
Human Herpesvirus Type 8 DNA Sequences in Cell-Free Plasma and Mononuclear Cells of Kaposi’s Sarcoma Patients

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Human herpesvirus (HHV) type 8 has been detected in both classical and AIDS-related Kaposi’s sarcoma, body-cavity lymphomas, and other types of tumors. HHV-8 has also been detected in DNA from peripheral blood mononuclear cells (PBMC) of some Kaposi’s sarcoma patients and more readily in B cell fractions derived from panned cell subpopulations. Two patients were followed using several methods: in situ hybridization, solution-based polymerase chain reaction (PCR), and in situ PCR. HHV-8 was intermittently detected in plasma, and detection correlated with detection in PBMC. In situ PCR demonstrated HHV-8 sequences in both peripheral blood B lymphocytes and, to a lesser extent, T lymphocytes. HHV-8 may undergo periods of viremia while at other times it is undetectable and infects circulating B cells and some T cells.

Nucleic acid sequences of Kaposi’s sarcoma (KS)-associated herpesvirus or human herpesvirus (HHV) type 8 have been detected in both classical and AIDS-related forms of KS, lymphoma, Castleman’s disease, and skin cancers from organ allograft recipients [1–4]. These sequences were also found in DNA extracted from the peripheral blood mononuclear cells (PBMC) of some KS patients [5, 6]. Detection of HHV-8 sequences in PBMC of human immunodeficiency virus (HIV) type 1-seropositive persons also correlates with an increased risk of development of KS [5]. Preliminary evidence suggests that the target cells in PBMC could be B lymphocytes [6]. HHV-8 was also detected in episomal form in B cell lymphoma cell lines by in situ hybridization [7], and more recently, in microvascular endothelial cells (MVEC) and spindle cells from KS lesions by in situ polymerase chain reaction (IS-PCR) [8]. We describe 2 patients, followed longitudinally, in whom HHV-8 was detected in tumor tissues, T and B lymphocyte fractions, and cell-free plasma.

Materials and Methods

Patients. Two patients, 1 with AIDS-associated KS and 1 with posttransplant KS, were followed longitudinally. Patient A was a 34-year-old man with AIDS (CDC stage IV) who presented with visceromegaly, diffuse adenopathy, and an elevated lactate dehydrogenase of 1100 U/dL. A lymph node biopsy revealed KS mixed with areas consistent with angioimmunoblastic lymphadenopathy. The patient was treated with intravenous foscamet for 2 weeks, resulting in gradual diminution of his tumor and normalization of his lactate dehydrogenase level [9]. After discontinuation of foscamet, his tumor slowly reappeared. The patient’s serum was tested for the presence of Epstein-Barr virus (EBV) antibody and was negative. In addition, the PBMC...
were also tested for EBV gene sequences by PCR (BCLF-1, imme-
diate-early gene) and shown to be negative (not shown).

Patient B was a 49-year-old HIV-1-seronegative man who had
had a renal transplant 7 years earlier for end-stage renal disease.
He was taking immunosuppressive drugs (cyclosporine, azathiop-
rine, and prednisone) and, 3 years after transplant, developed
lower-extremity cutaneous lesions, which proved to be KS on
biopsy. Reduction in the immunosuppressive therapy resulted in
slight improvement of his tumor. The patient’s PBMC were nega-
tive by PCR for EBV gene sequences.

Solution-based PCR. DNA was extracted from PBMC (1–2
× 10⁷), and tissue biopsies and PCR were done. DNA (500 ng)
was amplified by HHV-8–specific primers (5'-AGCCGAAAGGAT-
TCCACCATT-3' and 5'-TCCGTGGTTGTCTACGTCCAGA-
3'), using published conditions [10] and analyzed on 1% agarose/
ethidium-bromide gels. The predicted size of the specific amplified
band was 233 bp (nt 987–1219 of the published HHV-8 sequence)
[10]. To prepare a cell-free virus pellet, plasma was centrifuged
at 1800 rpm for 15 min to remove cells and further centrifuged at
3500 rpm for 20 min to remove debris. To pellet virus particles,
5 mL of cell-free plasma was centrifuged at 20,000 rpm using a
SW41 Beckman rotor at 4°C for 2 h [11], and pelleted material
was resuspended in PBS. The pellets were treated with DNase at
37°C for 30 min (another aliquot was untreated) to rule out possible
contamination by infected cellular DNA. This treatment was
effective, as it removed any exogenous viral plasmid DNA spiked
into a control serum. DNA from the pelleted viruses was then extracted
by phenol, followed by ethanol precipitation.

