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Immunity in the Vagina (Part II): Anti-HIV Activity and Antiviral Content of Human Vaginal Secretions

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Abstract

Problem—Whether the concentrations of antiviral proteins, and anti-HIV activity, within human vaginal secretions changes across the menstrual cycle is unknown.

Method of Study—Using a menstrual cup, vaginal secretions from premenopausal women were recovered at the proliferative (d6–8), mid-cycle (d13–15) and secretory (d21–23) stages of the menstrual cycle. Antiviral protein concentration was determined by ELISA, and anti-HIV activity assessed using the TZM-bl reporter cell line.

Results—CCL20, RANTES, elafin, HBD2, SDF-1 α and IL-8 levels were detectable in the secretions. Vaginal secretions had anti-HIV activity against specific clade B strains of HIV, with significant inhibition of IIIB and increased infectivity of transmitted/founder CH077.t. No significant differences in either antiviral protein concentration or anti-HIV activity with respect to menstrual cycle stage were measured, but marked differences were observed in both parameters over the course of the cycle between different women, and in consecutive cycles from the same woman.

Conclusion—The vagina contains a complement of antiviral proteins. The variation in anti-HIV activity demonstrates that immune protection in the vagina is not constant. Intra- and inter-individual variations suggest that factors in addition to sex hormones influence antiviral protection. Lastly, the menstrual cup is a new model for recovering undiluted vaginal secretions from women throughout their reproductive life.

Keywords

antiviral; chemokine; cytokine; Human Immunodeficiency Virus (HIV); menstrual cycle; vaginal secretions

Introduction

The female reproductive tract (FRT) is the primary site of host defense against sexually transmitted infections (STI). Annually, there are approximately 498 million new incidences of STI, including 2.7 million cases of Human Immunodeficiency Virus (HIV), that cause significant levels of morbidity and mortality in women ¹. However, the role of the menstrual cycle and its associated hormonal changes in regulating immune protection in the FRT is poorly understood ².

A key constituent of immune protection are the secretions bathing the entire lining of the FRT. In the vagina these secretions are composed of vaginal transudate, vaginal epithelial cell secretions, cervical mucus and uterine fluids that have flowed into the lower FRT. Previous studies have demonstrated that cervico-vaginal fluid possesses anti-HIV ³⁻⁶, anti-HSV2 ⁷ and antibacterial activity in premenopausal women ^{6, 8, 9}. Whether this antiviral activity changes across the menstrual cycle is unknown.

Contained within the vaginal secretions are antimicrobials, cytokines and chemokines that are responsible for its antiviral and antibacterial activity. Human beta defensin (HBD) 2, stromal-derived factor 1 (SDF-1 α), elafin, CCL20, IL-8 and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) are some of the antiviral proteins present in cervico-vaginal secretions. HBD2, produced primarily by FRT epithelial cells but also macrophages, monocytes and dendritic cells, inhibits HIV infection through direct interactions with the viral membrane, decreasing CXCR4 expression on target cells and inhibiting the early steps of intracellular viral replication ^{10, 11}. Elafin is a serine protease inhibitor involved in tissue remodeling but also implicated in protection against HIV and is secreted in large amounts by FRT epithelial cells ¹². It shares 40% homology with secretory leukocyte protease inhibitor (SLPI), another protein with anti-HIV activity ¹³. CCL20, a neutrophil chemoattractant, is produced by uterine epithelial cells and inhibits HIV infection via an unknown mechanism ¹⁴. RANTES, a ligand for CCR5, is also produced by both epithelial cells and stromal fibroblasts and prevents HIV infection by blocking HIV-CCR5 co-receptor interactions ¹⁵. SDF-1 α , a natural ligand for CXCR4, is produced by stromal fibroblasts and prevents infection of uterine NK cells by HIV ^{16, 17}. IL-8 is constitutively secreted by stromal fibroblasts and epithelial cells. It can reduce HIV infection by inducing CCR5 internalization and decreasing viral transcription and replication ¹⁸⁻²⁰.

