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Viral Load of Human Herpesvirus 8 in Peripheral Blood of Human Immunodeficiency Virus-Infected Patients with Kaposi's Sarcoma

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Viral load is an important marker of activity of viral diseases for a number of viruses. We wished to evaluate whether the viral load of human herpesvirus 8 (HHV-8) in peripheral blood was a consistent feature of Kaposi's sarcoma (KS) patients and whether the viral load correlated with human immunodeficiency virus (HIV) RNA levels, CD4 counts, and/or the HHV-8 seroreactivity. Fifty-four consecutive plasma samples from 14 patients with KS were evaluated for HHV-8 viral load by quantitative real-time PCR. Samples were analyzed at the start of highly active antiretroviral therapy (HAART) and at different intervals during treatments. The median HHV-8 DNA load before HAART treatment was 8,998 (ranging from 170 to 40,100) copies/ml and 12,270 (ranging from 40 to 142,575) copies/ml during HAART. There were both increasing and decreasing trends. There was an association between HHV-8 DNA and HIV RNA viral loads (odds ratio [OR] = 5.40; 95% confidence interval [95% CI], 1.54 to 18.98) and between HHV-8 viral load and CD4 cell counts (OR = 7.24; 95% CI, 1.30 to 40.35). High HHV-8 viral load was also correlated with the titers of antibodies to the lytic HHV-8 antigen detected with immunofluorescence ($P < 0.01$), but not with antibodies to the latent HHV-8 antigen. In conclusion, we found that HHV-8 viremia in KS is associated with HIV viral load, CD4 cell counts, and lytic HHV-8 serological reactivity. HHV-8 viral load monitored by real time PCR might be useful for determination HHV-8 viral load during the follow-up of KS patients.

Kaposi's sarcoma (KS)-associated herpesvirus or Human herpesvirus 8 (HHV-8) (13), is a γ-herpesvirus now widely established as a necessary cause of KS and also associated with body cavity-based lymphoma and multicentric Castleman's disease (5). These diseases were previously rare but are now brought to prominence by the AIDS pandemic. Detection of HHV-8 DNA in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type 1 (HIV-1)-infected persons is associated with an increased risk of subsequent development of KS (30, 43) and with KS clinical stage (10, 11).

Highly active antiretroviral therapy (HAART) is effective for inhibiting HIV replication, increasing CD4 cell counts, and delaying AIDS-associated opportunistic infections (26, 33). Case reports suggest that HAART may also be of benefit against AIDS-related KS as well as in eliminating detectable HHV-8 DNA from PBMCs of HIV carriers (15, 24, 28, 35).

Previous studies about the relationship between peripheral blood HHV-8 load and KS pathogenesis have been limited by the use of qualitative or semiquantitative estimates of HHV-8 load. Reproducible, sensitive, and specific quantitative techniques are needed to assess the HHV-8 DNA load and its correlation with different clinical conditions.

We have therefore developed a highly sensitive and specific real-time PCR assay for the quantification of the HHV-8 genomes in peripheral blood. The present study wished to evaluate the consistency over time of HHV-8 viral DNA loads among KS patients receiving treatment for HIV-1 infection. We also sought to determine whether HHV-8 DNA viral load was correlated with HIV RNA viral load, CD4 cell counts, and/or serological reactivity to HHV-8.

MATERIALS AND METHODS

Patients. Fourteen HIV-infected patients with histologically confirmed KS (13 male and one female; range of ages, 28 to 56 years) were monitored at the Department of Oncology & AIDS, Centro di Riferimento Oncolgico of Aviano, Aviano, Italy, over the period 1997 to 2000. All patients but one had advanced clinical stages of KS disease, with visceral and/or lymph node involvement. According to the Krown staging system (21), 13 patients were at stage T1, 7 patients were at stage I1, and 8 patients were at stage S1.

After informed consent was given by each patient, blood samples were collected at consecutive visits and HIV RNA viral load, CD4 T-cell count, HHV-8 serology for lytic and latent viral antigens and HHV-8 DNA viral load in peripheral blood were assessed. For each patient, one blood sample was collected before starting HAART, and then two or more samples were taken, depending on the number of control visits after the therapy had been initiated.

Thirteen patients were receiving combination therapy with one protease inhibitor and two nucleoside reverse transcriptase inhibitors; one patient was receiving one nonnucleoside reverse transcriptase inhibitor and two nucleoside reverse transcriptase inhibitors.

During the follow-up, three patients had also been treated with local radiotherapy; one patient had also been IFN treated for 9 months; four patients had also been treated with standard chemotherapy schedules.

