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RESEARCH ARTICLE

HHV-8/KSHV is Not Associated with AIDS-Related Primary Central Nervous System Lymphoma

Andrea Antinori[1], Luigi M. Larocca[2], Lucia Fassone[3], Paola Cattani[4], Daniela Capello[3], Antonella Cingolani[1], Giuseppe Saglio[5], Giovanni Fadda[4], Gianluca Gaidano[3], and Luigi Ortona[1]

[1] Department of Infectious Diseases, Catholic University, Roma, Italy
[2] Department of Pathology, Catholic University, Roma, Italy
[3] Division of Internal Medicine, Department of Medical Sciences, "Amedeo Avogadro", Eastern Piedmont University, Novara, Italy
[4] Institute of Microbiology, Catholic University, Roma, Italy
[5] Department of Clinical and Biological Sciences, University of Torino, Orbassano-Torino, Italy.

Primary central nervous system lymphoma (PCNSL) is a major complication of the late stages of human immunodeficiency virus (HIV) disease. Epstein Barr virus (EBV) infection is the only genetic lesion consistently associated with this neoplasia. Recently, it has been proposed that the pathogenesis of AIDS-related PCNSL (AIDS-PCNSL) may be associated with infection by human herpesvirus-8/Kaposi's sarcoma associated herpesvirus (HHV-8/KSHV), although at present such association remains controversial. In order to conclusively assess the link between HHV-8/KSHV infection and AIDS-PCNSL, we performed a comprehensive study based on multiple molecular assays on cerebral tissues and cerebrospinal fluid (CSF) as well as specific immunologic assays on patients' sera. A well characterized panel of 33 Italian patients with AIDS-PCNSL and 13 controls with other HIV-related brain focal diseases from the same geographical area was analyzed. No signs of HHV-8/KSHV infection were detected in cerebral tissues by single-step PCR. Cerebral tissues of all AIDS-PCNSL scored negative for HHV-8/KSHV DNA sequences also by nested PCR, with the exception of one single patient who was simultaneously affected by Kaposi's sarcoma. All CSF samples analyzed were consistently devoid of HHV-8/KSHV sequences by molecular assays. By serologic assays, detecting both latent and lytic HHV-8/KSHV antigens, a specific immunoreactivity was observed in 16/33 (48%) AIDS-PCNSL and in 6/13 (46%) controls (*P*=0.88). A significant correlation with HHV-8/KSHV serum reactivity was seen with a homosexual route of HIV transmission (*P*=0.018), but not with the presence of AIDS-PCNSL. The results of our analysis conclusively assess that HHV-8/KSHV infection is not a feature of AIDS-PCNSL.

Corresponding author:
Andrea Antinori, MD, Department of Infectious Diseases, Catholic University, L.go A. Gemelli 8 – 00168, Roma, Italy; Tel.: 39-6-30154945; Fax: 39-6-3054519; E-mail: iclmi@rm.unicatt.it

Introduction

Primary central nervous system lymphoma (PCNSL) is a frequent complication of the late stages of human immunodeficiency virus (HIV) infection, and represents a major cause of morbidity and mortality among HIV infected patients (25). The incidence rate of PCNSL among HIV infected individuals is 3000 to 7000 times higher than that of the general population (12). The pathogenesis of AIDS-related PCNSL (AIDS-PCNSL) is poorly understood. At present, the only genetic lesion consistently associated with AIDS-PCNSL is represented by infection of the tumor clone by Epstein-Barr virus (EBV). EBV transcripts are present in the tumor cells of almost all cases of AIDS-PCNSL (23, 30), and infection associates with expression of the virus-encoded latent membran protein-1 (LMP-1) in almost half of the patients (23). Because of the consistent association of AIDS-PCNSL with EBV infection, detection of EBV DNA in cerebrospinal fluid (CSF) of HIV-infected individuals is regarded as a reliable marker of this disease for minimally invasive diagnosis (4, 9), as well as for monitoring the response to chemotherapy (3).