Previous studies have demonstrated a role for hormonal regulation of antimicrobial and cytokine production in the FRT. Within endometrial tissue, HBD4 and HBD2 are highest during the proliferative and menstrual phases, respectively ^{21, 22}. Maximal SLPI levels are observed during the late secretory phase, while elafin concentration increases dramatically at menses ²². In contrast to the upper FRT, the profile of antimicrobials and cytokines in the lower FRT is very different. Previously, we found that in cervico-vaginal lavage (CVL) samples from premenopausal women, the concentrations of SLPI, HBD2, alpha defensins 1-3, lysozyme and lactoferrin were lowest at mid-cycle, when estradiol levels peak in the blood, relative to both the proliferative and secretory stages of the menstrual cycle ²³. However, other studies have shown no effect of menstrual cycle stage on antimicrobial and cytokine levels within FRT secretions. Using tampons to recover the vaginal fluid, Valore et

al. (2002) demonstrated that the concentrations of calprotectin, lactoferrin, lysozyme, SLPI, HBD1, HBD2 and the alpha defensins remained relatively constant across the cycle ⁹. Shust et al. (2009) reported no difference in the concentration of SLPI, lactoferrin, lysozyme, HBD2, IL-8 and RANTES between CVL samples recovered at the proliferative and secretory stages ⁷. Overall, the evidence for hormonal regulation of antimicrobial and cytokine concentration in human vaginal fluid across the menstrual cycle is unclear and requires further investigation.

In this study, we used a menstrual cup to recover vaginal fluid from the proliferative, mid-cycle and secretory phases of the menstrual cycle in a group of premenopausal women, prior to analyzing secretions for the presence of antimicrobials and cytokines that are associated with protection against HIV infection. Our results suggest that specific aspects of innate immune protection in the FRT change across the menstrual cycle, and are not controlled solely by the concentration of the sex hormones estradiol and progesterone.

Methods and Materials

Study Participants

Healthy, premenopausal women (n=7) were recruited at Dartmouth-Hitchcock Medical Center (DHMC), Lebanon, New Hampshire. All volunteers were HIV negative, and not on any form of chemical or oral birth control or using an intra-uterine device. All investigations involving human subjects were conducted according to the principles expressed in the Declaration of Helsinki and carried out with the approval from the Committee for the Protection of Human Subjects (CPHS) at DHMC, and with written informed consent obtained from the volunteers.

Recovery of Vaginal Fluid

Volunteers were provided with an InStead © softcup (menstrual cup) to recover their vaginal fluid at three points during the menstrual cycle: days 6–8, 13–15 and 21–23 after the start of menses (day 1) corresponding to the proliferative, ovulatory and secretory stages of the menstrual cycle respectively. Each volunteer would provide a single sample for each stage of the menstrual cycle. The menstrual cup was inserted into the vagina as per the manufacturer's instructions for 1 hour (<http://www.softcup.com/video-tutorials>). Upon removal at DHMC, the cup was placed in a 50ml tube and immediately brought to the laboratory where its contents were centrifuged (800g; 5 mins) to recover the vaginal fluid. The weight and volume of vaginal fluid in the tube was measured, diluted in 1X phosphate-buffered saline (PBS) (Roche Diagnostics, Indianapolis, IN) at a ratio of 1ml PBS:1g of vaginal fluid, briefly vortexed and centrifuged (1000g; 5 mins) to remove any cellular material including epithelial cells and/or debris. The resulting supernatant was stored at –80°C prior to further analysis, while the epithelial cells were recovered and analyzed separately for hormone responsiveness ²⁴.

Measurement of Protein

Total protein concentration of vaginal fluid samples was determined using a BCA protein assay (ThermoScientific, Rockford, IL) according to the manufacturer's instructions.

Quantification was based on a standard curve after OD measurements at 562nm on an ELISA reader (Dynex, Chantilly, VA, USA).

