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# Cervicovaginal Shedding of HIV Type 1 Is Related to Genital Tract Inflammation Independent of Changes in Vaginal Microbiota

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## Abstract

We examined the relationship of proinflammatory vaginal cytokines and secretory leukocyte protease inhibitor (SLPI) with genital HIV-1 shedding after controlling for genital coinfections. Fifty-seven HIV-1-infected women in Seattle, WA ($n = 38$) and Rochester, NY ($n = 19$) were followed every 3–4 months for a total of 391 visits. At each visit, plasma and cervicovaginal lavage (CVL) were tested for HIV-1 RNA using qPCR. Vaginal samples were tested for bacterial vaginosis, yeast, hydrogen peroxide-producing *Lactobacillus* colonization, *Trichomonas vaginalis*, *Neisseria gonorrhea*, *Chlamydia trachomatis*, CMV, and HSV shedding. CVL interleukins (IL)-1$\beta$, IL-6, IL-8, and SLPI were measured using ELISA. Linear regression with generalized estimating equations examined effects of cytokine concentrations on CVL HIV-1 RNA, adjusted for plasma HIV RNA, and measured coinfections. CVL IL-1$\beta$ and IL-8 were significantly associated with CVL HIV-1 RNA. This persisted after adjusting for plasma HIV-1 RNA. Higher levels of IL-1$\beta$ were associated with higher concentrations of HIV-1 RNA in CVL ($\beta = 0.25$, 95% CI 0.09, 0.42), as were higher levels of IL-8 ($\beta = 0.34$, 95% CI 0.17, 0.50). Adjusting for the presence of the coinfections described, this relationship was attenuated for IL-1$\beta$ ($\beta = 0.16$; 95% CI –0.01, 0.33) but still significant for IL-8 ($\beta = 0.29$; 95% CI 0.13, 0.45). The proinflammatory cytokines IL-1$\beta$ and IL-8 are associated with higher cervicovaginal HIV-1 RNA concentrations, even after controlling for plasma viral load and vaginal microbial cofactors. This association suggests that there may be additional, noninfectious causes of inflammation that increase cervicovaginal HIV-1 shedding.

## Introduction

Shedding of HIV-1 in genital secretions is an important determinant of the risk of sexual transmission of the virus. Even when plasma viral load is undetectable, transmission is still possible,[1,2] likely due to ongoing genital shedding of the virus. Plasma viral load is the primary predictor of HIV-1 shedding in the genital secretions of women,[3,4] but localized factors such as cervical, vaginal. and uterine infections also increase the likelihood of viral shedding.[5,6] A meta-analysis of over 24 studies of genital infection and HIV-1 shedding showed that *Neisseria gonorrhea*, *Chlamydia trachomatis*, yeast vaginitis, cervicitis, and genital ulcers were associated with increased odds of detecting HIV-1 in genital secretions.[7] The mechanism for this association is not entirely clear, but many suspect that inflammation related to infections promotes viral transcription from infected cells[8] and/or increases vascular permeability. Alternatively, bacterial or viral infections may draw HIV-1-infected T cells to the area,[9] increasing viral concentrations and shedding.

Proinflammatory cytokines such as interleukin (IL)-1$\beta$, tumor necrosis factor (TNF)-$\alpha$, IL-6, and IL-8 are increased in many genital infections[10–13] and with genital shedding of HIV-1.[14–16] Vaginal hygiene practices[17] and cryotherapy for cervical neoplasia[18] have also been associated with genital HIV-1 shedding, which many hypothesize is related to inflammation resulting from trauma to vaginal epithelium. If inflammation is a risk factor for HIV-1 shedding and is present in the absence of infections, treating infections alone may not reduce genital HIV-1 concentrations and risk to sexual partners. In this study, we used the proinflammatory cytokines IL-1$\beta$, IL-6, and IL-8 as markers of inflammation and

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secretory leukocyte protease inhibitor (SLPI) as an antiinflammatory molecule to evaluate the association between HIV-1 shedding and genital inflammation, adjusting for concurrent genital infections.

## Materials and Methods

This was a prospective, observational study of HIV-1-infected women in Seattle, WA ($n = 38$) and Rochester, NY ($n = 19$) between 2002 and 2007. Study participants were 18–50 years of age at study entry, were not pregnant or menopausal, had an intact cervix, and had no active genital infections at study entry. There were no entry restrictions with regard to CD4 cell count, plasma viral load, or antiretroviral therapy. The University of Washington and University of Rochester Institutional Review Boards approved the study, and all subjects provided written informed consent.

