Human Immunodeficiency Virus in Plasma and Genital Secretions during the Menstrual Cycle

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Six human immunodeficiency virus (HIV)-positive women were studied weekly over 8 weeks to detect HIV RNA in plasma and cervical secretions and proviral DNA in cervical, vaginal, and cervicovaginal lavage samples by polymerase chain reaction (PCR) amplification techniques. In cervical swab samples, cell-free HIV RNA was detected more frequently than cell-associated HIV proviral DNA (22/48 vs. 7/48, respectively). Cervical HIV RNA was consistently detected in 2 women with plasma HIV RNA >100,000 copies/mL but was not detected in 2 women with plasma HIV RNA <10,000 copies/mL, regardless of menstruation status. HIV-specific IgA was detected in the plasma of 2 women and in at least 1 cervicovaginal lavage sample from all 6 women. Thus, quantitation of cervical HIV RNA can be accomplished by PCR techniques and may be useful in evaluating genital viral shedding.

Transmission of human immunodeficiency virus (HIV) provides evidence that virus is present in genital secretions of women. HIV has been isolated from cells and cell-free fluids from female genital secretions [1–3] and cell-associated HIV proviral DNA has been detected by polymerase chain reaction (PCR) amplification of cells from the genital tract in association with oral contraceptive use, cervical mucopus, cervical ectopy, pregnancy, and inflammation [4, 5]. However, genital tract shedding of cell-free HIV RNA in virions has not been reported in HIV-infected women. Whether hormonal cycling and menstruation or local immune responses, including HIV-specific IgA antibodies in genital secretions [6], affect shedding of HIV RNA in genital secretions has not been defined. Six HIV-seropositive women were followed weekly over 8 weeks to detect HIV RNA in plasma and cervical secretions, HIV proviral DNA in cells from cervical and vaginal swabs and cervicovaginal lavage samples, and HIV-specific IgA antibodies in plasma and cervicovaginal lavage samples.

Methods

Study design. Six HIV-positive women were recruited at Stanford University and followed weekly for 8 weeks by the same investigator (C.G.). The women answered a detailed questionnaire that included demographics, behavioral risk factors, medical history, previous sexually transmitted diseases (STDs), sexual activity, medications, and gynecologic history. Criteria for inclusion required women to report normal menstrual cycles and agree to eight weekly examinations. At the initial pelvic examination, a pap smear and cervical swabs were obtained to detect Chlamydia trachomatis DNA (Amplicor PCR Diagnostics Chlamydia; Roche Diagnostic Systems, Branchburg, NJ) and Neisseria gonorrhoeae by culture on Thayer-Martin medium. Blood was obtained for detection of hepatitis B surface antigen (Auszyme; Abbott, Abbott Park, IL) and rapid plasma reagin (Impact RPR; Wampole, Cranbury, NJ). At weeks 1, 5, and 8, CD4 cells were counted by fluorescence-activated cell sorting (Becton Dickinson, Mountain View, CA).

Sample collection. Study genital samples were obtained as cervical and vaginal swabs and as a 5-mL saline lavage. A Dacron swab (Baxter Healthcare, McGaw Park, IL) was gently inserted 1 cm into the cervical os, rotated 360°, and placed in 2 mL of supplemented RPMI medium (BioWhittaker, Walkersville, MD) containing 1% t-glutamine, 20% fetal bovine serum, and 1% penicillin/streptomycin. Vaginal swabs were rotated 360° in four quadrants of the vaginal vault. Cervicovaginal lavage was done by directing 5 mL of sterile, nonbacteriostatic normal saline at the cervical os. The fluid was aspirated from the vaginal vault and added to 2 mL of supplemented RPMI.

Swabs were vortexed, rotated 360° against the inside of a tube to express fluid, and discarded. Swab supernatants, cervicovaginal lavage samples, and blood samples were centrifuged for 10 min at 800 g. The plasma or supernatants were removed, aliquoted into cryovials, and frozen at −70°C. Cell pellets from cervical, vaginal, and cervicovaginal lavage samples were resuspended in 2 mL of RPMI and stored at −70°C.

HIV RNA PCR. Plasma and cervical supernatants were assayed for HIV RNA (Amplicor HIV Monitor kit; Roche). A 142-bp sequence of the HIV gag gene was amplified with an internal quantification standard included in each sample to account for any inhibition of extraction, reverse transcription, or amplification [7].
Table 1. CD4 cells, plasma HIV RNA, and cervical HIV RNA and DNA from genital samples from 6 women.