In situ PCR and hybridization. Freshly isolated PBMC or sub-
sets (T and B lymphocytes) from both patients were transferred to
safelined slides containing a 20-mm single well. The cells were
air-dried overnight, dipped in absolute methanol for 5 min, and
then washed twice in 1 X PBS (Ca++ and Mg++ -free). Appropriate
concentrations of fluorescein isothiocyanate–conjugated mono-
clonal antibodies directed against T and B cell epitopes, anti-
CD3 and anti-CD19, respectively, were applied separately to each
individual slide. After a 1-h incubation at 37°C, the slides were
heat-fixed at 105°C for 10 s and then incubated in 4% paraformal-
dehyde for 2 h. Paraformaldehyde was inactivated by incubating
slides in 3 X PBS for 10 min; the slides were then washed twice in
1 X PBS, for 10 min per wash. These slides were incubated
overnight in 0.03% freshly prepared hydrogen peroxide in 1 X
PBS, to quench the endogenous peroxidase activity. This slides
were treated with proteinase K (6 μg/mL) at room temperature
for 12 min. Proteinase K was inactivated by incubating slides on
a heat block at 95°C for 5 min. The HHV-8 DNA was amplified in
situ with primers that anneal to nt 987–1006 and 1200–1219 [10].
Amplified DNA signals were detected by a biotinylated probe,
which anneals at nt 1078–1102 within the amplicons. In situ hy-
bridization was done at 37°C, using a reaction mixture containing
20 pmol of probe, 50% deionized formamide, 2X SSC (1 X stan-
dard saline citrate = 0.15 M NaCl, 0.015 M sodium citrate), 10X
Denhardt’s solution, 1 mg/mL ssDNA, and 0.1% SDS. Amplified
DNA was detected using streptavidin-peroxidase; subsequent color
was developed using 3-amino 9-ethylcarbazole, and counter-
staining was done with Gill’s hematoxylin [12, 13]. The percentage of
HHV-8-positive cells was determined by counting at least
10,000 cells/slide by 2 independent observers. All microscopic
analyses were done on coded slides.

PBMC were separated with immunomagnetic beads coated with
high-affinity monoclonal antibodies (Immunotech, Westbrook,
MA) into T and B lymphocyte fractions, using the manufacturer’s
suggested protocol. Fluorescence-activated cell sorting analysis of
the B and T cell fractions demonstrated 95% purity by CD19
and CD3 positivity, respectively. PBMC of patient B were also
separated by this technique into B and T cell fractions and found
to be 99.5% pure B or T cells. For positive internal controls, the
conserved region of HLA-DQAl, using primers HLA-DQ-GH26-
[11, 12]. Detection of HHV-8 sequences by polymerase chain reac-
tion (PCR) in patient cells and cell-free plasma.

<table>
<thead>
<tr>
<th>Time points</th>
<th>Date</th>
<th>PBMC cell-free plasma pellet T cells B cells</th>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4-17-95</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>4-24-95</td>
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</tr>
<tr>
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<td>9-19-95</td>
<td>+</td>
</tr>
<tr>
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</tr>
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</tr>
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</tr>
<tr>
<td>4</td>
<td>9-17-95</td>
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NOTE. Cell-free plasma was ultracentrifuged, and pelleted material
was tested for presence of HHV-8 sequences. PCR was done at selected time points,
when material was available. PBMC, peripheral blood mononuclear cells.

Results

Fluctuation in HHV-8 viremia determined by PCR analyses.
Seven blood samples were taken from patient A over 3 months,
and 4 were taken from patient B over 2 months (table 1). PCR
were done on both patients’ original tumor biopsies, PBMC, and
pelleted plasma (table 1). Both tumor biopsies were positive by
PCR. PBMC from patient A were positive for HHV-8 at the
first time point but only weakly positive at the second (table
1). PCR signals were not detected at time points 3 and 4 but
were at the next several time points (table 1). The negative
and positive samples were detected by Southern blot hybridization,
DNA extracted from pelleted material was pelleted viral DNA. 5 mL of patient A's serum was pelleted (see text). DNA was amplified from pelleted material (30 ng) was PCR-amplified, using 330 Bam fragment primer. Ethidium bromide-stained gel: lane 1, 330 Bam fragment, amplified from pelleted viral DNA; lane 2, negative control; lane 3, positive control. Figure 1. A, Solution-based polymerase chain reaction (PCR) of pelleted viral DNA. 5 mL of patient A's serum was pelleted (see text). DNA extracted from pelleted material (30 ng) was PCR-amplified, using 330 Bam fragment primer. A, Ethidium bromide-stained gel: lane 1, 330 Bam fragment, amplified from pelleted viral DNA; lane 2, negative control; lane 3, positive control. B, Southern blot analysis of gel, using labeled 330 Bam fragment as probe.

