Transcriptional Activity of HIV-1 and HHV-6 in Retinal Lesions From AIDS Patients

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This study determined the frequency of multiple viral (HIV-1, HHV-6, and CMV) infections in 26 retinas from 16 AIDS patients. Of the 12 retinas of 26 that tested positive for HIV-1 DNA sequences, seven also were positive for HHV-6 DNA sequences. Four of these seven retinas were culture positive for HIV-1 and two of the four contained CMV DNA sequences and antigens. Using RNA probes, HIV-1 and HHV-6 transcriptional activity was demonstrated in two of the four HIV-1 culture positive retinas. These retinas also contained CMV DNA sequences and antigens. The results demonstrate that more than 35% of AIDS patients suffer from at least two simultaneous viral infections and 15% suffer from three viral infections. The presence of transcriptional activity of HIV-1 and HHV-6 suggests an active infection.


Ocular infection, a common complication of the acquired immunodeficiency syndrome (AIDS), affects as many as 98% of all patients infected with human immunodeficiency virus type 1 (HIV-1). AIDS-associated retinitis leads to blindness in 70-80% of patients prior to death, further complicating an already lethal disease process. Although the presence of cytomegalovirus (CMV), herpes simplex virus type 1 (HSV-1), human herpesvirus type 6 (HHV-6), and varicella-zoster (VZV) has been demonstrated in retinal lesions from AIDS patients, the actual etiology of AIDS-associated retinitis is uncertain. Coinfection of the human retina with HIV-1 and herpesviruses may have implications with regard to the pathogenic mechanisms of AIDS-associated retinitis. Previously, we have reported the presence of HIV-1 and HHV-6 transcriptional activity in HIV-1 culture-positive retinal tissue from AIDS patients. The results suggest an active infection.

Materials and Methods

Globes

Twenty-six postmortem globes from AIDS patients, asymptomatic HIV-1 carriers, and HIV-1-negative donors were provided by the Lions Eye Bank of Houston, Texas. Globes were received in K-sol media (Cilco, Inc., Huntington, WV) within 24-120 hr (1-5 days) after the patients’ deaths. All donor material used in this study was collected and handled at the time of autopsy using the guidelines of the Baylor Institutional Review Board for Human Research, Houston, Texas. The globes were bisected and examined for lesions under a dissecting microscope. Large lesions were cut into three sections to be evaluated by cocultivation, polymerase chain reaction (PCR), in situ hybridization, immunohistochemical staining, and electron microscopy.

Cells and Viruses

CCRF-HSB2 T-lymphocytes and human embryonic lung cells (HEL) were purchased from ATCC.

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(Rockville, MD) and propagated and maintained as recommended by the supplier. The peripheral blood mononuclear cells (PBMC) were prepared by the Ficol Hypaque gradient method, as described previously.\textsuperscript{14-16}

**Recovery of Viruses by Cocultivation**

The retinal lesions were gently dissected from pigment epithelium and choroid. After it was rinsed three times in phosphate buffered saline (PBS), the dissected retinal tissue was minced and dounce homogenized in PBS. One half of each homogenate was cultured for the recovery of HIV-1 and HHV-6 in PBMC and CCRF-HSB2 lymphocytes, respectively. Culture medium from PBMC cultures was removed twice a week for 4 wk and tested for P24 HIV-1 antigen by antigen capture enzyme-linked immunosorbent assay (ELISA; NEN-DuPont, Boston, MA). Cultures were considered positive if two consecutive determinations of optical density by ELISA were greater than two times the mean of the negative control, with the second positive value greater than the first (peak value usually was greater than 100 pg/ml). CCRF-HSB2 cell cultures were monitored daily by light microscopy for the presence of large refractile cells that are characteristic of HHV-6 infection in lymphocytes.\textsuperscript{14-16}

**Amplification and Detection of HIV-1, HHV-6, and CMV DNA Sequences**

Portions of retinal tissues from the lesions and the remaining hemispheres were used for this section of the study. For positive and negative controls, infected and uninfected cell pellets (PBMC for HIV-1, CCRF-HSB2 cells for HHV-6, and HEL cells for CMV) were used for DNA extraction. Viral DNA from retinal lesions and cell pellets was extracted in lysing buffer, purified, and subjected to PCR for amplification, as described in Table I.\textsuperscript{16} The amplified products were electrophoresed on agarose gels, stained with ethidium bromide, and photographed. All amplified DNA samples were further analyzed by slot blot hybridization using \textsuperscript{32}p-labeled DNA probes.\textsuperscript{8,16}