The association of AIDS-PCNSL with herpesviruses other than EBV has been investigated to a certain extent. All such studies ruled out a role for HHV-6 and CMV in AIDS-PCNSL development (10, 20, 26, 41). During the last few years, a novel herpesvirus has been identified in

lesions of AIDS-related Kaposi's sarcoma (AIDS-KS) and termed human herpesvirus-8 (HHV-8) or, alternatively, Kaposi's sarcoma associated herpesvirus (KSHV) (8). Infection by HHV-8/KSHV has been demonstrated to associate with all clinical variants of KS (37) and, among lymphoproliferate disorders, with primary effusion lymphoma (7) and with AIDS-related multicentric Castleman's disease (47).

Recently, it has been proposed that the pathogenesis of AIDS-PCNSL may associate with infection by HHV-8/KSHV. In fact, some studies have detected HHV-8/KSHV DNA sequences in the tumor lesions of AIDS-PCNSL patients at a relatively high frequency (11, 13, 33). The hypothesis that AIDS-PCNSL may associate with HHV-8/KSHV infection has been reinforced by indirect evidence suggesting that AIDS-PCNSL shares several features with AIDS-KS. First, epidemiologic data have shown a strong association between KS and PCNSL in men during the AIDS era (1). Second, the incidence of AIDS-KS and AIDS-PCNSL has decreased most profoundly compared to other AIDS-related cancers after the introduction of highly active antiretroviral therapy (HAART) (21). Finally, animal models infected with HIV homologs, but devoid of HHV8/KSHV infection fail to develop KS and PCNSL, although they frequently develop other types of AIDS-related cancers (14, 31).

Despite these lines of evidence, the precise link between HHV-8/KSHV infection and AIDS-PCNSL remains controversial. In fact, a number of investigations have been unable to confirm the presence of HHV-8/KSHV DNA sequences in AIDS-PCNSL biopsies (15, 28, 39). These discrepancies may be due, at least in part, to the use of different molecular techniques for HHV-8/KSHV detection in the different reports available. In addition to molecular investigations, the precise link between HHV-8/KSHV infection and AIDS-PCNSL may be elucidated by investigating the HHV-8/KSHV serologic status of AIDS-PCNSL patients. However, such serologic studies have not been performed systematically in large panels of AIDS-PCNSL.

Here we aimed at conclusively clarifying the association between AIDS-PCNSL and HHV-8/KSHV infection. Toward this aim, we have subjected a well characterized panel of patients with AIDS-PCNSL or other AIDS-related brain lesions to a comprehensive analysis of HHV-8/KSHV infection by multiple molecular and serologic assays. Our results indicate that AIDS-PCNSL is not associated with HHV-8/KSHV infection.

Methods

CSF and pathology samples. This study was based on thirty-three cerebral tissue samples (24 at brain biopsy and 9 at autopsy) and corresponding CSF (n=29) and serum (n=33) samples from 33 Italian HIV-infected patients with a definitive diagnosis of AIDS-PCNSL observed at the Department of Infectious Diseases of the Catholic University of Rome in a prospective cohort of AIDS-related brain focal lesions (2, 9). For control purposes, the study also included 13 cerebral tissues, CSF and serum samples of Italian HIV-infected patients with other focal neurological disorders matched from the same cohort (3 cerebral mycetoma, 4 progressive multifocal leukoencephalopathy, 2 toxoplasmic encephalitis, 1 cerebral vasculitis, 1 cytomegalovirus encephalitis, 1 herpes simplex virus encephalitis). For each patient, demographic and epidemiologic features (CD4+ T-lymphocyte count, HIV transmission route, concurrent diagnosis of KS) were recorded. In all cases of AIDS-PCNSL, cerebral tissue, CSF and serum samples were collected before or in absence of specific therapy. Pathological specimens were classified according to the Working Formulation for non-Hodgkin's lymphoma (40) and to a Revised European-American Classification of lymphoid neoplasia (REAL) (19).

