Kaposi's Sarcoma (KS) - Associated Herpesvirus-Like DNA Sequences in Peripheral Blood Mononuclear Cells: Association With KS and Persistence in Patients Receiving Anti-Herpesvirus Drugs


Herpesvirus-like DNA sequences (KSHV/HHV-8) have recently been described in AIDS-associated Kaposi's sarcoma (KS) lesions. Many questions remain regarding the role of this virus in KS and the therapeutic implications of this finding. In the current study, KSHV/HHV-8 DNA was detected in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus (HIV)-infected patients with KS (34/98) more often than in HIV-infected individuals without KS (12/64, P = .03). The detection of KSHV/HHV-8 DNA did not correlate with the CD4 lymphocyte count. Five patients dem-

Kaposi's Sarcoma (KS) is a frequent complication of human immunodeficiency virus (HIV) infection. The following epidemiologic observations have suggested that an infectious agent in addition to HIV plays an important role in the pathogenesis of this tumor. Among HIV-infected individuals, KS is up to 20-fold more likely to develop in homosexual or bisexual men compared with those in certain other risk groups (eg, blood factor recipients). KS is also relatively more common among HIV-infected women whose sexual partners are bisexual men than in women with other risk factors for HIV. Finally, KS occurs in young homosexual or bisexual men who are not infected with HIV.

Chang et al recently identified unique herpesvirus-like DNA sequences in nearly all KS lesions of patients with AIDS. Such sequences were also detected in KS lesions of patients without HIV. It was hypothesized that this putative virus, henceforth called KS-associated herpesvirus/human herpesvirus-8 (KSHV/HHV-8), contributes to the pathogenesis of KS. However, KSHV/HHV-8 DNA sequences were also identified in a variety of non-KS lesions including skin cancers of transplant patients, body cavity lymphomas, and Castleman's disease. These cumulative observations imply that KSHV/HHV-8 is etiologic in both KS and non-KS diseases or, alternatively, that KSHV/HHV-8 infection is relatively common and its presence in certain pathological conditions is incidental and unrelated to pathogenesis.

If KSHV/HHV-8 were involved in the pathogenesis of KS, it would potentially represent an attractive therapeutic or preventative target. Indeed, a recent retrospective analysis of a large group of HIV-infected patients revealed that the incidence of KS was significantly lower in those who had been treated previously with foscarnet, a drug that blocks the replication of all previously studied human herpesviruses through inhibition of their DNA polymerases. There are, in addition, anecdotal reports of remissions of early KS following treatment with foscarnet. At the same time, many other KS patients have received foscarnet or other anti-herpesvirus drugs without any apparent resolution of their KS, and little is known about the response of KSHV/HHV-8 to the administration of these agents.

In an attempt to further characterize the relationship between KSHV/HHV-8 and KS, we examined the peripheral blood mononuclear cells (PBMCs) of HIV-infected patients with and without KS for the presence of KSHV/HHV-8.

Because two herpesviruses that are closely related to KSHV/HHV-8, Epstein-Barr virus (EBV) and herpesvirus saimiri, are known to infect circulating lymphocyte subpopulations, we hypothesized that analysis of circulating PBMCs of patients with and without KS for KSHV/HHV-8 DNA might both more conveniently and more accurately reflect the true relative prevalence of KSHV/HHV-8 in these populations than would skin biopsies. We sought for study, in particular, patients receiving anti-herpesvirus drugs to determine whether this therapy could eradicate or markedly reduce the quantity of KSHV/HHV-8 DNA sequences in their PBMCs.

MATERIALS AND METHODS

PBMC collection, storage, and processing. PBMCs were obtained from 162 HIV-infected adult patients enrolled in Institutional Review Board-approved research protocols at the National Cancer Institute (NCI) during the period from April 1985 until May 1995. Of these, 98 had documented KS, and 64 did not have evidence of KS. The KS patients were all homosexual men, while the others belonged to the following risk groups: 42 were homosexual men, 14 had received clotting factors, and 8 had other risk factors (see Table 1). PBMCs obtained from 11 HIV-negative individuals were also examined. The PBMCs were isolated from Ficoll-Hypaque (Pharmacia Biotech Inc, Piscataway, NJ) density gradient centrifugation and washed twice in phosphate-buffered saline (GIBCO, Grand Island, NY). The majority of the samples of PBMCs were cryopreserved and stored in liquid N2; other PBMC samples were analyzed fresh. In some cases, PBMCs were also isolated from normal HIV-negative, male and female heterosexual volunteers. For other studies (eg, of the relationship between anti-herpes therapy and the presence...
of KSHV/HHV-8), we used more than one specimen as follows: when multiple frozen PBMC samples were available, we tested the last sample available before starting on anti-herpes drugs and then the first sample, if available, after 3 weeks of therapy had been completed with acyclovir, and/or ganciclovir, and/or foscarnet; in two patients in whom no sample was available after 3 weeks of therapy, the last sample obtained after therapy was begun was used. PBMCs from HIV-infected hemophiliacs were obtained from patients participating on the Multicenter Hemophilia Cohort Study.12 HIV infection was confirmed serologically by enzyme-linked immunosay (ELISA) and Western blot.

