Relationship of Kaposi Sarcoma (KS)–Associated Herpesvirus Viremia and KS Disease in Zimbabwe

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The relationship between Kaposi sarcoma–associated herpesvirus (KSHV) viremia and KS disease was investigated in 500 subjects who received treatment in Harare, Zimbabwe. Subjects were grouped by results of human immunodeficiency virus (HIV) type 1 serological tests, KS diagnosis, and KS clinical stage. The plasma KSHV DNA concentration was associated with concomitant KS and HIV-1 infection (AIDS-KS; $P < .001$) and AIDS-KS clinical stage ($P = .01$). Plasma KSHV DNA levels were greater in AIDS-KS than in matched HIV-1–seronegative KS ($P = .04$). The plasma KSHV DNA level was not associated with age, sex, systemic symptoms, or CD4+ lymphocyte count. Plasma and peripheral blood mononuclear cell KSHV DNA concentrations were linearly related ($r^2 = .44; P < .001$), and the nucleotide sequence of the K1 gene highly variable region was identical in both compartments. These findings provide evidence that KSHV viremia is common in advanced AIDS-KS in Zimbabwe and suggest a relationship between KSHV lytic replication and untreated HIV-1 infection.

Prior to the onset of the AIDS epidemic, Kaposi sarcoma (KS) was an uncommon cancer among Zimbabwean men and a rare cancer among Zimbabwean women. Concurrent with the spread of HIV-1 in Zimbabwe, between 1987 and 1995 the incidence of KS increased >40-fold in Zimbabwean men and >200-fold in Zimbabwean women [1, 2]. Zimbabweans with AIDS-KS typically have high tumor burdens, rapid disease progression, and a median life expectancy of <6 months (C. L. Olweny et al., personal communication). Conventional chemotherapy and/or radiation therapy improves the quality of life for Zimbabwean patients with AIDS-KS but does not affect their survival. A better understanding of the factors that contribute to KS pathogenesis is needed if more effective strategies to prevent and treat KS in Zimbabwe and other African countries are to be developed.

Like other herpesviruses, KS-associated herpesvirus (KSHV; human herpesvirus 8) exhibits both latent and lytic (productive) patterns of gene expression [3]. Previous studies have provided evidence for a link between KSHV and HIV-1 replication and suggested a model in which KSHV lytic replication is important for KS pathogenesis. First, although KSHV gene expression in most KS tumor cells is restricted to the latent gene program [4, 5], KSHV-infected PBMC from persons with AIDS-KS have evidence of lytic KSHV replication [6]. Second, treatment with antivirals that inhibit KSHV lytic replication in cultured cells [7–9] is associated with
both a decreased risk of KS development for persons at high risk for KS [10–14] and regression of tumors in persons with established KS disease [15]. Third, the risk of AIDS-KS is greater after the acquisition of new KSHV infection in HIV-1–infected persons [16–18]. Finally, the treatment of AIDS-KS with antiretrovirals is correlated with reduced KSHV viremia [19].

If KSHV lytic replication is important for KS pathogenesis, then KSHV viremia should be associated with AIDS-KS disease. In support of this hypothesis, we have previously reported high levels of cell-free, DNase-resistant, KSHV DNA in Zimbabweans with AIDS-KS [20]. However, plasma KSHV DNA was not correlated with KS clinical stage or markers of HIV-1 disease. Because our prior study evaluated only a small number of subjects, most of whom had advanced AIDS-KS, potential relationships among KSHV viremia, HIV infection, and KS disease could not be excluded. Therefore, the present study was undertaken to test the hypothesis that cell-free KSHV DNA in the circulatory compartment is related to AIDS-KS disease.

**SUBJECTS AND METHODS**

**Study population.** A planned sample size of 500 Zimbabwean subjects with KS was estimated to have a 94% power to detect a relationship between plasma KSHV DNA prevalence and KS disease stage. All participants were recruited from an outpatient KS clinic at the Parirenyatwa Hospital, Harare, Zimbabwe. Any patient attending this clinic was eligible for participation. Each participant received a complete physical exam, liver function tests, a complete blood count, chest X-ray, HIV serological tests, and a CD4+ lymphocyte count. Subjects were asked whether they had fevers, night sweats, or weight loss. Biopsy samples were obtained from all subjects with suspected KS lesions. KS was defined as the presence of characteristic cutaneous or mucosal lesions that were confirmed by histopathologic tests. Visceral KS was defined as the finding of characteristic KS lesions by bronchoscopy or endoscopy. KS clinical stage was determined at study entry on the basis of clinical data by the following criteria [21]: stage 1, locally indolent cutaneous lesion; stage 2, locally aggressive cutaneous lesion with or without regional lymph node involvement; stage 3, multiple cutaneous lesions or generalized lymph node involvement, without palatal KS or radiographic evidence of pulmonary KS; and stage 4, palatal or visceral KS. A single investigator performed all KS staging. All samples were assigned an identification number, and the key that linked the subject information with the blood sample number was available only to the principal investigators. Investigators performing laboratory analyses were blinded to the clinical characteristics of study subjects. Data from 31 subjects have been reported elsewhere [20].