PCR detection of HHV-8/KSHV DNA in cerebral tissues. Analysis of HHV-8/KSHV infection in cerebral tissues was performed by single-step polymerase chain reaction (PCR) as well as by nested PCR. Single-step PCR was performed as previously reported using oligonucleotides $KS330_{233}$-F and $KS330_{233}$-R as primers (8, 16). For nested PCR studies (35), 2.5 μl of the PCR product obtained with primers $KS330_{233}$-F and $KS330_{233}$-R were added to the PCR mixtures containing primers $KS330_{233}$-FN and $KS330_{233}$-RN and amplified for an additional 35 cycles (15). The PCR products were visualized by ethidium bromide staining on a 2% agarose gel. Previous experiments (16) showed that the sensitivity of the single-step PCR assay allows the detection of 100 copies of HHV-8/KSHV target sequence in a background of 200,000 human haploid genome equivalents. Conversely, analogous experiments showed that the nested PCR assay allows the consistent and reproducible detection of 1 viral copy per 200,000 human haploid genome equivalents (15). Each tested DNA sample was analyzed in 5 independent experiments and only cases with a repeatedly positive result in all 5 experiments were considered positive for HHV-8/KSHV infection. To assess integrity and suitability of all DNA

samples, a 268-bp fragment of human β-globin gene was amplified (data not shown).

In order to closely monitor the occurrence of PCR false positive results, several negative controls were regularly analyzed in each PCR reaction. The PCR negative controls included reaction mixtures lacking any DNA template and reaction mixtures with HHV-8/KSHV negative human DNAs obtained from B-lymphoblastoid cell lines. Negative controls were also included both during sample preparation and DNA extraction. Procedures to prevent specimen contamination and PCR carryover were rigorously observed at every step (22).

PCR detection of HHV-8/KSHV DNA in CSF. CSF specimens were assayed for detection of HHV-8/KSHV DNA by a nested PCR. Outer PCR was performed using primers P1 and P2, corresponding to positions 711-728 and 1412-1430 of the published 1853-bp KS330 BamHI region (27) as already reported (6). For inner PCR, 2.5 μl of the outer PCR product were amplified for an additional 25 cycles using primers $KS330_{233}$-F and $KS330_{233}$-R (8), as previously described (6). The PCR products were visualized by ethidium bromide staining on a 2.5% agarose gel. The sensitivity of this PCR was determined by end-point dilution of the $KS330_{233}$ fragment cloned in pCR™ 2.1 vector (Invitrogen, San Diego, USA) mixed with known amounts of DNA from human fibroblasts (Eurobio, Les Ulis, France). This nested PCR allows the consistent and reproducible detection of 1 viral copy per 200,000 human haploid genome equivalents. Procedures to prevent specimen contamination and PCR carryover were rigorously observed at every step in this analysis (22).

Analysis of EBV infection in tissue samples and in CSF. EBER in situ hybridization (ISH) was carried out by using a cocktail of fluorescein-isothiocyanate labeled oligonucleotides complementary to the two nuclear EBER (1/2) RNAs (Dako, Glostrup, Denmark), according to the instruction of the supplier.

EBV-DNA was amplified by a nested PCR technique using primers derived from the EBNA1 gene region, as already described (9). The detection limit of the nested PCR was tested using serial dilutions of DNA extracted from Daudi cells, known to contain approximately 40 EBV-DNA copies per cell. Several negative and positive controls were used during each amplification run. Clinical samples were tested at least twice and only consistent results were considered. To assess whether CSF specimens with a negative EBV-DNA by PCR contained DNA suitable for amplification, the samples were tested using primers GH26 and GH27 from the HLADQA1 region. CSF samples scored negative by nested PCR were checked for the presence of *Taq* polymerase inhibitors. Ten EBV-DNA copies were put in the negative sample and amplified in parallel with 10 EBV-DNA copies in water: if the result with the tested CSF sample was again negative, the presence of PCR inhibitors could be assumed. If inhibitors were present, the CSF sample was serially diluted or, in parallel, DNA was extracted by column absortion (Qiagen) to detect a positive PCR result.

HHV-8/KSHV antibodies immunofluorescence assay (IFA). An IFA was developed using the HHV-8/KSHV-positive and EBV-negative B cell line BC3 (American Type Culture Collection, Rockville, Md.). The EBV producer cell line P3HR-1 (American Type Culture Collection, Rockville, Md.) and HHV-8/KSHV-negative and EBV-negative cell line Ramos (American Type Culture Collection, Rockville, Md.) were used as control. Cells were grown in RPMI 1640 medium (Eurobio, Les Ulis, France), supplemented with 10% heat-inactivated fetal calf serum, glutamine 200 mM and antibiotics. In BC3 and P3HR-1 cells, lytic replicative cycles of the virus (HHV-8/KSHV and EBV respectively) were induced incubating 10^6 cells/ml with 20 ng/ml of the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA, Sigma, St. Louis) for 72 hours.