**In Situ Hybridization**

Selected retinal sections containing lesions (2 x 2 mm) were fixed in 10% buffered formalin, processed, and embedded in paraffin. Four-micron sections were placed on RNase-free polylysine-coated slides and subjected to in situ hybridization using \textsuperscript{35}S-labeled RNA probes. The pBH10R3 plasmid containing a 9 kb fragment of HIV-1 DNA was digested with EcoRI, and the pZVH14 plasmid containing an 8.7 kb fragment of HHV-6 was digested with BamHI. The fragments were used to make \textsuperscript{35}S-labeled RNA probes with Riboprobe kits (Promega; Madison, WI), as described by Harper et al.\textsuperscript{17} Briefly, slides were rinsed in 2X saline sodium citrate (SSC). The proteins then were acetylated for 10 min to prevent nonspecific binding and were treated to glycinate aldehyde groups for 30 min. The slides then were washed in 2X SSC, dehydrated in increasing concentrations of ethanol, and air dried. The hybridization mixture containing the \textsuperscript{35}S-labeled probe was added to each section, covered, and incubated for 3 hr in a humidified 52°C incubator. The slides were rinsed in 2X SSC and incubated in prewarmed 50% formamide/2X SSC (vol/vol) for 20 min at 52°C with frequent agitation, rinsed in 2X SSC four times, and treated with RNase to digest single-stranded RNA. The slides were rinsed, dehydrated in increasing ethanol concentrations, and air dried.

For autoradiography, the slides were transferred to a darkroom, dipped in melted NTB-2 Kodak (Rochester, NY) emulsion, dried, transferred to black slide boxes, and exposed for 2-3 days. For developing, slides were warmed to room temperature and placed in chilled Kodak D-19 developer, water, and chilled fixative. After the slides were stained with Giemsa and mounted, they were screened for the presence of HIV-1 and HHV-6 transcripts using a BioRad (Richmond, CA) MRC-500 confocal laser scanning microscope.\textsuperscript{18}

**Electron Microscopy**

Glutaraldehyde-fixed 10 \( \mu \)m sections from the two retinas with positive RNA hybridization results for HIV-1 and HHV-6 were dehydrated, post-fixed in osmium tetroxide, and embedded in plastic. The sections then were stained with lead citrate and uranyl acetate. Fifty sections from each sample block were examined for the presence of intact particles.\textsuperscript{19} Five million PBMC cells from the positive retinal culture were fixed in glutaraldehyde, processed, and examined similarly.

**Western Blot Analysis**

Lymphocytes (H9) infected with HIV-1 and CCRF-HSB2 cells infected with HHV-6 were used to make positive control lysates as sources of antigens for HIV-1 and HHV-6, respectively. The lysates were electrophoresed on 10-12% sodium dodecyl sulfate polyacrylamide gels, blotted on nitrocellulose membranes electrophoretically, and treated with primary antibody (serum from the donor). After the unreacted antibody was removed by washing, the membrane was incubated in secondary antibody (goat antihuman horseradish peroxidase conjugated IgG). The membrane was washed to remove unreacted antibody and incubated in substrate solution [4-chloro-1-naphthol and hydrogen peroxide in methanol].\textsuperscript{20,21}
Immunoperoxidase Staining