Measurement of Cytokines and Antimicrobials

Cytokine and antimicrobial levels in vaginal fluid were measured by enzyme-linked immunosorbent assay (ELISA). The following ELISAs were performed according to the manufacturer's instructions: CCL20 (R&D Systems, Minneapolis, MN, USA), Elafin (R&D), HBD2 (Peprotech, Rocky Hill, NJ), RANTES (R&D), SDF-1 α (R&D) and IL-8 (R&D). Concentrations were quantified based on a standard curve after OD measurements at 450 nm on an ELISA reader (Dynex).

Measurement of anti-HIV activity

The two reference HIV-1 strains IIIB (X4) and BaL (R5) were obtained from Dr. Phalguni Gupta (Univ. of Pittsburgh, PA) while the two transmitted/founder HIV-1 strains CH077.t (R5) and CH058.c (R5) were provided by Drs. Christina Ochsenbauer and John Kappes (Univ. of Alabama at Birmingham, AL). The TZM-bl indicator cell line is a modified HeLa cell line that expresses high levels of the HIV receptor CD4 and co-receptors CCR5 and CXCR4, and was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc ²⁵. TZM-bl cells contain HIV long terminal repeat (LTR)-driven β -galactosidase and firefly luciferase reporter cassettes that are activated by HIV infection and subsequent Tat protein expression. For anti-HIV assays, TZM-bl cells were seeded in complete media supplemented with 20 mM HEPES (Invitrogen Life Technologies, Grand Island, NY), 2 mM L-glutamine (Invitrogen), 50 mg/ml primocin (Invivogen, San Diego, CA) and 10% charcoal-dextran stripped heat-inactivated Fetal Bovine Serum (FBS) (ThermoScientific, Logan, UT) at 2.5×10^4 cells per well in a 96-well plate and incubated at 37°C for 24 hours. Vaginal fluids from individual patients were diluted 1:20 in complete media and incubated with HIV at a multiplicity of infection (MOI) of 1 for 1 hour at 37°C in a final volume of 100 μ l. Following incubation, the combined virus and vaginal fluid (100 μ l) was added to the TZM-bl cells, in the absence of DEAE dextran, for 48 hours, after which the cells were lysed with β -Glo luciferase substrate solution (Promega, Madison, WI) and firefly luminescence was measured using a luminometer. Controls included incubation of TZM-bl cells with virus in medium alone, vaginal fluid in medium alone and complete media alone. Uninfected cells were used to determine background luminescence and data are expressed in relative light units (RLU).

Cell Viability Assay

TZM-bl cell viability was determined by the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) as per the manufacturer's instructions.

Statistical Analysis

All data were analyzed using GraphPad Prism version 5.0 (La Jolla, CA). Where appropriate data are presented as median (range) or mean and standard error. Comparison of cytokine concentration and anti-HIV activity between different stages of the menstrual cycle were

performed using either a repeated measures ANOVA with a Bonferroni post-test, or the Mann-Whitney U test. A p value of less than 0.05 was considered as significantly different.

Results

Recovery of vaginal secretions

Undiluted vaginal secretions were recovered using an InStead© cup at the proliferative, mid-cycle and secretory stages of the menstrual cycle. In general, the secretions were relatively viscous and adhered well to the plastic cup (Figure 1). We centrifuged each cup in a 50 ml tube, to efficiently remove the secretions and then determine their weight. As seen in Table 1, we observed a trend towards increased recovery of secretions at mid-cycle (1.17g) compared to the proliferative and secretory stages - approximately 1g each.

Protein analysis of vaginal secretions

Total protein concentration in the vaginal fluids was relatively constant across the menstrual cycle averaging 3624 (946–6205), 2998 (1977–8680) and 3123 (1060–4779) µg/ml at the proliferative, mid-cycle and secretory stages respectively (Table 1). Of the individual antiviral proteins we assayed, elafin (median: 12862 pg/ml) was the most abundant in the vaginal secretions followed by IL-8 (6143 pg/ml), HBD2 (2244 pg/ml), CCL20 (2331 pg/ml), SDF-1α (561 pg/ml) and RANTES (72 pg/ml). Similar to the total protein concentration RANTES, elafin, CCL20, SDF-1α, IL-8 and HBD2 (Table 1) did not demonstrate any significant changes over the cycle. However, there was a trend towards a decreased RANTES concentration at mid-cycle (34 pg/ml) compared to the proliferative (124 pg/ml) and secretory (81 pg/ml) stages. A similar profile was observed for HBD2 where mid-cycle values (1740 pg/ml) were lower than proliferative (2955 pg/ml) and secretory (2244 pg/ml) values. In contrast, IL-8 and CCL20 peaked at mid-cycle. Elafin levels were slightly higher at the secretory stage (19242 pg/ml) in comparison to the mid-cycle (11793 pg/ml) and proliferative stages (13235 pg/ml).