Uninduced and TPA-induced BC3 and TPA-induced P3HR-1 and Ramos cells were spotted on slides (inner diameter, 5 mm; 10 circles/slide; bioMérieux sa, Marcy L'Etoile, France), air-dried rapidly, and fixed in cold acetone. Cells (10^5/well) were incubated for 30 min at 37°C with 20 μl two-fold dilutions of human sera. The slides were washed three times in PBS for 10 min each time, incubated for 30 min at 37°C with a prestandardized dilution of Kallestad™ fluorescin-conjugated goat F(ab')2 fragment anti-human IgG (Sanofi Diagnostics Pasteur, Chaska, MN), washed again and examined under a fluorescence microscope. A typical speckled nuclear fluorescence, observed in both latently infected and TPA-induced BC3 cells, was referred to the reactivity of antibodies against latent antigens, as previously reported (24, 45). A cytoplasmic fluorescence pattern observed more frequently after TPA treatment, was presumed significant for the presence of antibodies against lytic antigens (24, 45-46). Specific reactivity at a dilution of 1:40 or more with uninduced and TPA-induced BC3 cells was considered positive for HHV-8/KSHV antigens.

Statistical analysis. Statistical comparisons between

Table 1.

Pts	CD4/μl	HIV transmission route	KS	Tissue samples			CSF samples		
				EBERS	HHV-8/KSHV DNA Single step PCR	HHV-8/KSHV DNA Nested PCR	EBV-DNA (Nested PCR)	HHV-8/KSHV DNA (Nested PCR)	BC3 IFA (titre)
P1	24	Homosexual	Yes	+	-	-	+	-	+ (1:80)
P2	32	Heterosexual	No	+	-	-	+	-	-
P3	42	Homosexual	Yes	+	-	+	+	-	+ (1:160)
P4	12	Homosexual	Yes	+	-	-	+	-	+ (1:80)
P5	5	Heterosexual	No	+	-	-	+	-	-
P6	5	Homosexual	No	+	-	-	+	-	-
P7	5	Heterosexual	No	+	-	-	+	-	-
P8	41	IVDU	No	+	-	-	+	-	-
P9	4	Homosexual	No	+	-	-	+	-	+ (1:80)
P10	52	Heterosexual	No	+	-	-	+	-	+ (1:320)
P11	3	Homosexual	Yes	+	-	-	-	-	+ (1:80)
P12	152	Heterosexual	No	+	-	-	+	-	+ (1:40)
P13	8	Heterosexual	No	+	-	-	+	-	-
P14	2	IVDU	No	+	-	-	-	-	+ (1:80)
P15	41	Heterosexual	No	-	-	-	-	-	-
P16	5	IVDU	No	+	-	-	+	-	-
P17	280	Heterosexual	No	+	-	-	+	-	-
P18	30	Homosexual	No	+	-	-	+	-	+ (1:640)
P19	4	Homosexual	No	+	-	-	+	-	-
P20	4	IVDU	No	+	-	-	+	-	+ (1:40)
P21	32	IVDU	No	+	-	-	n.a.	n.a.	+ (1:40)
P22	24	IVDU	No	+	-	-	-	-	+ (1:40)
P23	2	Homosexual	No	+	-	-	+	n.a.	+ (1:160)
P24	3	IVDU	No	+	-	-	+	-	-
P25	20	Heterosexual	No	+	-	-	+	-	-
P26	29	Homosexual	Yes	+	-	-	+	-	+ (1:160)
P27	8	Heterosexual	Yes	+	-	-	n.a.	n.a.	+ (1:320)
P28	15	IVDU	No	+	-	-	+	-	-
P29	4	IVDU	No	+	-	-	n.a.	n.a.	-
P30	4	Heterosexual	No	+	-	-	+	-	+ (1:80)
P31	2	Homosexual	No	+	-	-	+	-	-
P32	435	IVDU	No	+	-	-	n.a.	n.a.	-
P33	4	IVDU	No	+	-	-	+	-	-

Table 2.