using the labeled 233-bp fragment as a probe. The detection of HHV-8 also correlated with relapse and progression of patient A's tumor. Of note, the negative samples were drawn during foscarinet treatment. PBMC from patient B were positive for HHV-8 sequences initially but subsequently became negative (table 1). During this period, no treatment was given, and there was no change in his clinical status.

In order to study whether a cell-free phase of HHV-8 exists, solution-based PCR was done directly on plasma samples at different time points. Initially, HHV-8 signals were not detectable in the plasma. To increase sensitivity and minimize detection of contaminating cellular DNA, the virus particles were pelleted by high-speed centrifugation and treated with DNase before DNA extraction and PCR analysis. Strong positive signals were detected in the DNA extracted (30 ng) from the pelleted virus treated with DNase, as demonstrated by ethidium-bromide gels and by Southern blot analysis of the same gels, using a radiolabeled 330 Bam probe (figure 1A, B, respectively). As shown in table 1, HHV-8 signals were detected in both treated and untreated samples from patient A (time points 1, 5, and 7) and patient B (time point 1). PCR results were negative at other time points (patient A: time points 3 and 4; patient B: time point 2 and later), when PBMC were also negative.

Detection and localization of HHV-8 in PBMC subsets. To determine the cell type harboring HHV-8, in situ hybridization and IS-PCR were done. HHV-8 signals were found, by IS-PCR, in 20%–30% of the PBMC from patient A and 11%–23% of those from patient B (figure 2A, B, respectively). No such signals were detected in normal control PBMC from a disease-free HIV-1-seronegative person (data not shown). There were relatively fewer HHV-8-positive PBMC detected by standard in situ hybridization in the 2 patients (0.8%–0.3%; data not shown). Signals were localized near the nuclear membrane, consistent with the episomal nature of this virus, as previously described [7].

To determine which subpopulation of mononuclear cells harbor HHV-8, PBMC from patient A were first separated with immunomagnetic beads into T and B lymphocyte fractions, and PCR was done on extracted DNA from each fraction (table 1). HHV-8 signals were repeatedly detected (patient A: time points 6 and 7) in the B cell fraction; the corresponding T cell fraction was weakly positive (data not shown). This result supports recent findings of Ambroziak et al. [6] that HHV-8 signals are detected mainly in the B cell fraction. However, when T and B lymphocyte fractions were analyzed by highly sensitive IS-PCR, in which a single copy of a gene or fragment of a gene can be amplified in situ [12, 13], some T cells were also positive, although significantly less (range, 1.2%–2.2% in patient A and 0.1%–0.9% in patient B) than in the B cell fraction (range, 39%–48% in patient A and 27%–33% in patient B) (figure 2C, D).

Discussion

Until now, evidence of HHV-8 sequences in peripheral blood cells from KS patients has been based on PCR of DNA extracted from mononuclear cells. We detected HHV-8 signals by an in situ PCR method in PBMC. Although PCR analysis of panned lymphocyte populations detected signals in only the B cell fraction, we demonstrated that HHV-8 has a broader tropism and can infect T lymphocytes. It is possible that the HHV-8-positive cells contained in the T cell fraction were contaminating B cells. However, by combining an immunohistochemistry assay with in situ PCR [12, 13], we confirmed that small percentages of T cells were indeed infected with HHV-8 in the PBMC of both our patients. Detection of this sequence in B cells is consistent with a recent report of HHV-8-positive B lymphoma cell lines derived from body-cavity–associated lymphomas in AIDS patients [7]. Under circumstances of severe immunosuppression, infection by HHV-8 may directly or indirectly contribute to transformation of B cells. The direct role of HHV-8 in the development of body-cavity lymphomas is complicated by the coinfection of these cells by EBV. Detection of HHV-8 in plasma from DNase-treated and untreated samples suggests that the signal must originate from intact virus particles rather than from contaminating infected cellular DNA and that HHV-8 has viremic stages in which cell-free particles are found in the circulation. Negative PCR results from both fractions (plasma and PBMC) at other time points indicate that HHV-8 may also go through periods of latency, when it is extremely difficult to detect by PCR.