In four of our volunteers, we recovered samples from five consecutive stages of two menstrual cycles thus allowing us to analyze the changes across the cycle at a more individual level. There was an increase in pellet weight in 4/4 volunteers between the proliferative and mid-cycle stage in the first menstrual cycle (Figure 2A). This trend was maintained at the same transition point in the second cycle in 3/4 volunteers. There was a decrease in RANTES levels from the proliferative to mid-cycle in 3/4 volunteers (Figure 2D). Between the secretory stage of the first cycle and proliferative stage of the second cycle there was an increase in RANTES levels (3/4). Elafin increased between the secretory and proliferative stages in 4/4 volunteers (Figure 2E) while there was a decrease in HBD2 in 3/4 volunteers between mid-cycle and secretory stages (Figure 2F). IL-8 levels increased from mid-cycle to secretory (3/4) and decreased between secretory to proliferative stages (3/4) (Figure 2H).

Anti-HIV activity of vaginal secretions

We have previously demonstrated that CVL samples from both HIV+ and HIV– women are capable of inhibiting HIV infection of TZM-bl cells ^{3,4}. We tested the antiviral activity of

the vaginal fluids against three R5-tropic HIV clade B strains (BaL, CH077.t and CH058.c) and one X4-tropic strain (IIIB). As seen in Figure 3A, vaginal secretions had no statistically significant antiviral effect on BaL infection of TZM-bl cells reducing it by an average of 15% (range: 96% inhibition – 19% increase). The secretions significantly inhibited IIIB infection by an average of 33% (range: 96% inhibition – 16% increase) (Figure 3B). CH058.c infection was reduced by 11% (range: 66% inhibition – 58% increase) (Figure 3D). Interestingly, approximately half of our samples inhibited CH058.c infection, while the remainder enhanced infection of TZM-bl cells. In contrast, there was no inhibitory effect against CH077.t, with infection significantly increasing by an average of 101.54% (range: 2% inhibition – 227% increase) (Figure 3C).

We then analyzed the antiviral data with respect to cycle stage and found no significant effect of cycle stage on BaL infection of TZM-bl cells, with an average reduction of 7.5% (range: 83% inhibition – 16% increase), 11% (range: 96% inhibition – 16% increase) and 14% (range: 94% inhibition – 19% increase) in secretions recovered from proliferative, mid-cycle and secretory stages, respectively (Figure 4A). In contrast, proliferative and secretory secretions significantly reduced IIIB infection by 39.5% (range: 92% inhibition – 9% increase) and 48% (range: 95% inhibition – 22% increase) (Figure 4B). However, there was no significant anti-IIIB effect with secretions recovered at mid-cycle (31% reduction) (range: 95% inhibition – 12% increase) (Figure 4B). CH058.c infection was reduced by an average of 23% (range: 72% inhibition – 33% increase), 8% (range: 65% inhibition – 50% increase) and 23% (range: 60% inhibition – 50% increase) at the proliferative, mid-cycle and secretory stages (Figure 4D). This inhibitory trend was reversed with CH077.t in that samples increased HIV infection by an average of 61% (range: 2% inhibition – 120% increase), 100% (range: 54% increase – 165% increase) and 105% (14% increase – 210% increase) at the proliferative, mid-cycle and secretory stages (Figure 4C).