Deparaffinized retinal sections with lesions and appropriate positive and negative controls were incubated for 30 min at room temperature with 0.3% hydrogen peroxide and rinsed two times with distilled water. Slides then were treated with pepsin (3 mg/ml in 0.2 N HCl) for 40 min at 37°C, and the pepsin was removed by washing twice in PBS that contained 0.2% Triton X-100. The sections on slides were incubated with 10% goat serum at 37°C for 30 min. Monoclonal or polyclonal antisera (primary antibodies) were diluted 1:100 in PBS containing 0.03% Triton X-100. They were overlaid on tissue sections and incubated for 30 min at 37°C in a moist chamber. After the slides were rinsed with PBS containing 0.2% Triton X-100 to remove unreacted primary antibody, they were incubated for 30 min at 37°C with a biotin-labeled secondary antibody directed against the globulin fraction of the primary antiserum (goat antimouse IgG or goat antihuman IgG). After excess antiserum was removed by washing, slides were incubated 30 min at 37°C with the avidin-biotin horseradish peroxidase complex. Sites of peroxidase activity were visualized after incubation in 3-amino-9-ethylcarbazole with 0.01% hydrogen peroxide. After washing, slides were counterstained for 10 sec in fresh Mayer’s hematoxylin solution and rinsed in running tap water for 10 min. Two to six drops of crystal mount (Fisher Scientific, Springfield, NJ) was applied to each section and counterstained for 10 sec in fresh Mayer’s hematoxylin solution and rinsed in running tap water. Slides then were treated with pepsin (3 mg/ml in 0.2 N HCl) for 40 min at 37°C, and the pepsin was removed by washing twice in PBS containing 0.03% Triton X-100. The sections on slides were incubated with 10% goat serum at 37°C for 30 min. Monoclonal or polyclonal antisera (primary antibodies) were diluted 1:100 in PBS containing 0.03% Triton X-100. They were overlaid on tissue sections and incubated for 30 min at 37°C in a moist chamber. After the slides were rinsed with PBS containing 0.2% Triton X-100 to remove unreacted primary antibody, they were incubated for 30 min at 37°C with a biotin-labeled secondary antibody directed against the globulin fraction of the primary antiserum (goat antimouse IgG or goat antihuman IgG). After excess antiserum was removed by washing, slides were incubated 30 min at 37°C with the avidin-biotin horseradish peroxidase complex. Sites of peroxidase activity were visualized after incubation in 3-amino-9-ethylcarbazole with 0.01% hydrogen peroxide. After washing, slides were counterstained for 10 sec in fresh Mayer’s hematoxylin solution and rinsed in running tap water for 10 min. Two to six drops of crystal mount (Fisher Scientific, Springfield, NJ) was applied to each section and counterstained for 10 sec in fresh Mayer’s hematoxylin solution and rinsed in running tap water.

Results

Screening of Serum Antibody

Western blot analysis was used to screen for HIV-1 and HHV-6 antibodies in sera from tissue donors. Sera from all donors tested positive for HIV-1 and HHV-6 antibodies (Table 2). No HIV-1 or HHV-6 antibodies were detected in sera from two normal individuals.

Virus Recovery

The presence of infectious viruses was assessed by cocultivation in permissive cells of retinal lesions from HIV-1-infected patients. Twenty six retinas from AIDS patients and four from asymptomatic HIV-1 carriers were cultured for HIV-1 and HHV-6. Four of the 26 retinas from the 16 AIDS patients and none from asymptomatic HIV-1 carriers were culture positive for HIV-1 (Table 2). All retinal samples, when cultured, were negative for HHV-6. The normal retinas were negative for HIV-1 and HHV-6. The four culture-positive globes were received within 48 hr after enucleation, and the remaining globes were received between 3-5 days of enucleation. Thus, low recovery of HIV-1 may be a result of the instability of HIV-1 in K-sol media kept at 4°C longer than 48 hr. The absence of recovery of HHV-6 from these specimens suggests that, like most other herpesviruses, it is less stable than HIV-1 when stored under such conditions.

Table 1. Conditions used for PCR

<table>
<thead>
<tr>
<th>HIV-1</th>
<th>HHV-6</th>
<th>CMV</th>
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<tbody>
<tr>
<td>Denaturation</td>
<td>94°C (30 sec)</td>
<td>94°C (30 sec)</td>
</tr>
<tr>
<td>Annealing</td>
<td>65°C (1 min)</td>
<td>45°C (2 min)</td>
</tr>
<tr>
<td>Polymerization</td>
<td>70°C (4 min)</td>
<td>72°C (3 min)</td>
</tr>
<tr>
<td>(no extension)</td>
<td>(7 sec extension)</td>
<td>(7 sec extension)</td>
</tr>
<tr>
<td>No. of cycles</td>
<td>35</td>
<td>50</td>
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Viral Antigens

Using immunoperoxidase staining and purified monoclonal HIV-1 antisera, HIV-1 antigens were detected in lesions from two HIV-1 culture-positive retinas (Figs. 1A, B). The antigen-containing cells were scattered in all retinal layers. CMV antigens also were demonstrable in the same lesions using monoclonal antisera to CMV (Figs. 1C, D). No HHV-6 antigens were detectable in any of these tissues.