Evaluation of HIV-1 plasma viral load and $CD4^+$ cell counts. Human blood specimens (about 10 ml of peripheral blood) were separated by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) into plasma and

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PBMCs. Aliquots of plasma and PBMCs as a dry pellet of 5×10^6 cells were frozen at −80°C. HIV RNA viral load was measured by the branched DNA assay (version 3.0; Chiron Corp., Emeryville, Calif.), following the manufacturer's instructions.

CD4 lymphocyte counts were evaluated by a whole-blood lysing technique (16). Briefly, 100 μl of blood was added to the appropriate monoclonal antibody combination and incubated for 15 min; thereafter, the samples were lysed and fixed by a commercial preparation (Immunoprep, Beckman-Coulter, Milan, Italy). The four-color CD3-CD4-CD8-CD45 and CD3-CD45-CD56-CD19 monoclonal antibody combinations (Coulter, Milan, Italy) were used to stain peripheral blood lymphocytes. Cell suspension was then analyzed in an EPICS XL flow cytometer (Beckman-Coulter). Absolute lymphocyte subset numbers were calculated by using the Flow Count Beads System (Beckman-Coulter). Accurate pipetting was performed by a single, regularly calibrated, equipment, and replicate experiments using the two different $CD3^+$ tubes showed a coefficient of variation (CV) of 3.5% ± 1.4% (value given is mean ± standard deviation).

Pretreatment of samples before PCR. Aliquots (200 μl) of plasma from each patient were used. Frozen cell pellets were thawed and resuspended in 200 μl of phosphate-buffered saline. DNA was extracted from the plasma and PBMCs using the QIAmp blood kit (Qiagen GmbH, Hilden, Germany), following the instructions from the manufacturer. A final elution volume of 50 μl was used. Another commercially available DNA extraction kit (Cobas Amplicor CMV prep kit; Roche Diagnostics, Mannheim, Germany) was also evaluated.

Quantitative PCR. Real-time (TaqMan) PCR was used for detection and quantification of HHV-8 DNA. This method is based on a primer pair and an oligonucleotide probe with the reporter fluorescein dye (FAM) attached to the 5′ end and a rhodamine dye (TAMRA) quencher linked to the 3′ end (18). The exonuclease activity of the DNA polymerase releases the reporter molecule as an elongation of the DNA chain occurs. This is seen as an increase in reporter fluorescence, which can be detected by a luminescence spectrophotometer. A threshold cycle value (C_t) is calculated for each sample by determining the point at which the fluorescence exceeds the threshold limit chosen for the specific plate.

Primers and probes were designed with the Primer Express Software (PE Applied Biosystems, Cheshire, United Kingdom). The real-time PCR assay used forward (5′-GCTCGAGTCCAACGGATTTG-3′) and reverse (5′-AATAGCG TGCCCCAGTTGC-3′) primers and the fluorogenic Taqman probe (5′-TTCC CCATGGTCGTGCCGC-3′) (PE Applied Biosystems) to amplify and detect a 67-bp amplicon in the HHV-8 minor capsid protein gene (open reading frame 26).

To each well of a 96-well plate 5 μl of sample and 20 μl of PCR mixture consisting of 12.5 μl of Universal PCR Mastermix (PE Applied Biosystems) and primers and probe at concentrations of 300, 900, and 200 nM, respectively, were added. The Mastermix contained uracil-*N*-glycosylase, which eliminates previously amplified PCR products to protect against carryover contamination. Cycling parameters were 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s and 60°C for 1 min.

Negative control water samples (for Epstein-Barr virus [EBV] and for HHV-8), positive control HHV-8 DNA extracted from culture supernatant of the BCBL1 cell line, and negative control from the serum of an HHV-8-seronegative healthy donor were included in triplicate on each plate. The samples were analyzed in triplicate, including one inhibition control to which 0.5 μl of the positive control was added to control for inhibitory substances in the samples.