**Quantitation of KSHV DNA.** Whole blood was centrifuged in CPT tubes (Becton-Dickinson), according to the manufacturer’s instructions, to obtain plasma and PBMCs. Plasma was centrifuged at 500 g for 15 min to remove cell debris and stored at −70°C. The DNA present in 0.15–0.2 mL of clarified plasma was extracted and purified with the QIAamp Blood Kit after the addition of 10 μg of salmon sperm DNA as a carrier. PBMCs were washed in PBS and stored at −70°C as a dry pellet of ∼10⁶ cells. Cell pellets were resuspended in 200 μL of PBS, and DNA was extracted and purified with the QIAamp Blood Kit (Qiagen). Purified PBMC DNA was quantified by absorption spectroscopy at 260 nm, and only samples that contained ≥0.05 μg DNA/μL were analyzed. All DNA extractions and purifications were performed with aerosol-resistant pipettes in a containment hood located in a dedicated preparation room. Products of PCR were not allowed in the preparation room, and all personnel wore gowns and gloves while working in there. After DNA purification was completed, samples were removed from the preparation room, and PCR analysis was performed in a separate laboratory.

The amount of KSHV DNA present in DNA preparations obtained from PBMCs or plasma was determined by real-time PCR amplification of a conserved region of the open-reading frame (ORF) 26 minor capsid gene, as described elsewhere [22]. In all assays, there was a linear relationship between the value of threshold cycle (Ct) for the standards and the logarithm of minor capsid DNA copy number (r² = 0.98 for each assay). If the measured KSHV DNA was ≥1 copy, the PCR was considered to be positive. Negative controls for all assays included 2 PCRs that contained 2 μg of carrier DNA and 4 reactions that contained no DNA. In all assays, the measured fluorescence of the 6 negative controls did not exceed threshold after 40 PCR cycles.

**Qualitative detection of KSHV DNA.** Qualitative detection of KSHV DNA used PCR amplification and Southern blot hybridization, as described elsewhere [23]. Amplification was carried out in 50-μL mixtures that contained 20 μL of extracted DNA, 1.25 U of Pfu (Stratagen), 200 μM each dNTP, and 0.5 μM each primer, KS330 5′-AGCCGAAAGGATTCCACCAT-3′ and 5′-TCCTGGTTTGTCTACGGTCCAG-3′ [24], in Pfu buffer (Stratagen). Duplicate reaction mixtures were amplified for 45 cycles. PCR products were separated by electrophoresis in 3% agarose gels and transferred to nylon membranes. Membranes were hybridized with digoxigenin-labeled probe 5′-CCATGGTCGGCAGGCA-3′ [26]. Bound probe was detected with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and chemiluminescent substrate (Tropix). Each assay included 2 reactions without DNA as negative controls and 2 reactions with KSHV DNA as positive controls. Assays were considered valid only if controls yielded the expected results and if the patient replicates agreed. All PCR assays for the qualitative detection of ORF 26 DNA were performed in the University of Colorado Hospital Clinical Virology Lab-
Figure 1. Relationship of cell-free Kaposi sarcoma–associated herpesvirus (KSHV) DNA in plasma or serum to the presence or absence of KS disease and HIV-1 infection among Zimbabweans. A, Prevalence of KSHV DNA by group ($\chi^2$ test). The number of subjects in each group is indicated above the bars. B, Cell-free KSHV DNA levels ($\chi^2$, Kruskal-Wallis test). Median value (horizontal bars), 25th and 75th percentiles (black boxes), 10th and 90th percentiles (error bars), and 5th and 95th percentiles (black circles) are shown for each group. The horizontal line indicates the lower limit of detection (30 KSHV DNA copies/mL). The results of HIV-1 serological tests, KSHV latency-associated nuclear antigen serological tests, and KS diagnosis are indicated at the bottom.