Pts	CD4/μl	HIV transmission route	KS	Neurologic Disease	Tissue samples		CSF samples		
					HHV-8/KSHV DNA Single step PCR	HHV-8/KSHV DNA Nested PCR	EBV-DNA (Nested PCR)	HHV-8/KSHV DNA (Nested PCR)	BC3 IFA (titre)
C1	24	IVDU	No	Cryptococcosis	-	-	-	-	-
C2	403	IVDU	No	Aspergilloma	-	-	-	-	-
C3	100	Homosexual	No	PML	-	+	-	-	+ (1:40)
C4	58	IVDU	No	Candidosis	-	-	-	-	-
C5	35	IVDU	No	Vasculitis	-	-	-	-	+ (1:40)
C6	162	Heterosexual	No	Toxoplasmosis	-	+	-	-	+ (1:80)
C7	62	IVDU	No	HSV encephalitis	-	+	-	-	+ (1:40)
C8	41	IVDU	No	PML	-	-	-	-	-
C9	15	Heterosexual	No	Cryptococcosis	-	-	-	-	-
C10	27	Heterosexual	Yes	PML	-	-	-	-	+ (1:320)
C11	55	Homosexual	No	PML/Toxoplasmosis	-	+	-	-	+ (1:320)
C12	35	IVDU	No	CMV encephalitis	-	-	-	-	-
C13	15	Heterosexual	No	PML	-	-	-	-	-

different groups for proportion of discrete variables were expressed as relative risks and 95% confidence intervals (95%C.I.) and were performed using a Mantel-Haenszel chi-square test. A stratified asymptotic 2xK analysis using homosexual transmission route and HHV-8/KSHV serum reactivity as exposure and outcome variables and AIDS-PCNSL diagnosis as confounding variables was performed. The statistics of 3-way cross-tabulation tables were analyzed by a log-linear model after testing for partial and marginal associations (5). Significant level was established at less than 0.05 and all P valued were two-tailed.

Results

Descriptive analysis of the 33 AIDS-PCNSL cases and of the 13 controls is reported in Table 1 and in Table 2, respectively. Twenty-four AIDS-PCNSL patients and 11 controls were male (R.R. 0.84; 95%C.I. 0.59-1.20; *P*=0.47). Median age was 34 yrs (IQ range: 32-39) for AIDS-PCNSL cases and 37 yrs (IQ range: 31-45) for controls (*P*=0.17). CD4 count was <50 cell/μl in 4/33 AIDS-PCNSL and in 3/13 controls (R.R.1.30; 95%CI 0.67-2.54; *P*=0.35). No significant differences were detected for sexual HIV transmission route (R.R. 1.29; 95%CI 0.85-1.95; *P*=0.34) and for presence of Kaposi's sarcoma (R.R. 1.24;95%CI 0.88-1.79; *P*=0.65) between AIDS-PCNSL and controls.

All cases of AIDS-PCNSL were histologically classified as diffuse large B-cell lymphoma (B-DLCL). Depending on the presence of immunoblastic plasmocytoid features (23), tumor samples were further distinguished into 11 large noncleaved cell lymphoma (LNCCL), 18 immunoblastic plasmocytoid lymphoma (IBPL), and 4 with a mixture of LNCCL and IBPL features.

HHV-8/KSHV infection in tissue and CSF samples. Single step PCR analysis of HHV-8/KSHV DNA sequences in cerebral tissues scored consistently negative in all 33 AIDS-PCNSL and in all 13 control samples (see Table 1 and Table 2). Nested PCR analysis of HHV-8/KSHV DNA sequences scored negative in the cerebral tissues of 32/33 AIDS-PCNSL and of 9/13 control samples. Overall, positivity by nested PCR was restricted to 1 AIDS-PCNSL sample and 4 control lesions. The AIDS-PCNSL patient scored positive by nested PCR (P3 of Table 1) was an homosexual man with Kaposi's sarcoma diagnosed before the onset of AIDS-PCNSL. The control lesions scored positive by nested PCR included one PML, one toxoplasmic encephalitis, one HSV encephalitis, and one PML/toxoplasmosis coinfection (C3, C6, C7 and C11 of Table 2). Three out of four control patients with HHV-8/KSHV DNA sequences detected in tissue samples (C3, C6, C11 of Table 2) had their HIV infection acquired by a sexual route, but none of them had Kaposi's sarcoma. Patients with a non-lymphomatous inflammatory disease had a higher risk of harboring HHV-8/KSHV DNA sequences in their tissue compared with patients with AIDS PCNSL (R.R. 3.64; 95%CI 1.77-7.52; P=0.018).