The number of genomes in each sample was calculated from an EBV DNA standard. The DNA standard used was extracted (QIAmp blood kit) from the EBV-infected Burkitt lymphoma cell line Namalwa, which carries two integrated EBV genomes per cell. Each cell has a total DNA content of 6.7 pg, and by spectrophotometrical measuring of the DNA amount in the extracted material, the number of EBV genomes per microliter could be calculated (27). Four 10-fold dilutions (10^3 to 10^6 copies genomes/ml) of this DNA standard together with EBV primers (forward primer sequence, 5′-AAGGTCAAAGAACAAGG CCAAG-3′; reverse primer sequence, 5′-GCATCGGAGTCGGTGGG-3′) and probe (5′-AGGAGCGTGTCCCCGTGGAGG-3′) were included on each plate to amplify and detect a 65-bp amplicon in the EBV LMP-1 protein gene, giving a standard curve from which the number of genomes in the samples could be calculated (17). The standard curve was created by the ABI PRISM 7700 Sequence Detection System software by plotting the C_t values against each known concentration of the EBV standard. Comparability of the HHV-8 and EBV DNA detection systems was investigated by comparing the C_t values for serial dilutions of viral DNA and checking whether the curves were parallel to each other, which they were (plot of input DNA versus C_t for EBV − C_t for HHV-8: $r = -0.044$). The linear range of the standard curve was 10^3 to 10^6 copies/ml. Values below the linear range of the standard curve are presented, but need to be interpreted with caution.

TABLE 1. Analysis of plasma samples extracted in parallel by using the QIAmp blood kit and the Cobas Amplicor CMV prep kit

Sample no.	QIAGEN		ROCHE	
	HHV-8 (copies/ml)	% CV (intrassay)	HHV-8 (copies/ml)	% CV (intrassay)
1	10,280	0.4	1,315	10
2	36,900	48	23,990	70
3	1,059	15	395	34
4	28,321	9	8,995	65
5	9,615	7	1,830	12
6	2,228	14	535	57
7	2,530	9	500	30
8	3,868	19	1,815	47
9	71	35	72	36

Results were expressed as copies of HHV-8 genomes per milliliter of plasma or per 10^6 cells, when PBMCs were tested.

HHV-8 serology. A three-step mouse monoclonal antibody enhanced immunofluorescence assay (MIFA) (25) was performed on all the plasma samples, as described elsewhere (41, 42). This assay uses the BCBL-1 cell line for detection of antibodies directed against both latent and lytic HHV-8 antigens. HHV-8 titers by MIFA were determined by serial dilutions of the plasma samples beginning at a dilution of 1/10 and proceeding up to 1/640.

Statistical analysis. The Spearman correlation coefficient (r) was used to analyze the correlations between the following covariates: HHV-8 DNA viral load, HIV RNA viral load, CD4 cell counts, and plasma HHV-8 serology. Odds ratios (OR) and their corresponding 95% confidence intervals (CI) were obtained by unconditional multiple logistic regression models (6). The dependent variable was HHV-8 DNA viral load. The covariates, HIV RNA viral load, CD4 cell counts, and lytic and latent HHV-8 plasma reactivity, were entered in the model separated in two levels. When entered as continuous variables, the unit was set to twofold or fourfold units of measurement of each variable. The models included a term for treatment (pre- and during HAART). The observations at each assessment were treated as separate units. A total of 54 observations were obtained from 14 patients. The statistical analyses were performed using the SAS language (reference version 6.12; SAS Institute Inc., Cary, N.C.) program.

RESULTS

DNA extraction and assay validation. The QIAmp blood kit (Qiagen GmbH) and the Cobas Amplicor CMV prep kit (Roche Diagnostics) were compared in parallel analysis of 9 plasma samples extracted with the two different kits (Table 1). Because of its better intrassay reproducibility we decided to use the QIAmp blood kit for the study. The average yield of the HHV-8 DNA extraction with this method, as assessed by adding a known amount of HHV-8 DNA to negative plasma before extraction, was 51%. To estimate the extent of interexperimental variability, plasma aliquots from the same patients were subjected to several DNA extractions performed on different days and each DNA extract was tested twice by real-time PCR. We also tested the reproducibility by comparing freshly extracted samples with analyses of aliquots of the same extracted samples after storage at −20°C. The variability thus observed includes both variations associated with the extraction of DNA, the preparation of the PCR mixture, and the reaction and analysis procedures. The overall CV was 21.5%. However, when the load was around 10,000 copies, the reproducibility of the method was better (mean CV, 17%; range, 3 to 28%) than for samples with lower viral loads (1,000 copies/ml) (mean CV, 31%; range, 3 to 68%).