PCR amplification and Southern blot analysis of KSHV/HHV-8 DNA sequences. DNA was extracted from PBMCs using a QIAamp Blood Kit (QIAGEN Inc, Chatsworth, CA) according to the manufacturer’s instructions and stored in Tris-EDTA (TE) buffer at 4°C. PCR primers were synthesized by Lofstrand (Gaithersburg, MD) to amplify a 233 bp region of the KSHV/HHV-8 genome.6 The conditions for PCR were as follows: 94°C for 2 minutes (1 cycle); 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute (35 cycles); 72°C for 5 minutes (1 cycle). Each PCR reaction used 0.1 µg of genomic DNA, 50 pmol of each primer, 1 U of Taq polymerase (Boehringer Mannheim, Indianapolis, IN), 100 µM of each deoxyribonucleotide triphosphate, 10 mM/L Tris-HCl, 50 mM/L KCl, and 1.0 mM/L MgCl2. The quality of genomic DNA was confirmed in all cases by parallel PCR amplification of the G3PDH gene (CLONTECH Laboratories, Inc, Palo Alto, CA).

The PCR products were separated on 2% agarose gels in Tris-Acetate (0.4 mM/L) with Dsodium EDTA (0.02 mM/L) (TAE; ABI, Columbia, MD), and visualized using ethidium bromide. The PCR products were transferred to nylon membranes by capillary transfer, bound by UV crosslinking and 2-hour incubation at 80°C, and analyzed by Southern hybridization using a fluorescein-labelled probe (Boehringer Mannheim, Indianapolis, IN) except for a single substitution at base pair 47 (Fig 1), which has been previously reported.

None of the 11 HIV-negative normal volunteers were found to have detectable KSHV/HHV-8 in their PBMCs. By contrast, KSHV/HHV-8 DNA was detected in HIV-infected subjects and in accord with other recent reports,13 we detected these sequences more frequently in HIV-infected patients with KS than in those without KS (Table 1). Overall, among the HIV-infected patients, 35% (34/98) of those with KS had KSHV/HHV-8 sequences detected in their PBMCs as compared with 19% (12/64) of those without KS (odds ratio (OR) = 2.30; 95% confidence interval [CI] = 1-02-5.25; P = .03). It is important to note that whereas our HIV-infected population with KS consisted exclusively of homosexual men, the HIV-infected patients without KS included individuals from several risk groups. Within the subset of HIV-infected homosexual men, the difference in rates of detecting KSHV/HHV-8 DNA between the groups with and without KS was no longer significant: 35% (34/98) and 24% (10/42), respectively (OR = 1.70; 95% CI = 0.70-4.21; P = .2). Of the 14 heterosexual HIV-infected clotting factor recipients, only 1 had detectable KSHV/HHV-8 (P = .07; v homosexual men). Of the 8 patients in other risk groups, only 1 was positive for KSHV/HHV-8. Thus, the HIV-infected homosexual patients had a substantially higher KSHV/HHV-8 DNA detection rate as compared with the HIV-infected non-homosexual patients regardless of the presence of KS itself: 31% (44/140) and 9% (2/22), respectively (OR = 4.58; 95% CI = 1.03-41.88; P = .02).

In the present study, the HIV-infected patients with and without KS were similar with regard to their CD4 counts (median CD4 counts of 40 and 42 cells/mm3, respectively) (Table 1). Moreover, there was no statistical correlation between the CD4 count and the likelihood of detecting KSHV/HHV-8 DNA in homosexual men (Table 2). It is therefore unlikely that differences in the CD4 counts in the HIV-infected subjects contributed to the observed differences in the rate of detecting KSHV/HHV-8 DNA.