With respect to CSF samples, analysis of HHV-8/KSHV DNA sequences by nested PCR scored negative in 28/28 AIDS-PCNSL and in 13/13 control lesions tested.

EBV infection in tissue and CSF samples. All 33 but one patients with AIDS-PCNSL displayed EBV transcripts in their tumor tissue samples (Table 1). EBERs ISH confirmed that the viral sequences were harbored by the neoplastic population (data not shown). EBV-DNA was detected by nested PCR in 25/29 (86%) CSF samples of patients with AIDS-PCNSL, but in none of 13 CSF collected from control patients (R.R. 4.25; 95%C.I. 1.80-10.01; *P* <0.000001). Three out of four patients with an EBV-DNA undetectable in CSF harbored viral transcripts in their tumoral tissues (see Table 1).

Immunofluorescence assay. The results of the immunofluorescence assay with the HHV-8/KSHV positive B-cell line BC3 showed that 16/33 (48%) AIDS-PCNSL cases and 6/13 (46%) controls had serum reactive for lytic and latent HHV-8/KSHV antigens (R.R. 1.03; 95%C.I. 0.71-1.48; *P*=0.88). Specific reactivities and antibody titers are reported in Table 1 and in Table 2. An antibody titer of 1:40 or more was detected in 10/13 (77%) homosexuals, 6/15 (40%) heterosexuals, and 6/18 (33%) IVDUs. (Figure 1). The risk of having a serum specific reactivity for homosexuals was 3.13 fold than that for IVDUs (95%C.I. 1.06-9.21; *P*=0.018). No significant differences were detected for seroprevalence between patients with homosexual and heterosexual

Table 1. (Opposing page, top) Clinic and epidemiologic characteristics, EBV and HHV-8/KSHV molecular features, and results of serologic IFA assay in 33 HIV-infected patients with PCNSL.

Table 2. (Opposing page, bottom) Clinic and epidemiologic characteristics, EBV and HHV-8/KSHV molecular features, and results of serologic IFA assay in 13 HIV-infected patients with non-lymphomatous brain focal disorders.

