DETECTION OF LYMPHOTROPIC HERPESVIRUS DNA BY POLYMERASE CHAIN REACTION IN CEREBROSPINAL FLUID OF AIDS PATIENTS WITH NEUROLOGICAL DISEASE

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Summary. – Cerebrospinal fluid (CSF) samples from 49 acquired immunodefficiency disease syndrome (AIDS) patients with a central nervous system (CNS) disease were examined by polymerase chain reaction (PCR) to evaluate the association between the positivity for cytomegalovirus (CMV) and Epstein-Barr virus (EBV), and clinical diagnosis of a CNS disease. Frequency and clinical relevance of detection of DNA of human herpesviruses 6 (HHV-6), 7 (HHV-7) and 8 (HHV-8) were also determined. DNA of one or more of the following viruses was found in 26 of 49 patients (53%): CMV in 16 (33%), EBV in 13 (27%), human herpesvirus 6 (HHV-6) in 2 (4%), human herpesvirus 7 (HHV-7) in 1 (2%), and human herpesvirus 8 (HHV-8) in 1 (2%). The CMV detection was significantly associated with encephalitis and peripheral neuropathy (7/16 vs. 2/33, $p = 0.003$), while EBV with primary CNS lymphoma (P-CNSL) (8/13 vs. 0/36, $p < 0.0001$). HHV-6 DNA was found in CSF of two patients with neuroradiological features suggestive of cerebral lesions. HHV-8 or HHV-7 DNA was detected in the CSF of patients with unexplained neurological symptoms. This study confirms that the PCR analysis of CSF is a valid tool for the diagnosis of neurological diseases associated with CMV and EBV. On the other hand, HHV-6, HHV-7 and HHV-8, instead, were rarely detected in CSF of AIDS patients and have certainly no correlation with the CNS disease found.

Key words: AIDS; CMV; CNS disease; EBV; HHV-6; HHV-7; HHV-8;

Introduction

Among the neurological complications of the CNS occurring in patients with AIDS, opportunistic viral infections account for about a half of the cases (Petito *et al.*, 1986). These include infections caused by herpesviruses, such as herpes simplex viruses 1 (HSV-1) and 2 (HSV-2), varicella-zoster virus (VZV), CMV (mainly associated with encephalitis, meningitis, polyradiculopathy and neuropathy), EBV (very closely associated with P-CNSL) and papovaviruses, such as JC virus (JCV, associated with progressive multifocal leukoencephalopathy (PML)) (MacMahon *et al.*, 1991; Said *et al.*, 1991; Echevarria *et al.*, 1997; Cinque *et al.*, 1997; Weber *et al.*, 1997).

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Abbreviations: AIDS = acquired immunodeficiency syndrome; CMV = cytomegalovirus; CNS = central nervous system; CSF = cerebrospinal fluid; CT = computed tomography; EBV = Epstein-Barr virus; HHV-6, HHV-7, and HHV-8 = human herpesviruses 6, 7, and 8; HIV = human immunodeficiency virus; HSV-1 and HSV-2 = herpes simplex viruses 1 and 2; JCV = JC virus; MRI = magnetic resonance imaging; PCR = polymerase chain reaction; P-CNSL = primary CNS lymphoma; PML = progressive multifocal leukoencephalopathy; VZV = varicella-zoster virus

Furthermore, HHV-6 has been found in the brain or CSF of adults and children with AIDS, but its role in neurological disease is still unclear (Saito *et al.*, 1995; Knox *et al.*, 1995; Cinque *et al.*, 1996).

Recently, two other lymphotropic herpesviruses (HHV-7 and HHV-8) have been identified in CSF of AIDS patients, but their role in CNS disorders of these patients has not yet been clarified (Torigoe *et al.*, 1996; Brink *et al.*, 1998). The etiological diagnosis of a CNS disease is currently difficult because conventional diagnostic procedures are either insensitive or invasive. Recently, a PCR for the detection of herpesvirus DNA has significantly improved the diagnosis of neurological diseases (Cinque *et al.*, 1992, 1993, 1996; Fox *et al.*, 1995; Arribas *et al.*, 1995; Portolani *et al.*, 1997).

The purpose of this study was to correlate the clinical diagnosis of HIV-associated neurological disorders with CSF-positivity for specific herpesviruses, as determined by a nested PCR assay.