Analyzing the data across two consecutive menstrual cycles from our 4 volunteers demonstrated that there was considerable intra- and inter-subject variation with no consistent effect of menstrual cycle stage upon anti-HIV activity. The least variability in antiviral activity was observed against BaL at all points in the menstrual cycle (Figure 5A). IIIB demonstrated a similar trend (Figure 5B). In contrast, the overall effects on CH077.t and CH058.c were much more variable. For example, there was increased CH077.t infection in the first proliferative stage sample for volunteer 101 (Figure 5C) and an approximately 15% reduction in viral infection in the second proliferative sample. Similarly, volunteer 103 showed no anti-CH058.c activity in her first mid-cycle sample, but a 40% inhibition in her second sample (Figure 5D). None of the changes in antiviral activity correlated with the concentration of antimicrobials or cytokines measured.

Discussion

In this study, we used a menstrual cup to recover vaginal secretions from a group of premenopausal women in order to determine the effects of menstrual status on anti-HIV protein concentration, and anti-HIV activity within the lower FRT. Total recovery from the vagina was greatest at mid-cycle and lowest at the proliferative and secretory stages. Of the anti-HIV proteins we investigated, elafin was present at the highest concentration in vaginal

secretions followed by IL-8, HBD2, CCL20, SDF-1 α and RANTES. However, owing to the variations in anti-HIV protein levels between women, there was no significant change in their concentration between the different stages of the menstrual cycle. Antiviral activity depended on the strain of HIV used, with the secretions significantly increasing CH077.t infection of TZM-bl cells, but having no significant effect on BaL and CH058.c, and mild inhibitory effects on IIIB. Similar to antiviral protein levels, endogenous anti-HIV activity within the vaginal secretions did not significantly change with stage of the menstrual cycle, suggesting that factors in addition to sex hormones may modulate anti-HIV activity in the lower FRT.

Our use of a menstrual cup to collect vaginal secretions is a relatively novel and under-utilized technique to which there are several benefits. First, due to the nature of the insertion process that involves the cup being pushed up the length of the vagina, recovered secretions should be broadly representative of the entire vaginal compartment. In contrast, CVL recovery includes secretions from the entire vagina and the external surface of the cervix, while material recovered from swabs generally represents what is present in localized areas of the vagina or cervix. This is important as fluids are not distributed equally over the vaginal surface and thus discrete regions of the vagina may have different concentrations of antimicrobials and cytokines. Additionally, a recent study has shown that mucus collected from the vagina is phenotypically different from cervical mucus²⁶. Therefore, the menstrual cup may provide an accurate snapshot of the vaginal compartment. Second, the cup allows for the initial recovery of undiluted vaginal fluids. CVL generally involves a large initial dilution when the cervix and vagina are washed with up to 10ml of fluid. This in turn may limit the potential application of CVL. Third, unlike CVL, the menstrual cup can be used by volunteers themselves and does not require the participation of trained professionals or specialized equipment, thus greatly reducing the burden of participation upon the volunteers. However, we understand there are limitations to the menstrual cup, including user-to-user variability in accomplishing the insertion and removal procedure. Additional studies are needed to compare and determine which techniques provide the most accurate representation of the immune environment in the lower FRT.

Vaginal secretions contain a potent cocktail of antimicrobials, cytokines and chemokines required to maintain a protective environment in the lower FRT²⁷. Several studies have investigated the protein complement within lower FRT secretions. In comparison to these, our studies found that levels of total protein (3123 $\mu\text{g/ml}$) in secretions recovered by the menstrual cup were at least 3 to 37 times higher (89–1280 $\mu\text{g/ml}$)^{6, 23}. A similar trend was observed for IL-8 (6143 pg/ml), CCL20 (2331 pg/ml) and RANTES (72 pg/ml) where we observed concentrations that were 6 to 190, 24 to 122 and 1.5 to 31 times higher respectively, than values reported elsewhere^{3, 5–7, 28, 29}. In contrast, elafin (12.89 ng/ml) and HBD2 (2.24 ng/ml) were generally lower than previously published data. For example, elafin has been reported at average concentrations of 5.9, 35.9, 100 and 200 ng/ml in lower FRT secretions from HIV negative women, while HBD2 averaged 570, 117, 5.55 and 1.11 ng/ml ^{3, 5, 7, 9, 12, 30, 31}. Different collection protocols (tampon, swab and CVL) and sample populations may account for some of these inconsistencies in levels of measured protein⁶.