Participants had four study visits in the first year and three visits per year in subsequent years. Each study visit included a comprehensive face-to-face interview ascertaining demographic information, reproductive and medical history, sexual behavior, and medication use. Plasma was obtained for HIV RNA quantification. A pelvic examination was performed with collection of vaginal swabs. Cervicovaginal lavage (CVL) was collected by lavaging the ectocervix and vaginal walls with 7 ml of 10 mM lithium chloride (LiCl) solution, which was then collected from the vaginal pool, spun at 3000 rpm for 5 min to separate the epithelial cells, and stored at $-80°C$ in 1-ml aliquots. Urine was collected for *N. gonorrhea* and *C. trachomatis* detection by nucleic acid amplification (COBAS Amplicor PCR).

Plasma and CVL HIV RNA were quantified by an independently validated real-time polymerase chain reaction (PCR) assay described previously[19] with a lower limit of quantification of 30 c/ml. Herpes simplex virus (HSV) serology testing was done by Western blot. CVL was tested for genital shedding of HSV and cytomegalovirus (CMV) using PCR.[20,21] CVL was tested for interleukins (IL)-1$\beta$, $-6$, $-8$, and SLPI using an in-house ELISA with commercial antibodies as previously described[10] and values were corrected for dilution using a standard concentration of LiCl per ml of lavage fluid.[22] The presence and quantity of *Lactobacillus* and yeast were ascertained by vaginal culture. Hydrogen peroxide production was ascertained by subculture on tetramethylbenzidine (TMB) agar containing horseradish peroxidase as previously described.[23] Bacterial vaginosis (BV) was diagnosed from vaginal Gram stain using Nugent's criteria[24]; a score of 0–3 was considered normal flora, 4–6 intermediate, and 7–10 positive for BV. A dichotomous variable indicating abnormal vaginal microbiota (Nugent score 4–10) was created for the purposes of analysis. *Trichomonas vaginalis* was detected by culture using the InPouch system (Hardy Diagnostics, Santa Maria, CA).

Plasma and genital viral loads as well as cytokine concentrations were $\log_{10}$ transformed for the purposes of analysis. We used linear regression with generalized estimating equations and robust standard errors to compare quantities of cytokines between women with and without genital shedding of HIV-1 and to examine effects of $\log_{10}$ cytokine concentrations on $\log_{10}$ CVL HIV-1 RNA, adjusted for $\log_{10}$ plasma HIV RNA, and the presence of abnormal vaginal flora, $H_2O_2^+$ *Lactobacillus* colonization, yeast, *T. vaginalis*, CMV, and HSV shedding. The prevalence of *N. gonorrhea* and *C. trachomatis* was so low that these infections were not included in the model.

## Results

A total of 57 women enrolled in the study, 38 in Seattle and 19 in Rochester, and completed a total of 391 visits (median visits per person 6, range 1–15). In total, 312 of 391 visits (80%) had complete data for analysis. Participants ranged in age from 23 to 52 years, with a mean of $38 \pm 6$ years. At enrollment, the mean plasma viral load was $26{,}218 \pm 65{,}525$ c/ml, the CD4 count was $456 \pm 285$ cells/ml, and the use of antiretroviral therapy was reported by 31/57 (54%). The cohort was primarily white 33/57 (58%), with 13/57 (23%) reporting African-American race and 11/57 (19%) reporting another ethnicity. Just over half, 38/57(55%), had a high school education or less, 10/57(17%) reported some college or technical school, and 9/57 (16%) reported college education or masters degree. Mean gravidity was $6 \pm 8$ and mean parity was $3 \pm 2$.

The majority of samples (72%) were collected during the luteal phase of the menstrual cycle. At over half of the visits (52%) women had been sexually active in the past 30 days, and at a third of the visits (30%) women had been sexually active in the past week, but the median time since last intercourse was 13 days (IQR 5, 16). The majority of women (71%) reported sex in the past year with only male partners, while 5 (9%) reported only female partners and 6 (11%) reported both genders. Of 53 women who answered the question at the enrollment visit, 9 (17%) reported never using condoms (4 of whom reported sex only with women in the past year), while 24 (45%) reported always using condoms.