Phylogenetic analysis of KSHV ORF K1. KSHV ORF K1 DNA was amplified by PCR with nested primer sets, as described elsewhere [25]. Ethidium-stained agarose gel electrophoresis verified the presence of the expected 687-bp K1 PCR product. PCR-amplified DNA was purified (Wizard Prep; Promega) and directly analyzed by automated DNA sequence analysis. For one subject, molecular clones were generated by ligation of PCR product into pCR2.1-TOPO (Invitrogen) and analyzed by automated DNA sequence analysis.

For each PCR product or molecular clone, DNA sequence data for 607 nt that encompassed variable regions 1 and 2 (VR1 and VR2) were obtained with both forward and reverse primers. DNA sequences were manually edited with Sequencher 4.0.5 (Gene Codes) and aligned with CustalW in Bioedit 5.0 (available at: http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Inferred phylogenetic trees were constructed by neighbor-joining/UPGMA analysis of data generated by DNAdist run in Bioedit. As references, K1 VR1-VR2 nucleotide sequences of 32 KSHV isolates from all 3 major subtypes (A, B, and C) available through Genbank were incorporated into the inferred phylogenetic tree. Bootstrap analyses were performed with Seqboot software in the Phylip 3.6 package (available at: http://evolution.genetics.washington.edu/phylip.html). The graphic depiction of inferred trees was performed with Treeview 1.5 (available at: http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

Statistical methods. All statistical analyses used Statview (Abacus Concepts) and assumed a 2-sided significance level of $P = .05$. Because of nonnormal distributions of plasma KSHV DNA, nonparametric tests were used where appropriate. Between-group characteristics were compared using Fisher’s exact test for 2 groups of categorical variables, the $\chi^2$ test for independence of multiple groups of categorical variables, either the Wilcoxon signed-rank or Mann-Whitney test for 2 groups of continuous variables, and the Kruskal-Wallis test for multiple groups of continuous variables. Correlations were assessed with Spearman’s rank test. Least-squares regression was used to describe the relationship between plasma and PBMC KSHV DNA.

RESULTS

Subject characteristics. A total of 500 subjects with possible KS were recruited from the Parirenyatwa Hospital Outpatient Clinic. Forty subjects were excluded from the analysis because the results of either the HIV-1 serological or histologic tests of
Association of KSHV viremia and AIDS-KS. Cell-free KSHV DNA was detected in peripheral blood by real-time PCR amplification of ORF 26 DNA for 304 (66%) of 460 subjects. The specificity of real-time PCR amplification for the detection of ORF 26 DNA was evaluated by PCR amplification with an alternative set of ORF 26 primers, followed by the detection of PCR product by Southern blot hybridization. PCR products from 11 subjects who had a broad range of plasma KSHV DNA loads (<30 to >10^6 copies/mL) by real-time PCR were also evaluated by Southern blotting. In this subset of subjects, concordance between real-time ORF 26 PCR and the qualitative PCR/Southern blot test for the detection of KSHV DNA was 100%. Thus, the results of the real-time PCR assay were not due to amplification of non-KSHV DNA.

Both the prevalence and magnitude of cell-free KSHV DNA were strongly associated with AIDS-KS (figure 1). Cell-free KSHV DNA was detected in 298 (72%) of 415 subjects with AIDS-KS (median, 197 copies/mL; range <30 to >10^6 copies/mL). In univariate analyses of patients with AIDS-KS, the plasma KSHV DNA concentration was not associated with age (P = .28), sex (P = .28), the presence of systemic symptoms (fever, night sweats, or weight loss; P = .19), or CD4^+ lymphocyte count (figure 2). However, both the prevalence and magnitude of plasma KSHV DNA were associated with AIDS-KS clinical stage (figure 3).

The relationship between plasma and PBMC KSHV DNA loads was evaluated for 188 subjects with AIDS-KS (10 at stage 2, 66 at stage 3, and 112 at stage 4) from whom adequate amounts of cellular DNA were obtained from PBMC preparations. Among subjects in this subgroup, both plasma and PBMC KSHV DNA levels were associated with KS disease stage (P = .004 and P = .04, respectively). There was a significant linear relationship between plasma and PBMC KSHV levels that spanned a range of 4 log_{10} copies/mL of plasma KSHV DNA and 6 log_{10} copies/10^5 cells of cell-associated KSHV DNA (figure 4).