<table>
<thead>
<tr>
<th>Subject</th>
<th>CD4 cells/μL, mean (SE)</th>
<th>Plasma HIV RNA, log10 copies/mL, mean (SE)</th>
<th>RNA, log10 copies/mL, mean (SE)</th>
<th>RNA, no. of positive samples (n = 8)</th>
<th>DNA, no. of positive samples (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>575 (13)</td>
<td>3.66 (0.05)</td>
<td>&lt; 2</td>
<td>0</td>
<td>0</td>
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<tr>
<td>2</td>
<td>800 (29)</td>
<td>1.48 (0.46)</td>
<td>&lt; 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>284 (18)</td>
<td>4.14 (0.06)</td>
<td>&lt; 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>128 (8)</td>
<td>4.16 (0.04)</td>
<td>2.70 (0.63)</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>340 (32)</td>
<td>5.56 (0.11)*</td>
<td>4.11 (0.17)</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>450 (26)</td>
<td>5.24 (0.15)*</td>
<td>4.24 (0.16)</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

NOTE. CVL, cervicovaginal lavage pellets.
* Plasma culture positive.

assays ranged from 4.53 to 4.95 log10 copies/mL (mean, 4.71; SD, 0.15). Samples yielding >100 copies/mL HIV RNA were considered positive. Cervical HIV RNA is reported as the copies per milliliter plus 1 log10 to account for the average dilution of cervical secretions in the collection medium (data not shown).

**HIV DNA PCR.** DNA was extracted from cervical, vaginal, and cervicovaginal lavage cells (Isoquick kit; Microprobe, Garden Grove, CA). Nested PCR reactions were done on the envelope gene using 1 and 0.5 μg of purified DNA for each of the 144 samples. First-round reactions used primers DR7 and DR8 (modified ES7 and ES8 primers, respectively, to omit M13 forward and reverse primer sequences at the 5' end) as described [8]. Positive reactions were identified by electrophoresis of products on a 1% agarose gel. Template quantification of the 144 specimens was done in duplicate using a modification of the HIV assay (Amplicor; Roche) targeted to the envelope gene using primers DR7 and DR8. Each reaction contained a quantitation standard generated to contain irrelevant sequences but of the same G + C content and length as the consensus HIV-1 subtype B sequence expected to be derived using DR7 and DR8. Results are reported as the mean of the final set of determinations. Cervical HIV DNA is reported as the number of copies of proviral DNA per million cells calculated from the amount of DNA extracted from cell pellets.

**HIV-specific IgA antibody.** Plasma and cervicovaginal lavage supernatants during the first 4 weeks were screened for HIV-specific IgA antibody. To increase sensitivity, 0.3 mL of lavage was concentrated 6-fold to 50 μL (Centricon-100; Amicon, Beverly, MA). Potentially interfering IgG antibody was removed from the concentrated lavage and plasma samples by adsorption to protein G (1:8 dilution) (Quik-Sep; Isolab, Akron, OH). The final dilutions were 1:1.3 and 1:8 for the lavage and plasma specimens, respectively. HIV-specific IgA antibody was measured by indirect immunofluorescence assay [9] using an α-chain–specific anti-human IgA globulin conjugate (Organon Teknika, Durham, NC).

**HIV cultures.** For HIV cultures, cervicovaginal lavage supernatant, cells, plasma, and peripheral blood mononuclear cell (PBMC) samples were added to 5 × 10^6 phytohemagglutinin-stimulated donor cells from samples obtained at weeks 1 and 7. Cultures were done on week 2 from 3 subjects menstruating at the week 1 visit. Cultures were screened for reverse transcriptase activity in supernatants 11, 18, 25, and 32 days after inoculation as described [10].

**Results**

**Study subjects.** The 6 women were white, ranged in age from 30 to 48 years, and had regular menstrual cycles of 26–30 days. Risk factors reported for HIV infection were heterosexual contact (subjects 2, 4, and 5), intravenous drug use (subjects 3 and 6), and acupuncture needlestick (subject 1). HIV infection had been documented 4–7 years before study entry. Two women had >500, 2 had 300–500, and 2 had 100–300 CD4 cells/μL. Subjects were asymptomatic except for subject 5 who had easy bruising and thrombocytopenia. All had normal Pap smears, and tests for syphilis, C. trachomatis, and N. gonorrhoeae were negative. Three women (subjects 1, 2, and 6) were on no antiretroviral treatment and 3 were receiving antiretroviral treatment during the study. Subject 3 received azidovudine, pegolated interleukin-2, and α-thymosin; subject 4 received didanosine; and subject 5 received stavudine for weeks 1–5 and 7. Due to symptomatic thrombocytopenia, subject 5 was treated with danazol during weeks 2–8 and prednisone for weeks 6 and 8.

