Quotidian Changes of Genital Tract Cytokines in Human Immunodeficiency Virus-1-Infected Women During the Menstrual Cycle

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The role of hormonal changes throughout the menstrual cycle on genital tract inflammation during chronic human immunodeficiency virus (HIV) infection is not well defined, but it has implications for HIV prevention. We assessed daily levels of 26 vaginal cytokines and chemokines from 15 women infected with HIV-1. Taking into account coexisting sexually transmitted infections, behavioral factors, and menstruation, this study illustrates cyclic patterns of granulocyte macrophage colony-stimulating factor, interferon-α2, interleukin (IL)-6, IL-10, macrophage inflammatory protein (MIP)-1α, MIP-1β, and tumor necrosis factor (TNF)-α. Progesterone was associated with levels of granulocyte colony-stimulating factor, IL-1α, and monocyte chemoattractant protein-1. Interferon-α2, IL-6, MIP-1α, MIP-1β, and TNF-α levels predicted HIV shedding, but these associations were heavily influenced by the menstrual cycle.

Keywords. cytokines; hormones; human immunodeficiency virus; menstrual cycle.

The female genital tract (FGT) is an immunologically complex environment that undergoes significant physiological changes during the menstrual cycle (MC). During the periovulatory phase, the immune milieu in the upper FGT shifts from an inflammatory to immune tolerant state that is hormonally driven [1]. However, fluctuations in cytokine and chemokine profiles that underlie changes in the lower FGT during the MC are poorly defined, particularly in women who are human immunodeficiency virus (HIV) positive and have high levels of generalized immune activation due to their HIV infection. The effect of hormones either directly or indirectly through immune milieu has the potential to significantly impact HIV shedding by recruiting or activating relevant target cells [2] and increasing infectiousness [4].

Changes in cytokines at intervals likely to capture the dynamic nature of hormonal changes during the MC have not been examined in detail, although several studies have measured cytokines at weekly intervals [2-4]. Al-Harthi et al. [3] previously described weekly FGT cytokine profiles for a single cycle in women infected with HIV, and they found elevated levels of several proinflammatory cytokines during menstruation that correlated with HIV shedding. These data suggest a role for FGT cytokines influencing mucosal shedding during the MC, but it remains unclear whether shedding is independently influenced by the MC itself or by cyclic changes in cytokine levels.

To more precisely examine immune activation in the FGT during the MC in women with chronic HIV infection, we assessed 26 daily cytokine and chemokine levels in 15 women. This study provides a more comprehensive view of the cyclic patterns of cytokines and chemokines during the MC made possible by more frequent measures. Although we also found that a subset of proinflammatory cytokines were associated with virus shedding, this relationship was primarily mediated by the MC. These findings may provide a

biological explanation for prior studies showing associations between FGT cytokines and virus shedding [6-9].

METHODS

Study Design and Data Collection for the MC Cohort Study
A cohort of antiretroviral-naive women attending a Municipal Sexually Transmitted Disease clinic in Mombasa, Kenya were recruited to the study whether they reported regular MCs and no hormonal contraceptive use in the last 6 months. Dacron swabs of the vagina and endocervix were obtained daily between 7:00 and 11:00 AM beginning after the first day of menstruation and were stored at −80° in 1 mL freezing media (70% Roswell Park Memorial Institute medium 1640, 20% fetal calf serum, and 10% dimethyl sulfoxide). Luteinizing hormone (LH) levels were obtained from daily urine samples. Douching and sexual activity were assessed daily. Estradiol, progesterone, and HIV-1 RNA were measured 3 times per week, and sexually transmitted infections (STIs) were assessed weekly as previously described [10, 11]. Informed consent was obtained from all study participants. This research was approved by human subjects review committees at the Fred Hutchinson Cancer Research Center, the University of Washington, and the University of Nairobi.

Laboratory Analysis
Cytokine concentrations were determined for vaginal swab samples using the Milliplex MAP Human Cytokine/Chemokine Pre-mixed 26-Plex (Millipore Corporation, Billerica, MA) on a Luminex 200 (Luminex Corporation, Austin, TX). Upper and lower limits of detection were calculated per plate for each cytokine. To minimize the effect of plate-to-plate variability, all of the samples from a single woman were run on the same plate.