Detection of KSHV/HHV-8 DNA before and during anti-herpesvirus therapy. In light of anecdotal reports of KS regression during foscarnet therapy14 and one study noting the lower incidence of KS in HIV-infected patients receiving foscarnet therapy,19 we examined stored PBMCs from the 10 HIV-infected patients with KS who had received ganciclovir or both ganciclovir and foscarnet for the treatment of documented cytomegalovirus (CMV) infections and for whom such samples were available. All of these patients were homosexual men. Their median CD4 count at the onset of antiviral therapy was 8 cells/mm3 (Table 3). Seven of

RESULTS

KSHV/HHV-8 detection in PBMCs in different cohorts. We performed PCR amplification and Southern blot hybridization to detect the unique 233 bp KSHV/HHV-8 sequences in PBMCs obtained from 162 HIV-infected patients (including 98 with KS), and 11 HIV-negative controls. All of the HIV-infected patients had AIDS or symptomatic HIV infection (CDC group IV). Their median CD4 count was 42 cells/mm3 (range 0-953). To confirm that the PCR products identified as KSHV/HHV-8 sequences by Southern blot were indeed the designated KSHV/HHV-8 sequences, we sequenced the PCR product amplified from a representative patient with KS. This sequence was identical to that originally described by Chang et al6 except for a single substitution at base pair
these patients had also received acyclovir either for herpes simplex treatment or as a component of an experimental protocol. The samples tested were obtained within a median of 41 days after the start of ganciclovir (range 14 to 77 days) and 35 days after the start of foscarnet (range 7 to 151 days).

Five of 9 patients for whom samples of PBMCs were available before beginning anti-herpesvirus drug treatment had detectable KSHV/HHV-8 DNA before therapy (Table 3). Two of 8 patients for whom PBMCs were available during ganciclovir therapy, and 3 of 7 patients for whom PBMCs were available during therapy with foscarnet (either alone or in combination with ganciclovir) were positive for KSHV/HHV-8 DNA during therapy with these agents. Two patients who were positive for KSHV/HHV-8 DNA before anti-herpesvirus therapy were negative on ganciclovir, but both of these patients had detectable KSHV/HHV-8 DNA in their PBMCs later in their course when foscarnet therapy was added to ganciclovir. One patient who was positive before anti-herpesvirus therapy was negative on foscarnet alone. At the same time, two patients who were DNA-negative either at baseline or on acyclovir became positive during treatment with ganciclovir alone or in combination with foscarnet. Southern blots of PCR products from three representative patients are shown in Fig 2. Thus, several patients continued to demonstrate the KSHV/HHV-8 DNA sequences in their PBMCs while receiving potenter anti-herpesvirus therapy, and there was no consistent pattern of change in likelihood of KSHV/HHV-8 DNA detection once therapy with either ganciclovir or foscarnet was begun.

### Table 1. KSHV/HHV-8 Sequence Detection in PBMCs in Different Cohorts

<table>
<thead>
<tr>
<th>Patient Category</th>
<th>Median CD4 Count* (range)</th>
<th>Proportion Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-infected with KS</td>
<td>40 (9-953)</td>
<td>34/48 (35%)</td>
</tr>
<tr>
<td>HIV-infected without KS</td>
<td>42 (6-623)</td>
<td>12/64 (19%)</td>
</tr>
<tr>
<td>Homosexuals (all males)</td>
<td>104 (17-623)</td>
<td>10/42 (24%)</td>
</tr>
<tr>
<td>Other risk factors (4M, 4F)</td>
<td>100 (10-276)</td>
<td>1/8 (13%)</td>
</tr>
<tr>
<td>Clotting factor recipients (13M, 1F)</td>
<td>16 (0-42)</td>
<td>1/14 (7%)</td>
</tr>
<tr>
<td>HIV-negative (3M, 8F)</td>
<td>NA</td>
<td>0/11 (0%)</td>
</tr>
</tbody>
</table>

* Abbreviation: NA, not available.

** Table 2. Detection of KSHV/HHV-8 DNA Sequences in PBMCs: Relationship to CD4 Count**

<table>
<thead>
<tr>
<th>Patient Category</th>
<th>CD4 Count (cells/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-infected with KS</td>
<td>0-50 51-200 &gt;200</td>
</tr>
<tr>
<td>Homosexual men</td>
<td>17/52 (33%) 7/25 (28%) 10/21 (48%)</td>
</tr>
<tr>
<td>HIV-infected without KS</td>
<td>4/17 (24%) 3/16 (19%) 3/9 (33%)</td>
</tr>
<tr>
<td>Heterosexual women</td>
<td>0/1 (0%) 1/2 (0%) 0/1 (0%)</td>
</tr>
<tr>
<td>Heterosexual clotting factor recipients (13M, 1F)</td>
<td>1/14 (7%)</td>
</tr>
</tbody>
</table>

Abbreviation: NT, none tested.
Table 3. Detection of KSHV/HHV-8 DNA Sequences in PBMCs Obtained From AIDS-KS Patients Before and During Anti-Herpes Therapy