An unexpected finding was the lack of a consistent effect of menstrual cycle stage on protein concentration in the vaginal secretions. Our prior results using secretions from freshly isolated vaginal epithelial cells (VEC) in culture, demonstrated a consistent inhibitory effect of estradiol on HBD2 and elafin secretion²⁴. The absence of consistent mid-cycle decreases in HBD2, elafin and the other cytokines suggests that VEC, despite their ability to produce antimicrobials, are not exclusive or major contributors to the mid-cycle decline observed elsewhere²³. Unfortunately, previous publications provide a complicated backdrop against which to make comparisons, and it is still unclear whether a mid-cycle drop in cytokine, chemokine or antimicrobial levels occurs *in vivo*^{23, 32, 33}. For example, Keller et al. (2007) demonstrated that antimicrobials, cytokines and chemokines including SLPI, HBD2, IL-8 and lactoferrin decrease at mid-cycle in CVL recovered from healthy premenopausal women while total protein concentration remains relatively constant²³. Using a comparable CVL protocol MacNeill et al. (2012) also observed a significant decrease in IL-8 concentration between the proliferative and mid-cycle stages. However, we observed a different profile where IL-8 was greater at mid-cycle compared to the other stages, although this difference was not significant. Similar to our findings and in contrast to Keller et al., vaginal fluid, recovered from tampons from 5 different donors, failed to see significant difference in levels of HNP1-3, HBD2 and lysozyme from the proliferative to secretory phases of the cycle with no observable mid-cycle drop, or surge, in individual protein levels⁹. Cervical secretions recovered using Dacron swabs showed a mid-cycle surge in SLPI compared to the proliferative and secretory stages but no change in IL-8^{34, 35}. Our results are complemented by observations from the murine model, where Hickey et al. (2012) demonstrated that while antimicrobial and cytokine levels in uterine lavage decreased at diestrus, there was no change in their concentration in vaginal lavage samples suggesting that hormonal effects may be restricted to specific anatomical compartments³⁶.

Prior studies from our laboratory have shown that CVL from nonpregnant and pregnant women inhibit HIV infection of target cells by BaL, IIIB and CH077 by 29–79%, 66–82% and 75%, respectively^{3, 5}. Our results indicate that vaginal secretions recovered from the menstrual cup have anti-HIV activity, but that this activity is variable and depends upon the strain of virus. Furthermore, we found no correlation between cycle stage and antiviral activity which is consistent with results from another group²⁸. We believe that these differences arise because the secretions recovered by the menstrual cup are primarily from the vagina, while CVL includes a substantial cervical component due to the washing of the ectocervical epithelium and entrance to the cervical os. CVL may therefore contain additional antiviral factors that increase inhibition of HIV infection.

In contrast to our findings showing no inhibition against BaL and 33% inhibition against IIIB, a previous study using undiluted vaginal fluid recovered with a menstrual cup demonstrated 60–80% inhibition of BaL and IIIB infection of TZM-bl cells³⁷. One explanation for our differing results may be the way in which the vaginal secretions were handled. In their study, Venkataraman et al. (2005) sonicated undiluted vaginal fluid recovered from the menstrual cup and, in some cases, treated secretions with 5% acetic acid followed by resuspension in sodium phosphate prior to performing the antiviral assays³⁷. Our protocol used an equal volume of PBS to dilute the secretions followed by

centrifugation to remove any debris. In our protocol, antiviral components in vaginal secretions, such as vaginal mucus, would have been removed by centrifugation, thus leading to decreased anti-HIV activity. Venkataraman et al. determined that a large component of antiviral activity was due to the cationic fraction of vaginal fluid. We cannot rule out the possibility that PBS dilution and subsequent centrifugation may have selectively eliminated or inactivated elements of this cationic fraction and partially abrogated endogenous antiviral activity.