Statistical Methods
Cytokine, progesterone, and estradiol levels were $\log_{10}$-transformed. Cytokine measurements below the lower limit of detection (LLOD) were assigned the midpoints between zero and the per-plate LLOD for that cytokine. Hormone levels, linear splines, and days since the LH surge were used as independent predictors of cytokine levels in linear mixed-effects (LME) models with random intercepts. LME models to estimate the relationship between cytokines and viral shedding were limited to cytokines in which >80% of samples were above the LLOD. The Benjamini-Hochberg procedure was used to adjust for multiple comparisons. All analyses were performed in Stata 11 (StataCorp, College Station, TX).

RESULTS

Study Population
The majority of the women (80%) were enrolled on the second day of menses, and subsequently attended daily clinic visits for 1 full cycle (median, 27 days; range, 21–31) (Supplemental Table 1). Seven women (47%) had CD4+ T cell counts >350 cells/μL, with a cohort median of 354 cells/μL (range, 139–566). The cohort median serum viral load was 4.48 $\log_{10}$ copies/mL (range, 0.48–6.44), whereas median cervical and vaginal viral loads were 3.37 (range, 0.95–6.11) and 2.63 $\log_{10}$ copies/mL (range, 0.95–6.6), respectively. Twelve women (80%) experienced clinical symptoms of bacterial vaginosis and approximately half had 1 or more coexisting STIs.

Cyclic Patterns and Hormonal Control of Genital Tract Cytokines and Chemokines
The levels of cytokines and chemokines in vaginal swabs varied, with interleukin (IL)-8 detected at the highest concentrations (median, 1349 pg/mL) and IL-5 the lowest (median, 1.50 pg/mL) (Table 1). For 13 of the 26 cytokines tested, >80% of the samples had levels above the LLOD (Table 1).

The dataset was first fit using linear splines with a change point at the LH surge that split the cycle into the follicular and luteal phases (data not shown). The majority of cytokines and chemokines tested appeared to decrease during the follicular phase and then gradually increase after ovulation during the luteal phase, consistent with prior studies describing cyclic patterns of mucosal defenses [4, 12]. Because of this pattern, we decided to center the data using the LH surge and used days since the surge as the predictor in a single LME model to test whether each cytokine exhibited a cyclic pattern over the MC. Interleukin-6, macrophage inflammatory protein (MIP)-1α, tumor necrosis factor (TNF)-α, granulocyte macrophage colony-stimulating factor (GM-CSF), MIP-1β, interferon (IFN)-α2, IL-10, monocyte chemoattractant protein (MCP)-1, IL-15, IL-1β, eotaxin, and IL-7 levels were significantly associated with days since the LH surge; however, eotaxin and IL-7 were no longer significant after adjusting for multiple comparisons (Table 1). Multivariate analyses adjusting for bacterial vaginosis and other STIs, sexual activity, or douching did not substantially alter the original univariate estimates (data not shown), but adjusting for menstruation slightly attenuated the associations, with only 7 of the 10 cytokines remaining statistically significant (Table 1). Visually, the cyclic patterns for IL-6, MIP-1α, TNF-α, and GM-CSF were the most striking, whereas the averaged trends across women for MIP-1β, IFN-α2, and IL-10 were more subtle (Figure 1).

To assess whether there was a direct correlation between hormone levels and cytokine concentration, separate LME models were run for progesterone and estrogen. Progesterone levels were positively correlated with IL-1α and inversely correlated with granulocyte colony-stimulating factor (G-CSF) and MCP-1 after adjusting for multiple comparisons (Supplemental Figure 1A). Consistent with this finding, the slopes of the linear splines representing the luteal phase—a time when progesterone levels peak—were either negative in the case of G-CSF or close

Table 1. Cytokine Concentrations and Their Associations With Days Since the LH Surge, Sorted by Unadjusted *P* Value