Materials and Methods

Patients. For this study, patients with AIDS-associated neurological disorders were examined at the Santa Maria Annunziata Hospital in Florence between November 1996 and December 1999. At the time of diagnostic lumbar puncture and complete neurological examination 59 CSF samples were obtained from 49 patients with AIDS. All the patients tested in this study either had refused brain biopsy or had a performance Karnofsky index <50, for which a cerebral biopsy is not indicated. Clinical neurological examination and neuroradiological study by computed tomography (CT) or magnetic resonance imaging (MRI) represented the first step to define clinical neurological syndromes and to conduct differential diagnosis. CSF samples were examined for glucose and protein content, cell count; tests for bacteria, mycobacteria and fungi were made by cultivation; DNA of herpesviruses and JCV were identified by PCR. The identification of microbial antigens was not routinely used for the diagnosis of opportunistic organisms except for *Cryptococcus neoformans*. Meningitis and meningoencephalitis caused by *Mycobacterium tuberculosis* and *C. neoformans* were diagnosed by isolation from the CSF using conventional culture techniques or by detection of the polysaccharide antigen of *C. neoformans*. Toxoplasmosis was diagnosed on the basis of focal or multifocal ring enhancing lesions on CT or MRI scans and ^{201}T1-SPECT scan was used to distinguish a primary brain lymphoma from cerebral toxoplasmosis (Lorberboym *et al.*, 1996). However, diagnosis of encephalitis caused by *Toxoplasma gondii* infection was often made on the basis of the response to antitoxoplasma treatment. The presumptive diagnosis of cerebral lymphoma was made on the basis of combined results of MRI and ^{201}T1-SPECT scan. Progressive PML diagnosis was based on the association of focal or multifocal CNS symptoms and signs with corresponding non-enhancing white-matter lesions on CT or MRI scans. Among the 49 patients examined, 38 presented cerebral lesions as evidenced by CT and MRI, and 11 presented only neurological symptoms. The median $CD4^+$ cell count of these patients was 2×10^7/l (range, 1–300 $\times 10^6$/l). Patients were grouped on the basis of clinical diagnosis (Table 1). CSF from 10 seronegative subjects (4 normal and 6 bone marrow recipients) without neurological disorders and CSF from 5 patients with non-immunodeficiency-related (sporadic) P-CNSL were tested as controls.

CSF PCR assay. Two pairs of primers in a nested PCR (consisting of the first and the second PCR) were used to detect DNA of CMV, EBV, HHV-6, HHV-7, and HHV-8 in CSF samples. Some details of these specific nested PCR assays are given in Table 2. The assay of the CSF samples was performed blindly with regard to clinical diagnosis. Two or three samples were obtained from 7 patients at different time points. For DNA extraction the method described by Fox *et al.* (1995) was used. Briefly, CSF samples were pelleted at 15,000 x g for 5 mins; the supernatant was boiled for 10 mins and cooled on ice, and an 10 µl aliquot was added directly to the first PCR. For the second PCR, an aliquot of 2.5 µl (EBV), 2 µl (HHV-7), 1.5 µl (HHV-8), or 1 µl (CMV and HHV-6) of the first PCR product was used. An aliquot of 10 µl of the second PCR product was separated by electrophoresis in 1.5–2% agarose gel and stained with ethidium bromide. The sensitivity limit of each virus-specific PCR was assessed using purified DNA (CMV, HHV-7, and HHV-8), DNA extracted from EBV-infected Daudi cells (EBV) or plasmid DNA (HHV-6). Each DNA sample was diluted with a pooled CSF that did no contain detectable herpesvirus DNA. The sensitivity of each virus-specific PCR is given in Table 2 in terms of minimum number of genome copies required (53%) for positive detection. In each PCR, two aliquots of each CSF sample were tested, and the sample was regarded as positive or negative only in case of concordant results. When results were discordant, 2 aliquots were tested again, and the sample was eventually regarded as positive in the case of at least 2 positive results out of 4 examinations. In each assay the following positive and negative controls were included: human embryonic lung fibroblast cells infected and uninfected with CMV (AD169), Daudi cells infected and uninfected with EBV, HSB-2 cells infected and uninfected with HHV-6, SupT1 cells infected and uninfected with HHV-7, and BCBL-1 cells infected and uninfected with HHV-8. As a rule, also a DNA-free water sample was tested blindly in each assay. Precautions were strictly observed to avoid PCR contamination (Kwok *et al.*, 1989). PCR results were regarded as positive only in the case the negative controls gave negative results.