**Plasma and cervical HIV RNA and genital proviral HIV DNA.** HIV RNA was detected in 22 of 48 cervical supernatants from 3 of the 6 women and in 45 of 48 plasma samples (table 1). Mean plasma HIV RNA was 4.04 log10 copies/mL of plasma (range, 1.48–5.56). Mean cervical HIV RNA was 3.76 log10 copies/mL of cervical secretions (range, 2.70–4.24) in 3 women with measurable cervical RNA. Proviral DNA was detected in 16 of 144 cell pellets from genital samples (7/48 cervical swab, 8/48 cervicovaginal lavage, and 1/48 vaginal swab samples). Cervical proviral DNA was detected in 2 women; 1 had a mean of 3.10 log10 copies/10^6 cells (in 2/8 positive samples) and the other a mean of 2.92 log10 copies/10^6 cells (in 5/8 positive samples).

Quantitative plasma and cervical HIV RNA fluctuated 2- to 3-fold in each woman over the 8 weeks. After correction for dilution, the amount of cervical HIV RNA was 10-fold lower than the amount of plasma HIV RNA in 3 women (figure 1). Cervical HIV RNA was always detected in weekly samples in 2 women with plasma HIV RNA of >5 log10 copies/mL (16/
Subject 1: Mean CD4 = 575

Subject 2: Mean CD4 = 800

Subject 3: Mean CD4 = 284

Subject 4: Mean CD4 = 128

Subject 5: Mean CD4 = 340

Subject 6: Mean CD4 = 450

Legend: ■ Plasma HIV RNA
      ● Cervical HIV RNA
      ▲ Cervical proviral DNA
      ● Pt. was menstruating
      x Pt. had spotting

Figure 1. Plasma HIV RNA, cervical swab RNA, and cervical swab proviral DNA for each of 6 subjects are displayed over 8 successive weekly samples. Menstrual cycle day for each patient (Pt.) is based on first day of reported menstrual flow as day 1. Visible blood in cervix at time of sampling is indicated on x axis during menstruation and when spotting was reported. Quantitative RNA results are shown for samples with >2 log10 copies/mL of plasma or cervical secretions. Quantitative cervical DNA results are shown for samples with >2 log10 copies of HIV DNA/10^6 cells.
16 samples tested) and was not detected in cervical samples of the 2 women with plasma HIV RNA of <4 log10 copies/mL (0/16 samples tested).

**HIV-specific IgA antibodies.** Circulating HIV-specific IgA antibody was detected in 4 weekly plasma samples from 2 women (subjects 2 and 3). Genital HIV-specific IgA antibody was detected in 4 lavage samples from patient 2 and in ≥1 lavage specimen from each of the other 5 subjects.

**HIV cultures.** Virus was isolated from 6 PBMC samples from week 1, from 2 of 3 PBMC samples from week 2, and 5 of 6 PBMC samples from week 7. Plasma cultures at the same times were positive only from subjects 5 and 6 (the women with the highest levels of plasma HIV RNA) at week 7. All 12 cultures (2 time points for each of 6 women) of cellular and supernatant fractions of cervicovaginal lavage were negative.

**Discussion**

HIV RNA or proviral DNA was detected in genital samples of 4 of 6 women at one or more time points. Cervical HIV RNA was detected in most of the weekly samples from 3 women and not at all in 3 others. HIV proviral DNA was intermittently detected in cell pellets from cervical swabs and cervicovaginal lavage fluids and less frequently from vaginal swab samples. Of 48 cervical swab samples, 22 were positive for HIV RNA from 3 women and 7 were positive for proviral DNA from 2 women. Although observations in only 6 women must be interpreted with caution, repeated measurements over 2 months suggest that shedding of HIV RNA may be more consistently detected than HIV proviral DNA in cervical swab samples.

Quantitative plasma HIV RNA varied 2- to 3-fold over 8 weekly samples in each woman. Cervical HIV RNA was consistently detected in 2 women with plasma HIV RNA of >100,000 copies/mL. The relationship between plasma and cervical HIV RNA among the 6 women in 48 paired samples suggests that cervical HIV RNA shedding is associated with increased level of plasma HIV RNA. Detection of HIV DNA and quantitative measures of cervical HIV RNA did not appear to be affected by menstruation (figure 1).

Detection of HIV proviral DNA in cervical samples has been associated with cervical ectopy [4] and inflammation [5]. In the women studied here, absence of detectable STDs or inflammation on clinical examination and Pap smears may account for the relatively low frequency of HIV proviral DNA. Detection of HIV-specific IgA antibody in cervicovaginal lavage supernatant provides evidence for local HIV-specific IgA antibody production. Quantitation of HIV RNA in the genital tract throughout the menstrual cycle provides evidence that measurement of cell-free viral RNA may be useful in evaluating genital viral shedding.

**Acknowledgments**

We thank S. Kwok and J. Sninsky of Roche Molecular Systems for provision of reagents in the development of the DNA quantitation assay.

**References**