Viral DNA

The DNA extracted from retinal lesions was subjected to amplification by PCR. HIV-1 DNA was found in 12 of 26 retinas from AIDS patients and in one of the four retinas from the asymptomatic HIV-1 carriers. Seven of 12 retinas from AIDS patients that contained HIV-1 DNA sequences also were positive for HHV-6 DNA sequences (Figs. 2A, B). Four of these seven retinas were culture positive for HIV-1, and two of the four also contained CMV DNA sequences and antigens (Fig. 2C; Table 2).
Fig. 1. Immunoperoxidase staining. Positive cells demonstrate a red-brown reaction product. (A, top left) Retinal section from an AIDS patient (90-641) positive for HIV-1 antigen. (B, top right) Retinal section from an AIDS patient (89-275) positive for HIV-1 antigen. (C, bottom left) Retinal section from an AIDS patient (90-641) positive for CMV antigen. (D, bottom right) Retinal section from an AIDS patient (89-275) positive for CMV antigen. (Original magnification X400.)

Fig. 2. Amplified HIV-1, HHV-6, and CMV DNA sequences in retinal tissues. (A) Autoradiogram showing HIV-1 DNA sequences. 1, retinal tissue from an asymptomatic carrier of HIV-1; 2–13, retinal tissues from AIDS patients (2–89-275, 3–90-641). (B) Autoradiogram showing HHV-6 DNA sequences. 1–7, retinal tissues from AIDS patients (1–89-275, 3–90-641). (C) CMV DNA sequences in an ethidium bromide-stained agarose gel. 1, 2, 10–16, retinal tissues from AIDS patients (1–89-275, 2–90-641).
Table 2. Analyses of retinal tissue

<table>
<thead>
<tr>
<th>Donor status</th>
<th>Retinas analyzed</th>
<th>Serum antibody</th>
<th>DNA sequences, PCR</th>
<th>Viral culture</th>
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<tr>
<td></td>
<td></td>
<td>HIV-1</td>
<td>HHV-6</td>
<td>HIV-1</td>
</tr>
<tr>
<td>Asymptomatic (HIV+)</td>
<td>4 (2 pairs)</td>
<td>2/2</td>
<td>2/2</td>
<td>1/4</td>
</tr>
<tr>
<td>AIDS</td>
<td>26 (13 pairs)</td>
<td>13/13</td>
<td>13/13</td>
<td>12/26</td>
</tr>
<tr>
<td>Normal (HIV+)</td>
<td>10 (5 pairs)</td>
<td>0/5</td>
<td>0/5</td>
<td>0/10</td>
</tr>
</tbody>
</table>

Two of four HIV-1 culture positive retinas were positive for HIV-1 and HHV-6 transcriptional activity. The two retinas with HIV-1 and HHV-6 transcriptional activity also contained CMV antigens and DNA sequences.

Viral Transcriptional Activity

Using 35S-labeled RNA probes coupled with in situ hybridization and confocal laser scanning microscopy, transcriptional activity of HIV-1 and HHV-6 was detected in lesions from two HIV-1 culture-positive retinas. The label was found in the optic nerve areas as well as in the retinal layers (Figs. 3B, C; Figs. 4B, C). These two retinas also contained HIV-1, HHV-6, and CMV DNA sequences and HIV-1 and CMV antigens.

Virus Particles

Fifty sections from each of four sample blocks containing tissues from four culture-positive retinas were screened by electron microscopy for the presence of intact HIV-1 and HHV-6 particles. No HIV-1 particles were detected in the retinal sections. Intact HIV-1 particles and cytoplasmic tubuloreticular inclusions were readily visible in PBMC cocultivated with the retinal tissues (Figs. 5A, B). Cytoplasmic tubuloreticular inclusions are characteristic of HIV-1 infections.