Statistical analysis was performed using the Fisher's test.

Results

Upon clinical evaluation, at least one of the following CNS disorders were diagnosed in 38 of 49 AIDS patients: encephalitis, extracerebral (retinitis) and peripheral nervous system (polyneuropathy) disorders, P-CNSL, encephalopathy, toxoplasmosis, PML, cryptococcosis and mycobacteriosis. The remaining 11 patients showed only non-specific neurological symptoms. In Table 1, the PCR results obtained

Table 1. Correlation between the PCR results of detection of DNA of some herpesviruses in CSF and presumptive clinical diagnosis in 49 AIDS patients

No. of		PCR-positive cases				
Clinical diagnosis	Patients	CMV	EBV	HHV-6	HHV-7	HHV-8
CMV-related neurological disorders						
Encephalitis	4	4	0	0	0	0
Encephalitis and other disorders	1	1	1	1	0	0
Peripheral nervous system disorders[a]	2	2	0	0	1	0
Extracerebral disorders[b]	7	5	0	0	0	0
Extracerebral and other disorders	2	1	1	0	0	0
Total	16	13	2	1	1	0
Lymphoma						
Cerebral lymphoma	5	1	5	0	0	0
Cerebral lymphoma and other disorders	3	2	3	0	0	0
Total	8	3	8	0	0	0
Other neurological conditions	14	0	1	0	0	0
Unexplained neurological symptoms	11	0	2	1	0	1
Number (%) of CSF-PCR positive patients	49	16 (35%)	13 (27%)	2 (4%)	1 (2%)	1 (2%)

[a]Peripheral nervous system neuropathy. [b]Retinitis (6 patients) and lumbosacral polyradiculopathy (1 patient).
Note: other neurological conditions include: encephalopathy (2 patients), neurotoxoplasmosis (5 patients), PML (2 patients), cryptococcosis (6 patients), and tuberculosis (6 patients).

for each tested herpesvirus and the corresponding clinical diagnosis, are shown. None of the CSF samples from the 15 HIV-seronegative controls was positive for lymphotropic herpesvirus DNA.

In 26 (53%) of 49 patients the DNA of at least one herpesvirus was detected; the CMV and EBV DNAs were most common (33% and 27%, respectively). In 7 (14%) patients DNA of more than one herpesvirus was detected. None of the CSF samples from the 15 HIV-seronegative controls was positive for lymphotropic herpesvirus DNA.

Of the 16 patients with CSF positive for CMV DNA 7 presented a clinical diagnosis of encephalitis (5 cases) or peripheral nervous system disorders (2 cases), while 6 were affected by CMV-related extraneurological diseases (mainly retinitis). Among the 33 patients without detectable CMV DNA in their CSF, 2 had a diagnosis of encephalopathy, 3 had extraneurological CMV infection, and 28 were affected by CMV-unrelated neurological disorders. Presence of CMV DNA in CSF was significantly associated with the clinical diagnosis of a CMV-related neurological disease such as encephalitis or peripheral nervous system disorders (7/16 vs. 2/33, $p = 0.003$). Moreover, absence of detectable CMV DNA in CSF was significantly associated with the clinical diagnosis of CMV-unrelated diseases (28/31 vs. 5/18, $p < 0.0001$).

EBV DNA was detected in all the 8 patients with diagnosis of P-CNSL as determined by ^{201}T1-SPECT imaging, but only in 5 of the 41 patients without P-CNSL. Therefore, the presence of EBV DNA in CSF was significantly associated with the diagnosis of P-CNSL (8/13 vs. 0/36, $p < 0.0001$). In one patient with P-CNSL, radiotherapy resulted in both great improvement of clinical conditions and a negative ^{201}T1-SPECT imaging. Of the 2 CSF samples taken from this patient, one at the time of the diagnosis and another after radiotherapy, EBV DNA was detected in the first sample only.

HHV-6 DNA was detected in 2 patients. In both cases the hypodensity zone with abnormal cortical and subcortical signal was associated to cerebral lesions characterized by areas of demyelinization; moreover, the JCV-specific PCR was negative in these cases. In one patient, the CSF was positive also for CMV DNA, while in the other patient HHV-6 was the only opportunistic pathogen detected; in the latter patient mild neurological symptoms consisting of moderate fever, confusion and hyposthenia were observed.

