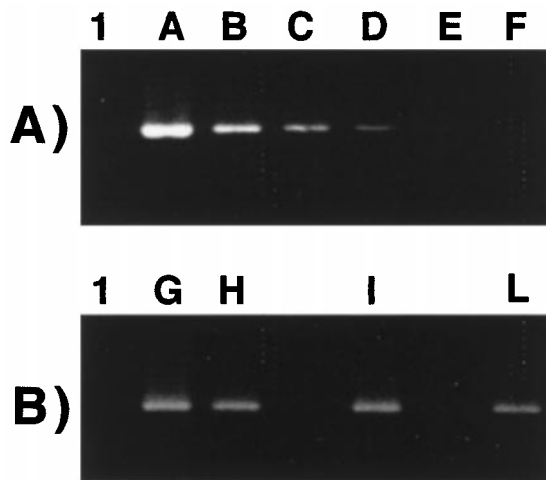
detected in 8/20 (40%) patients, and HHV-7 DNA was present in 18/20 patients (90%) (Figure 2). The prevalence of viral sequences were similar in MS patients and in controls from healthy donors. Positive signals for HHV-6 and HHV-7 were present only after nested PCR, suggesting that low amounts of virus were present in positive samples.

HHV-6 strains segregate in two different variants (HHV-6A and HHV-6B), closely related to each other but clearly distinguishable on the basis of biological and molecular characteristics (Di Luca *et al*, 1996). The HHV-6 variant present in positive samples was determined by restriction endonuclease cleavage of PCR products by *Hind*III and *Hinf*II. All positive samples harboured HHV-6 variant B DNA (data not shown).

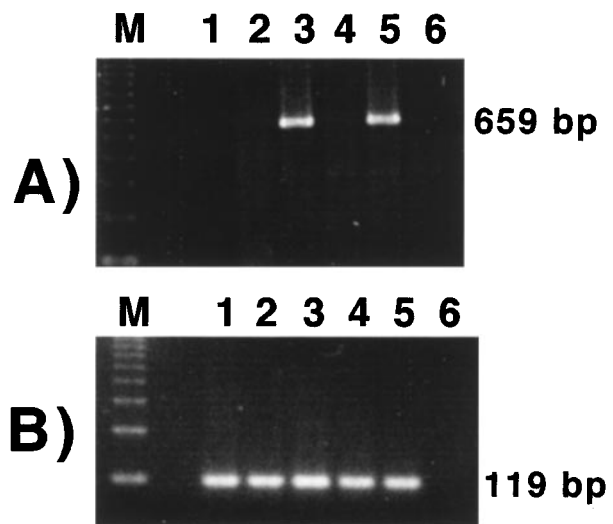
Samples for RNA analysis were available for six samples positive for HHV-6. Transcripts of three different genes (U94, U16/17, U91) were analysed by RT-PCR. All these transcripts belong to the immediate-early transcriptional class and are normally detected during all phases of lytic replication (Mirandola *et al*, 1998). Single step PCR amplification of cDNAs did not reveal the presence of detectable levels of viral transcripts. A nested round of amplification resulted in the detection of U94 mRNA in five samples, but no other viral transcript

**Table 1** Presence of HHV-6 and HHV-7 in PBMCs

	MS patients	Healthy controls
HHV-6	8/20 (40%)	11/30 (37%)
HHV-7	18/20 (90%)	26/30 (87%)



**Figure 1** Reconstruction experiment showing the sensitivity of single step (A) and nested (B) PCR for HHV-6. Dilutions of plasmids containing target sequences (A= $10^5$ , B= $10^4$ , C= $5 \times 10^3$ , D= $10^3$ , E and G= $5 \times 10^2$ , F and H  $10^2$ , I= $50$ , L= $10$  molecules, respectively) were subjected to PCR amplification and visualised on ethidium bromide stained agarose gels. Lane 1 is a blank PCR reaction, used as a control for contaminations.



**Figure 2** Ethidium bromide stained agarose gels showing the results of nested PCR reactions for HHV-6 (A) and HHV-7 (B). 1–6=PBMCs from MS patients, M=123 bp ladder used as molecular weight marker. The size of amplimers is expressed in base pairs (bp).

was observed. Furthermore, no residual DNA contamination of RNA samples was detected by analysing the same amount of RNA, without the initial reverse transcription reaction.

Likewise, eight samples positive for HHV-7 DNA were available for RNA analysis. Transcripts of three viral genes (U14, U16/17, U42) were searched by nested RT-PCR. Also in this instance, the transcripts belong to the immediate-early transcriptional class and are easily detected during productive infection (Menegazzi *et al*, 1999). However, no positive signal was detected.