FIGURE 3. Relationship of plasma Kaposi sarcoma–associated herpesvirus (KSHV) DNA to Zimbabwean AIDS-KS clinical stage. Data shown are for 414 subjects with AIDS-KS for whom the KS clinical stage was determined. A, The prevalence of KSHV DNA by group (χ^2 test; P = .03, χ^2 test; for stages 2, 3, and 4 only) is shown. The number of subjects in each group is indicated above the bars. B, Cell-free KSHV DNA levels are shown (Kruskal-Wallis test). The median value (horizontal bars), 25th and 75th percentiles (black boxes), 10th and 90th percentiles (error bars), and 5th and 95th percentiles (black circles) are shown for each group. The horizontal line indicates the lower limit of detection (30 KSHV DNA copies/mL). The KS clinical stage is indicated at the bottom.

FIGURE 4. Relationship of plasma and PBMC Kaposi sarcoma–associated herpesvirus (KSHV) DNA. Data for 180 Zimbabwean subjects with AIDS-KS and sufficient PBMC KSHV DNA were included in the analysis. Solid line, fit of data by least-squares regression (y = 0.55x + 1.9 log_{10} copies/mL; r^2 = 0.44; P < .001). Dotted lines, 95% CIs for the regression. Values that were below the limit of detection were included in the analysis and are indicated on the X- and Y-axes.
Figure 5. Relationship of Zimbabwean plasma and PBMC Kaposi sarcoma–associated herpesvirus (KSHV) to other KSHV strains. The phylogenetic tree was inferred by a neighbor-joining method. Bold indicates open-reading frame K1 sequences from paired Zimbabwean plasma and PBMC samples. Reference sequences were obtained from Genbank. Bootstrap values are shown as the percentage of times the groups distal to the node occurred in 1000 replicate trees.

The linear relationship between plasma and PBMC KSHV DNA was similar for subjects with stage 2 KS ($y = 0.46x + 1.5 \log_{10}$ copies/mL; $r^2 = 0.77$; $P < .001$), stage 3 KS ($y = 0.52x + 1.9 \log_{10}$ copies/mL; $r^2 = 0.35$; $P < .001$), and stage 4 KS ($y = 0.55x + 1.9 \log_{10}$ copies/mL; $r^2 = 0.45$; $P < .001$).

No evidence of intrasubject KSHV genetic heterogeneity. The possibility that real-time PCR results were due to sample contamination by extraneous DNA was further evaluated by PCR amplification and DNA sequence analysis of the highly variable regions in KSHV ORF K1 (VR1 and VR2). The VR1-VR2 nucleotide sequence was determined for paired plasma and PBMC specimens from 7 Zimbabwean subjects with AIDS-KS. All ORF K1 sequences from Zimbabwean subjects were unique and distantly related to KSHV present in the PEL cell lines (BCP-1, BCBL-1, BC-1, and BC-3) used in our laboratory (figure 5). Four Zimbabwean K1 sequences were most closely related to a Ugandan isolate previously designated as belonging to the A5 subgroup. The remaining 3 Zimbabwean K1 sequences grouped together within the B subgroup. The finding that Zimbabweans with AIDS-KS were infected with either subtype A5 or B KSHV is consistent with previous reports of the prevalence of these subtypes in other African populations [26–28]. In all cases, the K1 nucleotide sequence from paired plasma and PBMC specimens was identical. No evidence of intrasubject KSHV genetic heterogeneity was found by visual inspection of sequence chromatograms or, in one case, by DNA sequence analysis of multiple molecular clones.

Comparison of KSHV viremia in AIDS-KS and endemic
Our initial analysis suggested that KSHV viremia was significantly greater in subjects with AIDS-KS than in subjects with endemic KS (HIV−1−seronegative KS; figure 1). However, because cell-free KSHV DNA was associated with both KS clinical stage and PBMC KSHV, direct comparisons of the endemic KS (HIV-seronegative) and AIDS-KS (HIV-seropositive) groups are potentially misleading. Therefore, we compared plasma KSHV DNA in the 20 subjects with endemic KS (14 at stage 2 and 6 at stage 3) with the 150 subjects with AIDS-KS who had stage 2 or 3 disease (14 at stage 2 and 136 at stage 3). In this comparison, the plasma KSHV DNA was 4-fold greater in the AIDS-KS group (<30 vs. 112 copies/mL; P < .001).

To further control for differences in KS clinical stage and PBMC KSHV DNA in the endemic and AIDS-KS groups, a matched comparison was performed. The 20 subjects with endemic KS were matched to 20 subjects with AIDS-KS first by KS stage and second by PBMC KSHV DNA. The 14 subjects in the endemic KS group with stage 2 disease were paired with the 14 subjects with AIDS-KS who had stage 2 disease. Because there were only 14 subjects with stage 2 AIDS-KS in our study population, further matching by PBMC KSHV DNA was not possible. Six subjects with stage 3 endemic KS were matched to 6 subjects with stage 3 AIDS-KS by PBMC KSHV DNA. Compared with subjects who had endemic KS, the matched subjects with AIDS-KS were significantly younger, had fewer CD4+ lymphocytes, and had a greater plasma KSHV DNA level (table 1).