HHV-7 DNA was found only in the CSF of one patient with peripheral nervous system neuropathy. In this case, CMV DNA was also detected. Ganciclovir and foscarnet treatments performed against CMV infection resulted ineffective.

Out of all CSF samples tested for the presence of HHV-8 DNA only one was positive. This patient had a generalized Kaposi's sarcoma and unexplained neurological symptoms including drooling, expressive aphasia and reading/writing difficulties but neither neuroradiological findings of CNS lymphoma nor encephalitis; there were no indications suggesting other pathogens.

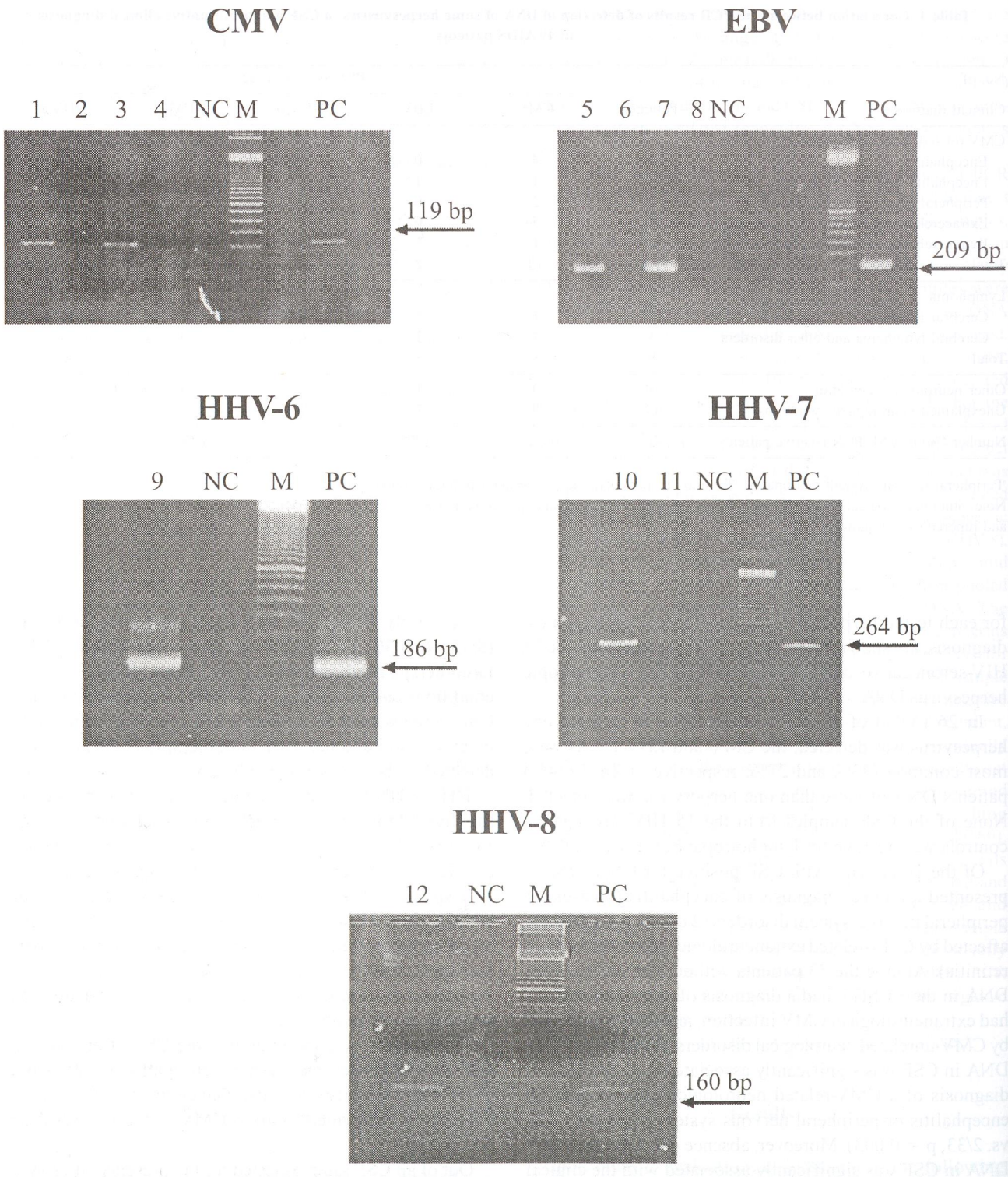

Fig. 1

Agarose gel electrophoresis of the products of the nested PCR

CSF samples from two patients with encephalitis and polyneuropathy, respectively, positive for CMV-DNA (lanes 1 and 3); negative control samples (lanes 2 and 4); CSF samples from 2 patients with primary CNS lymphoma positive for EBV DNA(lanes 5 and 7); negative control samples (lanes 6 and 8); CSF sample from a patient with neuroradiological features strongly suggestive of demyelinating lesions (lane 9); CSF sample from a patient with polyneuropathy (lane 10); negative control sample (lane 11); CSF sample from a patient with KS (lane 12). NC = negative control; PC = positive control; M = size marker (100 bp ladder).

Table 2. Characteristic of the nested PCR

Virus DNA assayed (reference)	Upstream primers (5′→3′) (nucleotide positions) [μmoles]	Downstream primers (5′→3′) (nucleotide positions) [μmoles]	Amplicon length (bp)	Sensitivity (No. of genomes)
EBV (Cinque *et al.*, 1993)	EB-3: AAGGAGGGTGGTTTGGAAAG (109332-109351) [0.15] EB1: ATCGTGGTCAAGGAGGTTCC (109353-109372) [0.30]	EB-4: AGACAATGGACTCCCTTAGC (109609-109628) [0.15] EB-2: ACTCAATGGTGTAAGACGAC (109542-109561) [0.30]	297 209	4
CMV [modified] (Ruger *et al.*, 1987)	A: ACGTTGATGCTGGGGATGTTCAGCA (120655-120679) [0.2] C: TGGGGCAGATGCTTCGGCCC (120725-120744) [0.2]	B: CGCTGATCTTGGTATCGCAGTACAC (120853-120877) [0.2] D: CGACGCCATGCCACCGCG (120827-120844) [0.2]	222 119	10
HHV-6 (Klotman *et al.*, 1993)	P0: CCGCAATCGAATCCATCCTAGCGG (47254-47277) [0.2] P1: CCCATTTACGATTTCCTGCACCACCT CT CTGC (47421-47452) [0.2]	P4: GTGAGAACGGATTCGACCAGTGCTG (47664-47689) [0.2] P3: TTCAGGGACCGTTATGTCATTGAGCATGTG (47578-47607) [0.2]	435 186	10