Cytokine/ chemokine	Median (pg/mL)	IQR (pg/mL)	% Above LLOD	Unadjusted (n = 14[a])		Adjusted for Menstruation (n = 14)	
				Reg Coef[b] (95% CI)	*P* value[c]	Reg Coef[b] (95% CI)	*P* value[c]
IL-6	11.5	3.16–38.5	81%	0.036 (0.023–0.048)	**<.001**	0.021 (0.008–0.034)	**.002**
MIP-1α	16.0	6.03–43.1	87%	0.027 (0.017–0.037)	**<.001**	0.020 (0.010–0.031)	**<.001**
TNF-α	10.4	3.89–40.3	80%	0.033 (0.021–0.045)	**<.001**	0.028 (0.015–0.042)	**<.001**
GM-CSF	12.2	4.37–34.7	78%	0.028 (0.018–0.039)	**<.001**	0.020 (0.008–0.031)	**.001**
MIP-1β	49.6	23.4–100	97%	0.018 (0.008–0.028)	**<.001**	0.014 (0.003–0.024)	**.015**
IFN-α2	39.8	20.9–69.2	98%	0.014 (0.006–0.023)	**.001**	0.011 (0.002–0.021)	**.016**
IL-10	3.98	1.59–14.8	63%	0.017 (0.007–0.028)	**.001**	0.013 (0.001–0.024)	**.027**
MCP-1	42.7	12.2–132	97%	0.020 (0.006–0.034)	**.004**	0.014 (−0.001 to 0.030)	.061
IL-15	3.89	1.76–8.13	65%	0.012 (0.003–0.021)	**.008**	0.010 (−0.0003 to 0.019)	.059
IL-1β	45.7	13.2–178	95%	0.022 (0.004–0.039)	**.014**	0.014 (−0.004 to 0.033)	.128
Eotaxin	44.7	29.5–66.1	99%	0.008 (0.001–0.015)	.023		
IL-7	11.2	5.50–22.4	92%	0.009 (0.0003–0.018)	.041		
IL-8	1349	660–3162	98%	0.014 (−0.001 to 0.029)	.075		
IL-5	1.57	1.46–1.91	25%	0.005 (−0.001–0.010)	.125		
IL-4	9.12	3.89–20.7	78%	0.008 (−0.002 to 0.019)	.128		
IFN-γ	5.56	1.60–20.7	72%	0.005 (−0.005 to 0.015)	.331		
G-CSF	389	138–776	99%	−0.006 (−0.020 to 0.008)	.383		
IL-17	5.89	1.93–21.9	64%	−0.006 (−0.019 to 0.008)	.425		
IL-13	3.85	1.74–14.3	60%	0.004 (−0.006 to 0.014)	.456		
IL-12p40	14.8	5.62–36.7	77%	0.003 (−0.008 to 0.014)	.609		
TNF-β	2.35	2.00–6.31	41%	−0.002 (−0.011 to 0.007)	.671		
IL-1α	1148	501–1905	99%	0.002 (−0.011 to 0.016)	.724		
IL-2	5.07	1.97–13.3	69%	−0.002 (−0.011 to 0.007)	.808		
IP-10	347	126–1230	98%	0.001 (−0.011 to 0.014)	.824		
IL-3	3.47	1.97–7.00	59%	−0.0004 (−0.006 to 0.005)	.890		
IL-12p70	3.47	1.78–10.4	60%	−0.0003 (−0.010 to 0.010)	.956		

Abbreviations: CI, confidence interval; GM-CSF, *granulocyte macrophage colony-stimulating factor*; G-CSF, *granulocyte macrophage colony-stimulating factor;* IFN, interferon; IP, induced protein; IQR, interquartile range; IL, interleukin; LH, luteinizing hormone surge; LLOD, lower limit of detection; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; Reg Coef, regression coefficient; TNF, tumor necrosis factor.

[a] Excludes woman without detectable LH surge.

[b] Interpreted as the change in $\log_{10}$ pg/mL cytokine concentration per day since the LH surge.

[c] Nominal *P* value is reported, and those that are in bold remained significant after Benjamini-Hochberg adjustment for multiple comparisons.

to zero for MCP-1 and IL-1α (Supplemental Figure 1B). No associations between estrogen and cytokine levels were observed (data not shown).

Impact of Cytokines and Chemokines and the MC on FGT HIV Shedding

Macrophage inflammatory protein-1α, IL-6, TNF-α, IFN-α2, and IL-7 were all significantly associated with cervical shedding after controlling for multiple comparisons (Supplemental Table 2) and adjusting for serum viral load, coexisting STIs, and other behavioral factors (data not shown). The strongest association observed was with MIP-1α, where every 1 $\log_{10}$ pg/mL increase corresponded to a 0.25 $\log_{10}$ copies/mL increase in cervical HIV RNA. However, the association between cytokines and shedding was no longer significant after controlling for

days since the LH surge. There were no detectable associations between cytokines and vaginal HIV shedding (data not shown).