The host range for HHV-8 has not been clearly defined. A recent study has demonstrated that the spindle and MVEC of the KS lesions are positive for HHV-8 sequences by IS-PCR [8]. We have also found infection of MVEC, spindle
cells, and various layers of dermis in the tissues of all three major types of KS (i.e., classical, African endemic, and AIDS-associated; unpublished data). The data in this report support the contention that HHV-8 is a unique viral agent able to infect PBMC with an intermittent viremic phase. There may be a primary infection of mononuclear cells, followed by infection of MVEC or transient viremia and active replication of the virus from latent site(s) during immunosuppression. Our findings that infection of PBMC by HHV-8 may be cyclical could also explain why this agent cannot consistently be detected within PBMC in all KS patients [5]. It will be important to study a larger longitudinal panel of patients to determine the relationship between HHV-8 viremia, clinical status, and progression to the development of Kaposi’s sarcoma.

References
Interleukin-12 Administered In Vivo Decreases Human NK Cell Cytotoxicity and Antibody-Dependent Cellular Cytotoxicity to Human Immunodeficiency Virus–Infected Cells

Steve Kohl, Mahvash Sigaroudinia, Edwin D. Charlebois, and Mark A. Jacobson

Persons infected with human immunodeficiency virus (HIV) have cellular cytotoxicity defects. Interleukin (IL)-12 is a potent stimulator of cytotoxicity. Fifteen HIV-infected patients were studied in a phase 1, single-dose escalation trial of human recombinant IL-12. One day after subjects received an IL-12 dose of 300 or 1000 ng/kg, they had a reduction in absolute lymphocyte count and peripheral blood mononuclear cell recovery. In evaluable patients 24 h after IL-12 administration, there was a 31% reduction overall in NK cell cytotoxicity (NKC) to HIV-infected cells at all doses and a 52% reduction in antibody-dependent cellular cytotoxicity (ADCC) at doses of 300 and 1000 ng/kg. In vitro incubation of patients' cells with IL-12 (before IL-12 administration) for 24 h increased NKC but had no effect on ADCC. The paradoxical acute reduction in cell number and cytotoxicity in vivo may be due to NK cell trafficking or regulatory cytokine mechanisms not apparent in vitro.

Interleukin (IL)-12 is a recently characterized cytokine [1], and human IL-12 is a potent stimulator of human NK cell cytotoxicity (NKC) in vitro [2]. Persons infected with the human immunodeficiency virus (HIV) have NKC defects [3]; IL-12 can increase NKC activity from HIV-infected patients after their NK cells are incubated in vitro with IL-12 [4]. Here we report the in vivo immunologic effect of recombinant human IL-12 in HIV-infected patients.

Materials and Methods

Patients and IL-12. We studied 15 18- to 50-year-old HIV-infected men with 100–500 CD4 cells/μL. They had no serious underlying medical illnesses or AIDS-related opportunistic infections. All were hospitalized and injected subcutaneously with a single 30- to 1000-ng/kg dose of human recombinant IL-12. IL-12 used in vitro was batch RBO12892 (supplied by S. Wolf, Genetics Institute, Cambridge, MA). The biologic activity of the IL-12 was 3.3 × 10^6 U/mg.

Cell quantification. White blood cells and absolute lymphocytes were counted in a clinical hematology laboratory using an automated cell counter. Peripheral blood mononuclear cells (PBMC) were quantified in a standard hemocytometer.

Leukocyte preparation. PBMC were isolated by density-gradient centrifugation of heparinized blood with Ficoll-Paque (Pharmacia, Piscataway, NJ) as previously described [5]. Before use in the cytotoxicity assay, we cultured 10^6 PBMC in a 100-μL cell suspension in microtiter plates for 18 h with or without 30 ng/mL IL-12 at 37°C in 5% CO₂-enriched air.

Cytotoxicity assay. Antibody-dependent cellular cytotoxicity (ADCC) and NKC of HIV-infected cells were measured, as pre-