Fig. 3. In situ hybridization showing HIV-1 transcriptional activity in optic nerve sections (original magnifications, x200). (A) Normal optic nerve. (B) Optic nerve sections from an AIDS patient (89-275) (arrow shows label in the retinal cells—highlighted). (C) Same section as in (B)—confocal laser image.
Fig. 4. In situ hybridization showing HHV-6 transcriptional activity in retinal sections (original magnifications, ×200). (A) Normal retina. (B) Retinal section from an AIDS patient (90-641) (arrow shows label in the retinal cells). (C) Same section as in (B)—confocal laser image.

in PBMC and have been observed in lymph nodes and lymphocytes of AIDS patients.\textsuperscript{19,22–25}

Discussion

Although several opportunistic agents (herpes group viruses) have been routinely observed in AIDS-associated retinitis, the actual etiology of the process has not been determined. Currently unknown is whether HIV-1 alone or in conjunction with HHV-6 is associated with the pathologic changes observed. It has been suggested that HIV-1 may directly or indirectly damage retinal tissue and interact with opportunistic pathogens, leading to the variety of ocular abnormalities observed with AIDS.\textsuperscript{12} Interestingly, HIV-1 has been isolated from and demonstrated within lesioned and nonlesioned retinal tissue.\textsuperscript{11} These data suggest that HIV-1 alone may not cause clinically recognized retinal lesions. In our study, HIV-1 and HHV-6 were confined to the lesion-containing areas of the retina.\textsuperscript{8}

The diversity of responses to HIV-1 infection suggests that multiple cofactors are likely involved in the pathogenesis of the disease. One of these cofactors may be herpes group viruses. Several human herpesviruses, such as HSV-1,\textsuperscript{26–27} CMV,\textsuperscript{28,29} and Epstein-Barr virus\textsuperscript{30} have been shown to transactivate HIV-1 long terminal repeat (LTR)-directed gene expression in vitro by a tat independent mechanism. However, none of these viruses are capable of invading CD4\textsuperscript{+} T-lymphocytes. Therefore, their mechanism of activation of HIV-1 is unclear. HHV-6, on the other hand, has been shown to be capable of infecting CD4\textsuperscript{+} T-lymphocytes, leading to an accelerated cytopathic effect compared to HIV-1 infection alone.\textsuperscript{31–36} It also has been shown that HHV-6 infection induces nuclear factors that specifically bind to the enhancer region of the HIV-1 LTR.\textsuperscript{31}

Recently, Martin et al identified sequences in immediate-early locus of HHV-6 that were able to cause activation of long terminal repeat of HIV-1 in vitro.\textsuperscript{37} On the other hand, Carrigan et al reported the suppression of HIV-1 replication in the presence of HHV-6. However, in their study, too, the rate of cell death was increased significantly if HHV-6 was
around in HIV-1-infected cultures. Because replication of HHV-6 itself is cytopathic to CD4+ T-lymphocytes, it might accelerate the rate of the disease process. Our data support this hypothesis. Two of the four HIV-1 culture-positive retinas, which also were positive for HIV-1 and HHV-6 transcriptional activity, showed a rise in HIV-1 P24 antigen activity as early as day 4. The remaining two retinas that contained only the HHV-6 DNA sequences showed an increase in HIV-1 P24 antigen activity much later (data not shown). This suggests that activation of HIV-1 (at least in vitro) may require active coinfe-

Fig. 5. Electron micrographs of PBMC from positive retinal culture. (A) HIV-1 virions (arrows; ×54,600). (B) Cytoplasmic tubuloreticular inclusions (arrows; ×29,400).
tion with HHV-6. The finding of HIV-1 and HHV-6 in combination with CMV in the retinas of AIDS patients may have etiologic implications regarding AIDS-associated retinitis. Infection with HIV-1 or HHV-6 alone, or in combination, may predispose retinal tissue to infection by other agents, such as CMV. That CMV retinitis is a late manifestation of AIDS and a poor prognostic sign further supports this hypothesis. A suitable animal model is urgently needed to firmly establish the relationship of HIV-1, HHV-6, and CMV to AIDS-associated retinitis.

Key words: AIDS, HIV-1, HHV-6, CMV, retina

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