## Discussion

The first description in 1995 that HHV-6 might be associated to MS (Challoner *et al*, 1995) stirred considerable interest and prompted several studies to confirm the association and to elucidate a possible viral role. Several years have elapsed and the situation remains undetermined. Some studies confirmed viral findings in MS patients, other investigations failed to indicate a viral involvement. The discrepancy could be ascribed to different techniques employed in the studies and to different patients' populations. Anyway, it is always difficult to establish aetiologic associations when the viral agent infects the majority of the population, when it establishes latent infections, and when it has a low pathogenetic potential in the immunocompetent host. As we recently proposed (Rotola *et al*, 1998), the analysis of viral transcription might be helpful in discriminating between latent and active infection. We therefore analysed the transcription pattern of HHV-6 and HHV-7 in PBMCs of patients with multiple sclerosis. We analysed PBMCs because most likely the virus is carried to the CNS by monocytes/macrophages and activated lymphocytes participate in demyelination (Rayne and Scheinberg, 1988). Furthermore, HHV-6 activation in the blood of MS patients has been suggested on the basis of increased levels of IgM and the presence of viral DNA in serum of patients (Soldan *et al*, 1997). Therefore, HHV-6 infection in the CNS might be caused and reflected by an active replication in the peripheral blood. Mayne *et al*, (1998) have recently reported that HHV-6 is rarely found in PBMCs of MS patients. However, it is possible that even low levels of productive infection by HHV-6 may cause clinical disease (Carrigan and Knox, 1995) and PCR cannot discriminate between latent and chronic low level infection. Consequently, the determination of viral replication through transcription analysis in peripheral blood could represent an important marker for at least a subset of patients.

The prevalence of HHV-6 was similar in MS patients and in healthy individuals. Also the analysis of distribution of HHV-6 variants failed to show differences, and HHV-6B was prevalent in MS

patients, as already described in the healthy population (Di Luca *et al*, 1996). We recently reported that latency of HHV-6 is associated to the presence of U94 mRNA in the absence of other mRNAs transcribed during the IE phase of infection (Rotola *et al*, 1998). Here, we unequivocally show that HHV-6 is latent in PBMCs of MS patients, since U94 is the only transcript found, and all other IE genes, transcribed with high levels during productive and restricted infection (Mirandola *et al*, 1998) were not detected.

The analysis yielded similar results for HHV-7, showing no association with MS. The prevalence of viral DNA was the same in MS patients and in healthy controls, and no footprint of viral transcription was detected in MS patients.

In conclusion, our studies on prevalence and transcriptional activity of HHV-6 in PBMCs suggest that these cells do not represent a significant reservoir of infection in the course of MS. Finally, we would like to point out that our results do not dismiss the possibility that HHV-6 is involved in the pathogenesis of MS. In fact, viral expression could be confined to the CNS, or the virus might play a role at the early onset of the disease. Additional studies are needed before drawing any definitive conclusion.

## Materials and methods

Twenty patients with relapsing-remitting MS were enrolled in the study. PBMCs obtained from each patient were purified on Ficoll gradients and DNA was extracted by conventional procedures. When possible, specimens were stored at  $-80^{\circ}\text{C}$  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 U31 gene (Mirandola *et al*, 1998), analysing 1  $\mu\text{g}$  of DNA, corresponding to 150 000 cells. Particular care was taken to avoid contamination of samples and blank reactions, consisting of the extraction mixture alone, were interspersed within experimental samples (to control for possible cross-contamination of DNA samples). HHV-6 variants were characterized by restriction enzyme cleavage, as previously described (Di Luca *et al*, 1996). The presence of HHV-7 DNA was searched by nested PCR, specific for U14 gene (Menegazzi *et al*, 1999). Aliquots of the PCR reactions were electrophoresed in agarose gels and analysed with ethidium bromide staining. Sensitivity of PCR reaction was determined by reconstruction experiments, amplifying known amount of cloned target molecules.

RNA was extracted from PBMCs with RNazol B (Biotecx) and treated with DNase to ensure that DNA was not present in the sample. After phenol/chloroform purification, the RNA was stored at  $-70^{\circ}\text{C}$  in ethanol/sodium acetate until utilised for reverse transcription (RT) and PCR amplification.

Immediately before reverse transcription, the RNA 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 HHV-6 and HHV-7 mRNAs as recently described (Mirandola *et al*, 1998; Mene-gazzi *et al*, 1999). The efficiency of retrotranscription was assessed by analysis of dilutions of cDNA with PCR specific for  $\beta$ -actin. To ensure that viral

DNA was not contaminating the RNA samples, positive specimens were analysed by nested PCR without retrotranscription.

PBMCs, obtained from healthy blood donors, were included as controls in the study.

## Acknowledgements

This study was supported by grants from Istituto Superiore Sanità (AIDS project), European Community Biomed2 BMH4 CT95, MURST 40% and 60% and from Italian CNR.

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