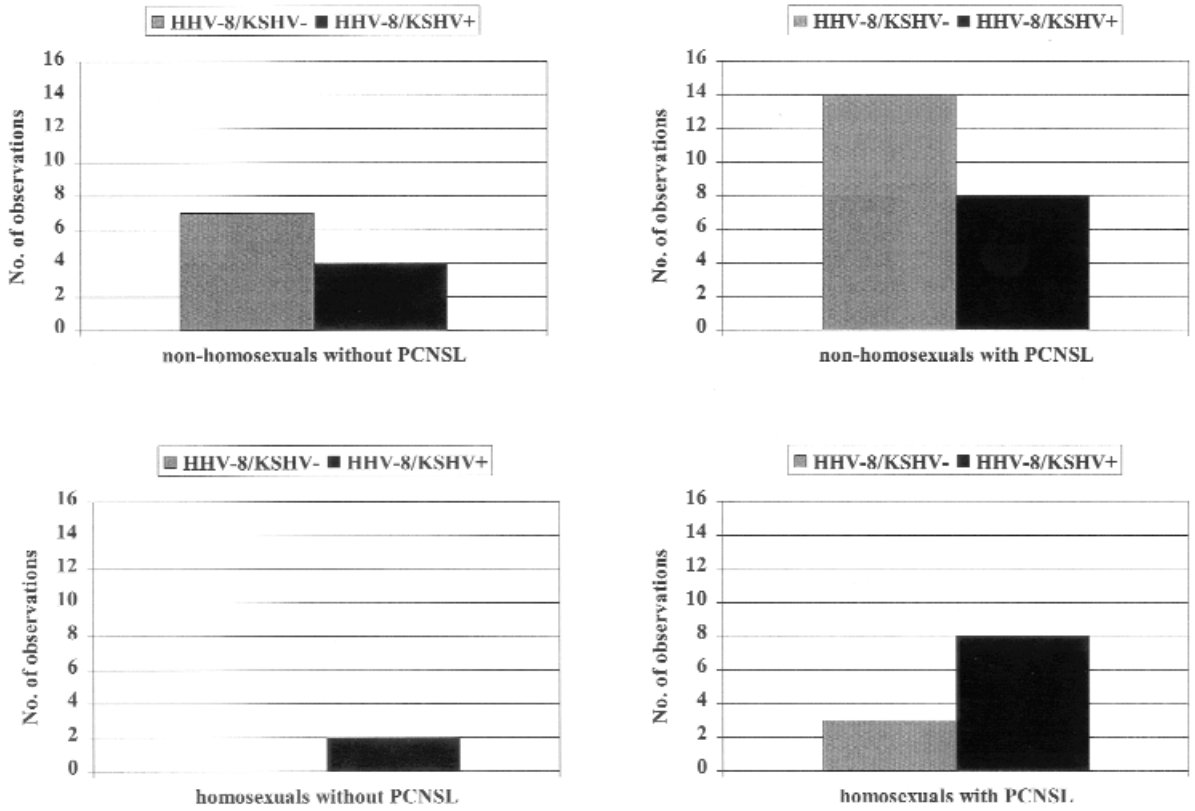

Figure 1. Categorized histograms of homosexual route of HIV transmission and PCNSL diagnosis for HHV-8/KSHV serum reactivity in 46 HIV-infected patients with AIDS-related brain focal disorders.

HIV route transmission, as well as for seroprevalence rates among cases and controls stratified for type of transmission route (data not shown). The significant correlation between HHV-8/KSHV serum reactivity and homosexual route of HIV transmission was confirmed also after stratified 2xK analysis employing AIDS-PCNSL as a confounding variable (Asymptotics statistics for all 2 strata combined: O.R. 7.07; 95%C.I. 1.29-43.88; P=0.009). The log-linear analysis did not reveal any significant interaction between AIDS-PCNSL and homosexual route to determine a HHV-8/KSHV serum reactivity (Maximum likelihood chi-square of fitted model: 1.36; P=0.71). Only a significant correlation at tests of marginal (P=0.009) and partial (P=0.01) association was observed between HHV-8/KSHV serum reactivity and homosexual route (see Figure 1). In all 5 patients (one with AIDS-PCNSL and 4 controls) who yielded a positive HHV-8/KSHV DNA nested PCR product in their cerebral specimens, antibodies to lytic and latent antigens were detected by IFA.

A typical speckled nuclear fluorescence, referred to antibodies against latent antigens, was seen in both uninduced and TPA-induced BC3 cells (24-45). A cytoplasmic fluorescence pattern was observed in a much higher proportion of cells after TPA-induction, probably due to lytic cytoplasmic antigens which are expression of the replicative products of HHV-8/KSHV (24, 45-46). No immunofluorescence background was observed when BC3 cells were tested with only the secondary antibody. Sera positive for HHV-8/KSHV did not react when tested with HHV-8/KSHV and EBV-negative B-cell line (Ramos) and Hep2 cells, routinely used for detection of antinuclear autoantibodies. HHV-8/KSHV titers were not modified after absorption of the positive sera with EBV producer P3HR1 TPA-induced cells. All sera negative for HHV-8/KSHV display specific immunoreactions against multiple viral antigens (HIV, HSV, CMV).

Discussion

This study was prompted by the recent concern that AIDS-PCNSL may be associated with HHV-8/KSHV infection (11, 13, 33). In order to conclusively assess this issue, an adequately sized panel of AIDS-PCNSL

has been tested by multiple experimental strategies, including molecular analysis of HHV-8/KSHV DNA in patients' tumor lesions and CSF as well as serological studies of patients immunoreactivity status against HHV-8/KSHV. For comparative purposes, the study also included a panel of HIV-infected patients with brain lesions due to opportunistic infections which had been matched to AIDS-PCNSL patients in a prospective cohort of AIDS-related focal lesions of the central nervous system. The results of our comprehensive analysis unequivocally document that HHV-8/KSHV infection is not a feature of AIDS-PCNSL.