The use of the inhibition control revealed that for some

TABLE 2. OR[a] of HHV-8 DNA viral load and corresponding 95% CI according to HIV RNA viral load and CD4 cell counts

Variable	No. (%) of samples with indicated HHV-8 DNA copies/ml		OR (95% CI)	OR (continuous) (95% CI)
	<40	≥40		
HIV-1 RNA (copies/ml)				
<50	13 (65.0)	8 (23.5)	1[b]	
≥50	7 (35.0)	26 (76.5)	5.40 (1.54–18.98)	1.15[d] (1.04–1.26)
CD4[c]/cell count[c] (cells/μl)				
<200	9 (52.9)	8 (28.6)	1[b]	
≥200	8 (47.1)	20 (71.4)	7.24 (1.30–40.35)	1.12[e] (0.66–1.89)

[a] Estimates from multiple logistic regression models including a term for treatment.
[b] Reference category.
[c] The sum does not equal the total number of samples because of a few missing values.
[d] OR for a twofold increment of HIV RNA viral load.
[e] OR for a twofold increment of CD4 cell counts.

samples there was partial inhibition occurring during the PCR assay, implying that the extraction methods used did not completely remove all inhibiting activity. The median inhibition was 67% (range, 24 to 99%).

HHV-8 DNA viral load evaluation of plasma samples. The HHV-8 DNA load was investigated by the real-time PCR (TaqMan) on 54 plasma samples from the 14 KS patients at different points during the clinical course. Samples were analyzed at the start of HAART and at different intervals during therapy treatments. Two of fourteen patients had undetectable HHV-8 DNA loads at all the visits. One patient had a very low HHV-8 DNA load (101 copies/ml) at the first visit and no detectable viral DNA during the follow-up. For one patient that was HHV-8 negative at the first visit, a low viral load (238 copies/ml) could be detected at the second visit, but the patient was HHV-8 negative again in subsequent visits. For the other 10 patients, the median HHV-8 DNA load was 8,998 copies/ml (ranging from 170 to 40,100 copies/ml) and 12,270 copies/ml (ranging from 40 to 142,575 copies/ml), considering samples before and during HAART, respectively. There were both increasing and decreasing trends during the follow-up visits.

When analyzed by Spearman correlation analysis, both HHV-8 ($r = -0.10$; $P = 0.46$) and HIV ($r = -0.16$; $P = 0.25$) viral loads were decreasing over time, albeit not a statistically significant decrease, possibly because of the small number of patients.

HHV-8 DNA viral load evaluation on PBMCs samples. For 10 of 14 patients, PBMCs stored at −80°C were available. The levels of HHV-8 DNA were low (mean, 1,978 copies/10^6 cells) or undetectable. Only one patient had a sample with high viral load (89,986 copies/10^6 cells). For this patient, 2 months later, only 14 copies/10^6 cells were detected, and both HHV-8 DNA in plasma and HIV RNA in plasma showed decreasing trends after the start of HAART (from 36,900 to 2,535 and from 2,231 to 500 copies/ml, respectively).

HHV-8 DNA viral load, HIV RNA viral load, and CD4 cell count comparison. HHV-8 plasma DNA levels correlated with plasma HIV RNA levels ($r = 0.45$; $P < 0.001$). Both high HIV RNA loads and high CD4 cell counts were associated with increased probability for detectable presence of HHV-8 DNA in plasma (Table 2). Results were consistent across strata of treatment. In particular, a twofold increment of HIV viral load was associated with an increased risk of 15% for presence of detectable HHV-8 DNA.

Although based on small numbers, note that three patients who had significant regression of their KS lesions while on HAART showed decreases in either HIV RNA or HHV-8 DNA viral load concomitant with an increasing CD4 cell count by time, whereas three patients who responded neither to HAART nor to chemotheraphy had increasing trends of HHV-8 DNA and decreasing CD4 cell counts.

HHV-8 DNA viral load and HHV-8 serology. All the plasma samples were tested for immunoglobulin G antibodies against both latent and lytic viral antigens on MIFA. All the samples were HHV-8 seropositive, with plasma antibody titers ranging from 1/40 to >1/640. Six of fourteen patients had antibodies only against the HHV-8 lytic antigens, not to the latent antigens. Only three patients showed a drop in the HHV-8 immunoglobulin G antibody levels, after the initiation of HAART. Antibody levels in the other patients' samples remained unchanged.