Genital shedding of HIV-1 RNA was detected in CVL at 56/312 (18%) of the visits. The most common genital infection was BV, diagnosed at 89 (29%) of 312 visits, followed by yeast, which was present at 64/312 visits (21%). Trichomoniasis was diagnosed at a small number of visits (15/312, 5%), as were *N. gonorrhea* and *C. trachomatis* (4/312, 1%). Although 38/45 (84%) of participants had positive serology for HSV-2, genital shedding of HSV-2 was detected at only 7/312 (2%) of visits. CMV shedding was detected at 10/312 (3%) of visits. $H_2O_2$-producing lactobacilli were detected at the majority of visits (193/312; 62%). At 46/312 (15%) visits women had intermediate Nugent scores (4–6). Nonspecific cervicitis, diagnosed by the presence of more than 30 white blood cells per high-powered microscopy field in a cervical swab, was present at 123/312 (39%) of visits.

IL-1$\beta$ was detectable at 278/312 (90%) visits, IL-6 at 161 (52%), IL-8 at 308 (99%), and SLPI at 232 (74%). In univariate analysis, the presence of abnormal vaginal flora, trichomoniasis, and HSV-2 shedding (Table 1) were associated with higher levels of IL-1$\beta$, while the presence of $H_2O_2$-producing lactobacilli was associated with lower levels. Yeast vaginitis and HSV-2 shedding were associated with higher levels of IL-8, while the presence of an abnormal Nugent score was associated with lower quantities of SLPI.

Values of IL-1$\beta$ and IL-8 were significantly different between visits with and without HIV-1 genital shedding detected (Table 2). In regression analysis CVL IL-1$\beta$ and IL-8 were significantly associated with concentration of CVL HIV-1 RNA and this persisted after adjusting for plasma HIV RNA (Table 3). After adjusting for the presence of $H_2O_2^+$

Table 1. Results of Univariate Regression Analysis for Association Between Cytokines Measured in Cervicovaginal Lavage and Presence of Genital Infections at 391 Visits from 57 HIV-1-Infected Women[a]

| | *Regression coefficient value for model with $log_{10}$ cytokine concentration as the dependent variable* | | | |
|---|---|---|---|---|
| *Coinfection* | *IL-1β* | *IL-6* | *IL-8* | *SLPI* |
| $H_2O_2$-producing lactobacilli | **−0.06 (−0.10, −0.02)** | −0.01 (−0.04, 0.02) | −0.003 (−0.04, 0.03) | −0.001 (−0.03, 0.03) |
| Abnormal Nugent score | **0.58 (0.34, 0.82)** | 0.001 (−0.17, 0.17) | −0.10 (−0.33, 0.14) | **−0.29 (−0.46, −0.12)** |
| Yeast | 0.09 (−0.24, 0.42) | −0.09 (−0.32, 0.13) | **0.41 (0.15, 0.67)** | 0.19 (−0.03, 0.42) |
| Trichomoniasis | **0.60 (0.29, 0.92)** | 0.22 (−0.10, 0.54) | 0.22 (−0.09, 0.52) | **−0.33 (−0.61, −0.05)** |
| CMV | 0.10 (−0.22, 0.42) | −0.06 (−0.42, 0.30) | 0.35 (−0.10, 0.80) | 0.03 (−0.25, 0.31) |
| HSV | **0.60 (0.24, 0.95)** | 0.43 (−0.23, 1.09) | **0.59 (0.20, 0.99)** | −0.32 (−0.71, 0.06) |

[a]Numbers in bold represent statistically significant associations.

*Lactobacillus*, abnormal Nugent score, yeast, *Trichomonas*, and CMV or HSV-2 genital shedding, this relationship was attenuated for IL-1*β* but was still significant for IL-8. Adjusting for sexual activity in the past month, time since sex, time since menses, antiretroviral use, or HSV-2 serology did not change the results of the analysis (data not shown).

## Discussion

In this study we evaluated the association between proinflammatory cytokines IL-1*β*, IL-6, and IL-8, the antiinflammatory molecule SLPI, and cervicovaginal shedding of HIV-1, controlling for plasma viral load and the presence of genital coinfections. In this analysis, IL-8 is associated with higher cervicovaginal HIV-1 RNA concentrations even after adjusting for the presence of several coinfections and plasma viral load. This association suggests that there may be additional, noninfectious causes of inflammation that increase cervicovaginal HIV-1 shedding.

Genital shedding of HIV-1, while primarily driven by plasma HIV-1 load,[3] is likely also mediated by local factors as evidenced by the fact that up to 33% of women with undetectable HIV-1 in plasma continue to shed virus in cervicovaginal secretions.[25] Several groups have shown that local, genital markers of inflammation are associated with increased HIV-1 genital shedding.[12,15] Anderson *et al.* showed that the presence of white blood cells in CVL is associated with increased detection of HIV-1 in CVL, even after controlling for plasma viral load,[26] but did not control for the presence of genital coinfections. Higher rates of genital shedding of HIV-1 are seen in women with concomitant genital tract infections such as herpes[27] or BV.[28,29] Treatment of these infections as well as *Candida vaginitis* and *Trichomonas* has been shown to decrease HIV-1 shedding.[5,30] Many hypothesize that the proinflammatory cytokines associated with infections promote HIV-1 replication and thus increase shedding.