HHV-7 [modified] (Okuno *et al.*, 1995)	H7-1: CACAAAAGCATCGCTATCAA (88173-88192) [0.5] H7-3: GACTCATTATGGGGATCGAC (88228-88247) [0.5]	H7-2: AGTTCCAGCACTGCAATCG (88562-88580) [0.5] H7-4: CGCATACACCAACCCTACTG (88472-88491) [0.5]	408 264	10
HHV-8 [modified] (Bigoni *et al.*, 1996)	KS-1: AGCCGAAAGGATTCCACCAT (47287-47307) [0.2] NS-1: ACGGATTTGACCTCGTGTTC (47321-47341) [0.2]	KS-2: TCCGTGTTGTCTACGTCCAG (47500-47520) [0.2] NS-2: AATGACACATTGGTGGTATA (47461-47481) [0.2]	233 160	4

The first and second line for each virus DNA refer to the first and second PCR, respectively. The corresponding nucleotide sequence for each herpesvirus was retrived from the GeneBank database, either from the complete sequence of the virion genome, as in the case of EBV (Acc. No. V01555; EBNA-1 gene), CMV (Acc. No. X17403; late gp 64 gene), HHV-6 (Acc. No. S57540; ZVH14 fragment), and HHV-7 (Acc. No. AF037218; immediate-early gene) or from the sequence of the capsid protein gene, as in the case of HHV-8 (Acc. No. U75698).

Discussion

In this study, a nested PCR was used to detect DNA of lymphotropic herpesviruses in the CSF of HIV-infected patients with neurological disorders. Nine AIDS patients were clinically diagnosed with neurological disorders (encephalitis, neuropathy, myelitis and polyradiculopathy) previously associated with CMV infection (McCutchan *et al.*, 1995; Cinque *et al.*, 1997). Seven of these patients had CMV DNA in their CSF. CMV DNA was also detected in 9 of 40 patients with neurological disorders not clinically related to CMV infection. These findings are in agreement with a previous prospective study (Fox *et al.*, 1995) carried out according to the same clinical diagnostic criteria as ours and with comparable PCR sensitivity. The lack of CMV DNA detection in 2 cases of encephalopathy could be explained by the presence of HIV-associated encephalopathy (Simpson *et al.*, 1995), which is indistinguishable from CMV-associated encephalopathy on the basis of clinical criteria only. The presence of CMV DNA in the CSF of patients with neurological disorders in association with other opportunistic pathogens may represent a subclinical CMV infection of the CNS. Moreover, we found a highly significant association between the absence of CMV DNA in CSF by PCR and the diagnosis of CMV-unrelated neurological or extraneurological disease. This result suggests that a negative PCR result can also be useful in etiologic diagnosis.