The search for HHV-8/KSHV DNA sequences in tumor lesions of AIDS-PCNSL has been performed by multiple PCR assay harboring different sensitivity thresholds. Overall, our molecular analysis allows the detection of 1 viral copy in a background of 200,000 human haploid genome equivalents. This threshold of sensitivity is 10^5-10^6 fold higher than the burden of HHV-8/KSHV DNA commonly detectable in tumors associated with HHV-8/KSHV infection (7). Because one study suggested that, in the context of AIDS-PCNSL, HHV-8/KSHV may reside predominantly in cells at the edge of the tumor lesion (11), we were careful to include the tumor margins in most samples analyzed. Our results show that, with one exception, tumor tissues of all AIDS-PCNSL are devoid of HHV-8/KSHV DNA sequences by all molecular approaches adopted. The one AIDS-PCNSL which has been scored positive by nested PCR, though not by single step PCR, was represented by a homosexual man with a diagnosis of AIDS-KS preceding the emergence of lymphoma. It is conceivable that the low amount of HHV-8/KSHV DNA detected in the cerebral tissue of this patient may be ascribed to infection of cell types related to AIDS-KS, namely circulating blood cells which frequently harbor HHV-8/KSHV in AIDS-KS patients (38, 49). Conceivably, circulating inflammatory blood cells were also the source of HHV-8/KSHV DNA positivity in the cerebral tissues of control patients scored positive by nested PCR, since the inflammatory features of the brain lesions increased the probability of harboring HHV-8/KSHV-infected bystander cells.

The lack of association between AIDS-PCNSL and HHV-8/KSHV infection is reinforced by our molecular analysis of the patients' CSF as well as by our immunologic studies of the patients' sera. With respect to CSF, we reasoned that AIDS-PCNSL, if infected by HHV-8/KSHV, might shed the viral DNA into the CSF in a fashion similar to that reported for the DNA of EBV infecting these lymphomas (9). Also, considering that the AIDS-PCNSL cellular microenvironment, such as astrocytes, microglia and migrant peripheral lymphocytes, is characterized by a strict anatomical and functional relationship with the CSF, the detection of HHV-8/KSHV DNA should be expected in the CSF if the virus infected predominantly these cellular populations. On the contrary, our results show that the CSF of all AIDS-PCNSL patients tested are consistently devoid of HHV-8/KSHV DNA sequences.

With respect to immunologic studies, seropositivity for HHV-8/KSHV is a consistent feature of patients affected by tumors infected by HHV-8/KSHV such as AIDS-KS (17, 24, 34, 45). We reasoned that if AIDS-PCNSL were associated with HHV-8/KSHV infection, the seroprevalence for HHV-8/KSHV antibodies should be higher in AIDS-PCNSL patients than in matched controls. Because it has been suggested that available ELISA assays exploiting HHV-8/KSHV recombinant antigens may fail to recognize particular variants of HHV-8/KSHV associated with specific types of diseases (45), we have adopted a conventional and well standardized IFA assay which allows the detection of both latent and lytic phase antigens of HHV-8/KSHV. Our results demonstrate that the seroprevalence rate for HHV-8/KSHV of AIDS-PCNSL patients does not significantly differs from that of matched controls. The stratified contingency table analysis and the log-linear model indicate that the high seroprevalence rate detected in the population studied is consistent with the diffusion of HHV-8/KSHV infection among homosexual men and, in part, reflects the relatively high background seroprevalence documented in several Italian regions (18, 43,50).

The results presented in the current investigation are at variance with recent studies reporting on the frequent detection of HHV-8/KSHV DNA sequences in AIDS-PCNSL biopsies (11, 33). Such studies have suggested that the validity of previous investigations failing to detect HHV-8/KSHV in AIDS-PCNSL was hampered by the use of low sensitivity assays for viral detection and/or the exclusion from the analysis of specific anatomical regions of the tumor biopsy (11, 15, 39). Differences in the experimental strategy adopted cannot explain the discrepancy observed between the current investigation and the studies reporting a positive association between AIDS-PCNSL and HHV-8/KSHV (11, 33). At present, the reasons for such discrepancy are unclear and may be of multiple nature, as recently exemplified by the discrepancies among studies on the relationship between HHV-8/KSHV and multiple myeloma (29, 32, 42, 44, 48, 51). Hovewer, independ-

ently of the precise reasons accounting for the observed discrepancies, it should be stressed that defining a positive association between a tumor and a virus based solely on the results of high sensitivity molecular assays, in the absence of serological evidence, may be misleading (36). Rather, such definition requires that multiple indicators of viral infection may be simultaneously detected in the same patient.

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