DISCUSSION

This is the first study to longitudinally assess the daily FGT immune milieu by examining a wide array of vaginal cytokines and chemokines during the MC of women infected with HIV. Proinflammatory cytokines IL-6, IFN-α2, TNF-α, GM-CSF, IL-15, IL-1β, and chemokines MIP-1α, MIP-1β, MCP-1, as well as one anti-inflammatory cytokine, IL-10, displayed cyclic patterns over the MC that were independent of coexisting STIs and behavioral factors. There was a moderate influence of menstruation on these associations, with levels of MCP-1, IL-15, and IL-1β no longer reaching statistical significance in a
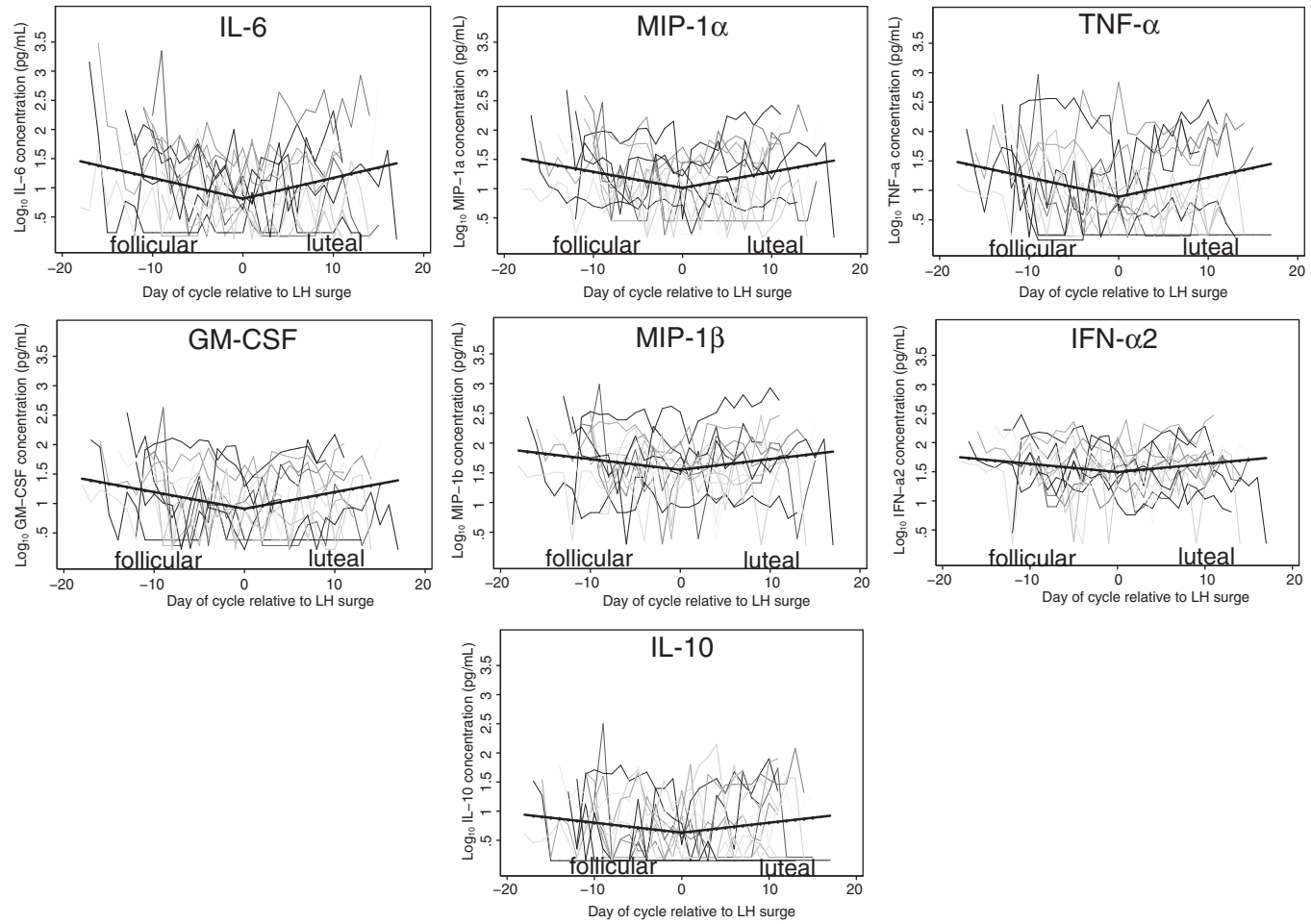
Figure 1. Pattern of cytokine and chemokine levels across the menstrual cycle, centered on the luteinizing hormone (LH) surge.

multivariate model. The cyclic patterns observed were not solely due to changes in secretion volume, because many cytokines did not vary over the cycle. Overall, these findings suggest a strong hormonal influence on cytokines and chemokines in the FGT.