We also found that the presence of EBV DNA in CSF is significantly associated with the diagnosis of P-CNSL as determined by ^{201}T1-SPECT. EBV DNA was detected in all the 8 patients with typical lesions of P-CNSL and in the 5 patients without diagnosis of lymphoma, supporting the high PCR sensitivity. It is still unclear whether the EBV DNA detection in CSF could be considered a preclinical marker as suggested in an earlier study (Cinque *et al.*, 1993), and whether an elevated sensitivity of the PCR method could be advantageous. In one of the 8 patients with lymphoma, EBV DNA detection in CSF preceded the neuroradiological diagnosis. In another patient with lymphoma, positive for EBV DNA in the CSF, radiotherapy improved the patient's clinical conditions and made him CSF-negative for the EBV DNA. This confirms the specificity of the PCR used by us in this study. Our data also suggest that the EBV-specific

PCR of CSF and ^{201}T1-SPECT could be used in combination to support clinical diagnosis mainly in patients unresponsive to antitoxoplasma treatment, although P-CNSL and encephalitis caused by *T. gondii* are not mutually exclusive and both conditions may coexist in the same patient. Of the 5 patients with detectable EBV DNA and without diagnosis of P-CNSL, 4 were aggressively treated with a combination of anti-retroviral drugs including protease inhibitors. On the other hand, none of the P-CNSL patients with EBV DNA in the CSF were subjected to the same anti-retroviral treatment protocol. These data suggest that a drastic reduction of the HIV load in the CSF produced by the newest generation of therapeutics (Stellbrink *et al.*, 1997) also prevents the development of P-CNSL even in the presence EBV DNA.

HHV-6 can reactivate during HIV infection (Iuliano *et al.*, 1997) and can be associated with demyelinization in AIDS patients (Knox *et al.*, 1995). In our study, HHV-6 DNA was found in the CSF of 2 patients, both with neuroradiological features suggestive of demyelinating lesions; however, there is no proof that the lesions were indeed demyelinating because inflammatory and demyelinating lesions can look alike on imaging. Since Cinque *et al.* (1996) detected HHV-6 DNA in CNS in absence of active infection it remains to be clarified whether the presence of HHV-6 in the CSF is always associated with demyelinating lesions.

To our best knowledge this is the first report of HHV-7 DNA detection in CSF of HIV-infected subjects. HHV-7 was already found to be associated with CNS disorders in infants (Torigoe *et al.*, 1996). In the CSF of a patient affected by polyneuropathy, we detected HHV-7 DNA together with CMV DNA. Recently, the HHV-7 presence was associated with the progression of CMV disease in immunocompromised patients (Osman *et al.*, 1996; Chan *et al.*, 1998). Therefore, HHV-7 could worsen the neurological disorder caused by CMV.

HHV-8 is the last discovered lymphotropic herpesvirus associated with Kaposi's sarcoma and body cavity-based-lymphomas (Levy, 1997). In this study, HHV-8 was detected as the unique opportunistic pathogen in the CSF of a patient affected by non-specific neurological symptoms and generalized Kaposi's sarcoma; however, we do not exclude the possibility of contamination by passenger HHV-8-positive blood lymphocytes in the CSF. In agreement with an earlier study (Brink *et al.*, 1998), this patient did not have CNS lymphoma or other neurological disease. On the other hand, a recent report on the association of HHV-8 with encephalitis in HIV-infected patients (Said *et al.*, 1997) suggests a possible tropism of this virus for the CNS.

In conclusion, we confirm that the PCR analysis of CSF is a valid tool for the diagnosis of neurological disorders associated with pathogens such as CMV and EBV both of which are frequently detected in the CSF of AIDS patients. The pathogenic role for HHV-6 and HHV-7, as with HHV-8, in neurological diseases of AIDS patients remains to be established.

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