HHV-8 DNA viral load was correlated with lytic antigen serological reactivity ($r = 0.29$; $P = 0.03$). In particular, a twofold increment of serological reactivity to the lytic HHV-8 antigens was associated with a nearly threefold-increased risk for presence of detectable HHV-8 DNA (Table 3). There was

TABLE 3. OR[a] of HHV-8 DNA viral load and corresponding 95% CI according to plasma reactivity against lytic (Ab LYT) and latent (Ab LAT) HHV-8 viral antigens

Variable and antibody titer	No. (%) of samples with indicated HHV-8 DNA copies/ml		OR (95% CI)	OR (continuous) (95% CI)
	<40	≥40		
Ab LYT				
≤160	11 (55.0)	5 (14.7)	1[b]	
>160	9 (45.0)	29 (85.3)	13.37 (2.56–69.81)	2.90[c] (1.27–6.61)
Ab LAT				
≤40	11 (55.0)	21 (61.8)	1[b]	
>40	9 (45.0)	13 (38.2)	0.79 (0.25–2.46)	1.00[c] (0.77–1.32)

[a] Estimates from multiple logistic regression models including a term for treatment.
[b] Reference category.
[c] OR for a twofold increment of serological reactivity to HHV-8 antigens.

no correlation with reactivity to the latent HHV-8 antigens ($r = 0.03$; $P = 0.85$). Results were consistent across strata of treatment.

DISCUSSION

Several reports on regression of KS after treatment with HIV therapies have been published (2, 12, 24), and the activities of both the HIV and the HHV8 infection affect the KS (19). Only a few studies have compared HIV and HHV-8 viral loads in vivo (8, 23, 31), and there are also not many studies regarding the clinical utility of quantitative assessment of the systemic HHV-8 DNA load as a marker for the development of KS and for monitoring the response to therapies used to treat KS (4, 8, 9, 22, 44). Techniques that provide accurate and reproducible measurements of the amount of HHV-8 in the circulatory compartment are needed. Taqman real-time quantification of specific PCR products allows accurate determination of the amount of DNA template present at the start of the reaction. We evaluated both cell-free and cell-associated HHV-8 DNA in sequential clinical samples from KS patients and found satisfactory reproducibility. We also found that HHV-8 viral loads have an association with HIV viral load in KS HIV-infected patients, suggesting a synergistic interaction between HHV-8 and HIV, as also reported in another study (29). However, HHV-8 viral loads are variable and fluctuate over time. Elevated HIV RNA levels may play a role in inducing HHV-8 reactivation through either increased immunosuppression or direct activation of HHV-8 itself. Alternatively, increased levels of HHV-8 activation could stimulate HIV replication. Since other herpesviruses have been shown to *trans*-activate HIV and probably to contribute to progression of HIV infection (34, 36), it is conceivable that HHV8 may have a similar effect.

HHV-8 viral load was found to be correlated with CD4 cell counts above 200 cells/μl. These findings are in agreement with the effects of HAART that has been shown to act on peripheral blood HIV RNA levels, as well as increasing $CD4^+$ T cells (26, 33), and might also affect HHV-8 viremia (15, 24, 28, 35).

It is thought that the virus persists in a latent form in most spindle cells of KS lesions and in some infected PBMCs (37). PBMCs in the circulatory compartment are more often prone to contain HHV-8 undergoing lytic viral replication (3, 14), and HHV-8 plasma viremia is an important event in KS pathogenesis. Detection of herpesvirus DNA in leukocytes could possibly represent latent infection, while detectable DNA in serum or plasma is usually associated with disease: this is seen for EBV, cytomegalovirus, and HHV-6 (1, 7, 38). For these reasons, quantitation of cell-free HHV-8 DNA may be an accurate marker in determining active HHV-8 replication. Lytic infection is present in KS (32), and correlation of antilytic antibody levels with the presence of plasma HHV-8 DNA argues that active virus replication is necessary for spread of virus or viral DNA to plasma.

Since the significance of seroreactivity to the lytic HHV-8 antigen is still debated, the correlation between HHV-8 viremia and lytic antibody reactivity is a novel finding that supports the usefulness of this serological marker. A relation between the titer of antibodies against HHV-8 latent antigens and HHV-8 viral load has been reported (20, 39, 40), but, similar to our study, no relation was found in a study where HHV-8 viral load was determined by a quantitative kinetic PCR assay on plasma and PBMCs from KS HIV-infected patients (9).

The real-time PCR system is a reproducible and sensitive method for DNA quantification that allows a high throughput of samples and is less labor-intensive and more informative than an ordinary nested PCR. It would be of interest, with the help of this system, to investigate whether quantification of HHV-8 DNA and HIV RNA may provide additional information in longitudinal studies of potent antiretroviral drug combinations and, potentially, the administration of drugs inhibiting HHV-8 replication. Such studies could also help to clarify the roles played by each virus, providing strategies to reduce the frequency of KS in HIV infection.

In conclusion, we find that HHV-8 viremia in KS is associated with HIV viral load, CD4 cell number, and serological reactivity against lytic HHV-8 antigens. HHV-8 viral load might be useful for monitoring the response to the therapies used to treat KS.

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