However, noninfectious events are also associated with higher levels of inflammatory markers. Two weeks after cryotherapy of cervical neoplasia, while the cervix was inflamed, Lawn *et al.* showed that TNF-$\alpha$ and IL-6 were increased, as was HIV-1 RNA in genital secretions.[31] Prakash *et al.* showed that in the 3 days following sexual intercourse, the number of $CD4^+$ T cells in the vaginal epithelium was higher than when women were sampled more than 3 days after intercourse.[32] These studies suggest that epithelial trauma—whether from sex, vaginal hygiene practices, or surgical procedures—can cause inflammation and may facilitate HIV-1 shedding. Our results also suggest this; in a model adjusted for the presence of many common genital tract infections, IL-8 (a neutrophil chemoattractant) was independently associated with an increased quantity of HIV-1 shedding.

One of the limitations of our study is that women were seen only every 3–4 months, and proximate behavioral causes of genital inflammation such as frequency and timing of sexual intercourse were measured as an average over that time; thus we have a limited ability to identify what factors are associated with increased genital levels of IL-8. Only 80% of visits had complete data (HIV plasma and CVL values, CVL cytokines, CMV and HSV testing) for analysis, which may create some bias, though there did not seem to be a pattern in which subjects or visits were excluded. It is possible that the association is due to an infectious cause of inflammation that we did not measure, but we tested for the most common causes of vaginitis, genital ulcers, and cervicitis and so the contribution of other infections is likely to be small. HIV-1 shedding was detected at only 18% of visits, which may have limited our

Table 2. Median Cytokine Values at Visits with and without Genital Shedding of HIV-1 RNA ($N = 391$ Visits from 57 Women)[a]

| | *Visits with shedding* (n = *56*) | *Visits without shedding* (n = *256*) | p-*value*[b] |
|---|---|---|---|
| IL-1*β* (pg/ml), median (95% CI) | 26.4 (15.6, 55.5) | 10 (7.8, 13.4) | **0.02** |
| IL-6 (pg/ml), median (95% CI) | 5.4 (2.5, 8.94) | 3.9 (2.5, 5.4) | 0.28 |
| IL-8 (pg/ml), median (95% CI) | 481.3 (311.8, 707.3) | 224.8 (175.8, 261.1) | **<0.01** |
| SLPI (pg/ml), median (95% CI) | 45,702 (31,189, 80,824) | 78,019 (63,447, 94,258) | 0.21 |

[a]Numbers in bold represent statistically significant associations.
[b]*p*-value for linear regression using generalized estimating equations for the association between the presence of genital shedding and cytokine values.

Table 3. Multivariate Analysis of Association Between Cervicovaginal Lavage Cytokines and HIV-1 Genital Shedding[a]

| | Regression coefficient for model with $log_{10}$ CVL HIV-1 as dependent variable (95% CI) | |
|---|---|---|
| *Cytokine* | *Adjusting for PVL* | *Adjusting for PVL, $H_2O_2$ lactobacilli, abnormal Nugent score, yeast, trichomoniasis, HSV and CMV shedding* |
| IL-1$\beta$ | **0.25 (0.09, 0.42)** | 0.16 (−0.01, 0.33) |
| IL-6 | 0.26 (−0.02, 0.55) | 0.23 (−0.02, 0.48) |
| IL-8 | **0.34 (0.17, 0.50)** | **0.29 (0.13, 0.45)** |
| SLPI | −0.10 (−0.30, 0.11) | −0.11 (−0.30, 0.08) |

[a]Numbers in bold represent statistically significant associations. CVL, cervicovaginal lavage; PVL, plasma HIV-1 viral load.

power to detect associations but makes the associations that we did detect much more robust.

Genital tract inflammation, and specifically that characterized by the presence of the neutrophil chemoattractant IL-8, is associated with HIV-1 genital shedding independent of the presence of other infections. This suggests that in addition to treatment or sexually transmitted infections, antiinflammatory therapies may be a potential mechanism to decrease HIV-1 shedding and the risk of sexual transmission.

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## Author Disclosure Statement

No competing financial interests exist.

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