**DISCUSSION**

In the present study, cell-free KSHV DNA in peripheral blood was common among persons with AIDS-KS. Because techniques for the cultivation of infectious KSHV from clinical specimens are not available, we were not able to determine the relationship between infectious viral titer and plasma KSHV DNA or KS disease. However, previous studies have demonstrated that plasma KSHV DNA is resistant to DNase degradation [20] and is correlated with antibody titers to lytic, but not latent, KSHV antigens [19]. Thus, the available evidence suggests that plasma KSHV DNA is a measure of cell-free KSHV virions and a marker of lytic viral replication.

Among persons with AIDS-KS in the present study, KSHV viremia was associated with KS clinical stage. Because the clinical staging criteria used in our study are a crude measure of the KS tumor burden, this finding implies that the KS tumor burden and KSHV lytic replication are directly related. Our finding that cell-associated KSHV DNA was also associated with KS stage is consistent with previous findings [20, 29, 30]. The relationship between plasma and PBMC KSHV DNA among subjects with AIDS-KS in the present study (figure 4) was similar to the relationship reported elsewhere for a smaller group of subjects [20]. Our finding that the relationship between cell-free and cell-associated KSHV DNA was similar is subjects with limited cutaneous disease (stage 2), multiple cutaneous lesions (stage 3), and visceral KS (stage 4) suggests that this relationship is constant throughout KS disease progression.

The linear relationship between plasma and PBMC KSHV DNA in AIDS-KS suggests that KSHV-infected cells in the circulatory compartment contribute to KSHV viremia. This interpretation is consistent with the finding that KSHV-infected cells in the PBMC compartment have evidence of lytic viral replication [6]. Although the relationship between circulatory compartment cell-free and cell-associated KSHV DNA was significant, changes in PBMC KSHV DNA explained only ~40% of the variation in plasma KSHV DNA. Possible explanations for the lack of a stronger correlation between plasma and PBMC KSHV DNA include KSHV lytic replication outside the circulatory compartment (i.e., lymphoid tissue) and contributions of both lytic and latent viral replication to cell-associated KSHV DNA.

KSHV viremia in AIDS-KS could result from decreased control of KSHV replication due to HIV−1−induced immunosuppression or activation of KSHV replication through the production of cytokines or other factors by HIV−1−infected cells.

### Table 1. Matched comparison of Zimbabwean endemic and AIDS−Kaposi sarcoma (KS).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Endemic KS</th>
<th>AIDS-KS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>KS stage, II/III</td>
<td>14/6</td>
<td>14/6</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>45 (20−72)</td>
<td>34 (23−53)</td>
<td>.05</td>
</tr>
<tr>
<td>No. of men/women</td>
<td>19/1</td>
<td>16/4</td>
<td>.34</td>
</tr>
<tr>
<td>No. of CD4+ lymphocytes/mm³</td>
<td>564 (87−878)</td>
<td>162 (50−778)</td>
<td>.002</td>
</tr>
<tr>
<td>PBMC KSHV DNA load, copies/10⁶ cells</td>
<td>19.6 (&lt;0.35–840)</td>
<td>2.8 (&lt;0.35–73,891)</td>
<td>.40</td>
</tr>
<tr>
<td>Plasma KSHV DNA load, copies/mL</td>
<td>&lt;30 (&lt;30–436)</td>
<td>41 (&lt;30–11,231)</td>
<td>.04</td>
</tr>
<tr>
<td>Plasma KSHV DNA prevalence, %</td>
<td>20</td>
<td>50</td>
<td>.10</td>
</tr>
</tbody>
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**NOTE.** Data are median (range), except where indicated. P was determined by the Wilcoxon signed-rank test. KSHV, Kaposi sarcoma−associated herpesvirus.

* Fisher’s exact test.
Our findings provide evidence that KSHV viremia is common in advanced AIDS-KS in Zimbabwe and suggests a relationship between KSHV lytic replication and untreated HIV-1 infection. Although our data support a connection between HIV-1 coinfection and augmented KSHV replication, they do not support a significant contribution of HIV-1–induced CD4+ lymphocyte count and plasma KSHV DNA argues against a direct relationship between HIV-1–induced immunosuppression and KSHV viremia.

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References