Prior studies of cytokine changes in the MC have focused on weekly samples, allowing comparison of cytokines during specified phases but not consistently over the entire MC. A previous study of HIV-positive women suggested that proinflammatory cytokine levels in cervicovaginal lavage (CVL) did not substantially change between the follicular and luteal phases but were elevated during menstruation [3]. However, in smaller studies of HIV-negative women, proinflammatory cytokines IL-6 and IL-1β have consistently been shown to be higher in CVL during the follicular phase than the luteal phase, whereas data on other cytokines over the cycle have been more variable [2, 4]. Our ability to detect changes in multiple proinflammatory cytokines over the MC that were not seen in prior studies likely reflects the fact that we examined daily changes in relation to the LH surge, providing more power to detect cyclic fluctuations that may be masked when comparing fixed time points in the cycle. Indeed, when we limited our analysis to weekly measurements, our associations were greatly attenuated (data not shown). Other study design differences, such as the collection method (swabs versus CVL), may also contribute to these discrepancies.

We found that progesterone levels were associated with IL-1α, G-CSF, and MCP-1 levels. Because progesterone is low during the follicular phase and then peaks during the luteal phase, it is not surprising that 2 of these analytes were not associated with the MC when these data were centered on the LH surge. Although no studies have directly compared vaginal cytokine concentrations with individual hormones, the inverse associations we observed between proinflammatory analytes (MCP-1, G-CSF) and progesterone are supported by prior studies, suggesting that progesterone promotes an anti-inflammatory state [13, 14]. Our data also showed that the increase in proinflammatory cytokines during the luteal phase was more gradual than the decrease observed during the follicular phase by linear spline, most likely due to the influence of progesterone (data not shown). Given this result, the positive association between the proinflammatory cytokine IL-1α and progesterone levels was surprising, and it is unclear whether this association is a spurious result or a novel finding, perhaps unique to HIV-positive women.

Since female hormones can alter the immune environment and thus potentially impact HIV replication, we performed an exploratory analysis of HIV shedding, focusing on contributions of both the FGT cytokine and chemokine milieu and the MC. We did not observe any associations between cytokine and vaginal HIV RNA levels, potentially because vaginal RNA concentrations were more variable and there were fewer vaginal samples in which HIV RNA was detectable (71% above the lower limit) compared with cervical samples (92% above the lower limit), resulting in reduced power. Concentrations of proinflammatory cytokines IL-6, MIP-1α, TNFα, MIP-1β, IFN-α2, and IL-7 were correlated with the level of cervical HIV RNA. These cytokines were among a subset that showed similar relationships with shedding in at least 1 of several recent cross-sectional studies using the same Luminex technologies [6-9]. Interleukin-6 and MIP-1β, in particular, are 2 analytes that have consistently been found to be predictive of viral shedding [3, 6-9]. Some of the other variability in cytokine associations could be explained by the fact that our study included longitudinal sampling from fewer individuals than the previous cross-sectional studies.

Consistent with the findings reported here, Al-Harthi et al. [3] showed that elevated FGT cytokine levels during menstruation, including IL-6 and MIP-1β, closely predicted viral shedding. However, the focus of this study did not address whether shedding was independently influenced by the MC itself or by cyclic changes in cytokine levels, and the mechanism by which cytokines impacts HIV-1 shedding during the MC has not been defined in any study. In the present study, multivariate analysis adjusting for days since the LH surge strongly attenuated the proinflammatory cytokine-shedding relationship, suggesting that although cytokine levels may influence HIV shedding, their effect is likely driven by hormonal changes during the MC.

The results from this study must be framed with a few limitations. First, only a single cycle was observed for each woman and it is likely that additional within-subject monthly variation could occur. However, daily swabs were collected during the same hours each morning to reduce within-person variability, and ovulatory cycles were verified with LH measurements. Second, we used a multiplexed assay that has considerable plate-to-plate variability [15]. To minimize this issue, we ran each woman's samples on a single plate.

This study demonstrates the quotidian changes of mucosal cytokines and chemokines during the MC in chronically infected HIV-positive women. Using cytokines and chemokines as a proxy for genital inflammation, we show that although a group of proinflammatory cytokines is associated with viral shedding, this activity is largely mediated by fluctuations during the MC. Overall, these findings highlight the importance of future mechanistic studies to determine the clinical impact of endogenous and exogenous hormones on the immunologic environment in the genital tract of HIV-positive women.

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