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CD4 Count*</th>
<th>Pretreatment</th>
<th>ACV (days)†</th>
<th>GCV (days)</th>
<th>FOS ± GCV (days)†</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>61</td>
<td>Pos</td>
<td>NT</td>
<td>Neg (14)</td>
<td>Pos (7)</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>Pos</td>
<td>NT</td>
<td>Pos (41)</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>Pos</td>
<td>NT</td>
<td>Neg (28)</td>
<td>Pos (31)</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>Pos</td>
<td>NA</td>
<td>NA</td>
<td>Neg (151)</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>Pos</td>
<td>Neg (77)</td>
<td>Pos (77)</td>
<td>NT</td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>Neg</td>
<td>Neg (31)</td>
<td>Pos (31)</td>
<td>Neg (35)</td>
</tr>
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<td>Neg</td>
<td>NT</td>
<td>Neg (49)</td>
<td>Pos (21)</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>Neg</td>
<td>Neg (1147)</td>
<td>NA</td>
<td>Neg (36)</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>Neg</td>
<td>NT</td>
<td>Neg (48)</td>
<td>NT</td>
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<tr>
<td>10</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>Neg (41)</td>
<td>Neg (36)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>5/9</td>
<td>0/3</td>
<td>2/8</td>
</tr>
</tbody>
</table>

Abbreviations: NT, not treated; NA, not available.
* CD4 count (cells/mm³) at the start of specific antiviral drug treatment.
† The numbers in parentheses refer to the time period (in days) from the initiation of therapy to sample collection.
‡ All tested patients were on combination therapy with foscarnet and ganciclovir except patient number 4 who was on foscarnet alone.

The method we used to detect KSHV/HHV-8 DNA almost certainly underestimates its true prevalence in the population. Indeed, before undertaking the current analysis, we examined PBMCs obtained from a subset of patients on up to 14 different dates. Although some of the patients showed 100% concordance of KSHV/HHV-8 DNA detection on all test dates, others exhibited a degree of variability in KSHV/HHV-8 DNA detection over time (data not shown). This quite possibly reflects fluctuating quantities of circulating KSHV/HHV-8 genome. Relevant to these issues is the experience with PCR for EBV, in which its genome is detectable by PCR in PBMCs from healthy volunteers only 12% of the time, despite a >90% seroprevalence. 14, 16 Our demonstration of KSHV/HHV-8 DNA in PBMCs of HIV patients is in agreement with the recently published results of Whitty et al.14 although we did not observe as striking a difference as they had in rates of its detection between patients with and without KS. Moreover, we did not detect KSHV/HHV-8 DNA more readily in patients with lower CD4 counts. Our study differs from that of Whitty et al in that the vast majority of our HIV-infected patients (with or without KS) had >200 CD4 cells/mm³, and all of our patients were symptomatic (CDC group IV). It is possible that a relationship between CD4 count and KSHV/HHV-8 DNA detection is only apparent when a broader range of CD4 counts and disease stages are examined, but our experience does indicate that any such analysis should be performed while controlling for the HIV risk group.

A key and novel aspect of the present work is our demonstration of the persistence of KSHV/HHV-8 in PBMCs during intravenous treatment with antiherpesvirus drugs. Although we could not detect KSHV/HHV-8 DNA in all subjects on treatment, our inability to uniformly detect the virus in serial samples off treatment, as previously noted, leads us to surmise that the antiviral drugs had no major impact. The results with foscarnet are of particular interest because, unlike acyclovir or ganciclovir, foscarnet does not require phosphorylation by viral kinases and directly inhibits viral polymerase-dependent DNA replication of all herpesviruses tested to date. Also, there had been suggestions that foscarnet might either prevent or ameliorate KS.

Antiherpesvirus drugs such as ganciclovir and foscarnet are only effective during active viral replication, and it is possible that KSHV/HHV-8, like EBV, persists in PBMCs in a form that is not dependent on viral replicative enzymes. Indeed, the experience with EBV may be particularly telling, in that acyclovir does not alter the viral load in circulating B cells but does inhibit productive EBV replication and provide effective treatment for oral hairy leukoplakia, an EBV-associated condition in which virus replication is di-

Patient No. 1 2 3
Treatment P G F/G P G P F/G

233 bp

Fig 2. Representative Southern hybridization of PCR products from AIDS-KS patients before and during antiherpesvirus therapy (patients 1 to 3 from Table 3). PCR amplification of the samples from each patient were performed at the same time. All samples were blotted and hybridized together; however, a blank section between the samples from patients 2 and 3 was deleted from the image for ease of visualization. Abbreviations: pretreatment (P), ganciclovir (G), combination foscarnet/ganciclovir (F/G), positive control (POS), and negative control (NEG).
rectly pathogenic. Thus, although we could not show eradication of KSHV/HHV-8 from the peripheral blood, we have not excluded the possibility that antiviral drugs may block the replication of this virus in other sites or that they may yet prove to be of some clinical benefit in the prevention or treatment of KS in KSHV/HHV-8 infected patients. Also, additional studies will be needed to determine whether prophylaxis against KSHV/HHV-8 infection by anti-herpes drugs or even a KSHV/HHV-8 vaccine might prevent the development of KS.

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