Previous studies have determined the minimum *in vitro* HIV inhibitory concentration for HBD2 (9000–20,000 ng/ml), elafin (0.01–10 ng/ml), RANTES (3000 pg/ml), CCL20 (2000–200,000 pg/ml), SDF-1 α (200,000 pg/ml) and IL-8 (500–50,000 pg/ml)^{12, 14, 18–20, 38–41}. Our measurements of HBD2, RANTES and SDF-1 α were considerably lower than these values with CCL20 in the lower range. In contrast, elafin and IL-8 were present at inhibitory levels. Recognizing that these proteins can function in an additive or synergistic manner, we were surprised not to observe greater antiviral activity in our system^{42, 43}. One explanation for this may be that measuring the overall quantity of antimicrobials or cytokines in the secretions does not provide a complete picture of their biological activity. Several proteins are processed from precursor molecules to active metabolites by proteases and other enzymes present in the vaginal secretions. For example, matrix metalloproteases are required to activate SDF-1 α , and the N-terminus of Trappin-2 is cleaved by mast cell tryptase to generate elafin. Our ELISAs do not differentiate between the precursor and processed form of protein. Thus, we cannot assess the ratio of active:inactive protein. In addition, vaginal secretions contain enzymes capable of inactivating the antimicrobials, such as Cathepsin D, which inhibits the function of CCL20. It is likely that these enzymes required for activation/inactivation are key regulators of the overall antiviral activity present in the vaginal secretions and are important for future studies to consider^{44–47}. It is also possible that while hormonal status may not affect antimicrobial levels directly, it could alter the activity of these activating/inactivating enzymes and thus indirectly modulate the quantity of biologically active antimicrobials. For example, Cathepsin D is induced by estradiol suggesting that it may increase in vaginal secretions at mid-cycle when estradiol levels surge and this may translate into greater inhibition of CCL20⁴⁸.

Often overlooked in studies of lower FRT secretions are the multiple functions of many of its protein constituents. Several of these proteins both inhibit and enhance HIV infection *in vitro*. For example, RANTES, IL-8 and SDF-1 α are proven inhibitors of HIV infection. However, they also capable of increasing HIV infection. Their precise effect probably depends upon their concentration, viral tropism (X4 or R5) or the *in vitro* system used. RANTES (50,000–500,000 pg/ml) increases HIV replication in monocytes and macrophages^{49, 50}. IL-8, at concentrations ranging from 500–50,000 pg/ml stimulates HIV replication in T lymphocytes and macrophages⁵¹. SDF-1 α between 50,000–2,300,000 pg/ml can both inhibit X4 viral entry into P4C5 HeLa cells (CD4+ CCR5+ CXCR4+), and promote Tat-mediated R5 proviral transcription⁴⁰. While the concentration of RANTES and SDF-1 α in secretions collected from the menstrual cup is considerably lower than that required to enhance HIV infection, our recovery of IL-8 is easily within the concentration

range over which enhancement occurs. Further, our recovery of IL-8 is substantially higher than that reported elsewhere. If IL-8 enhanced HIV infection of TZM-bl cells, this could explain why the secretions we collected had lower anti-HIV activity than expected.

We used established definitions of the proliferative, mid-cycle and secretory stages based on an idealized 28-day menstrual cycle. However, this may not be applicable to all women and the volunteers presented in Figures 2 and 5, had cycle lengths ranging from 27–32 days. There is substantial variation not only in the total length of a woman's cycle, but also in length of each stage. Only 10% of women with a 28-day cycle have a classical 14-day proliferative and secretory phase⁵². The proliferative phase ranges from 10–23 days and the secretory phase from 7–19 days. The variability in cycle length was largely attributed to changes in the length of the proliferative phase⁵³. While the overall decrease in cycle length is only 2–3 days, our “window” for each stage of the cycle was 72 hours⁵². Given the dramatic changes in serum hormone levels that occur over short periods, especially at ovulation when estradiol levels surge and recede over the span of 72–96 hours, even a shift of 2–3 days may represent a substantially different endocrine environment.

In conclusion, the menstrual cup is a novel and effective technique for recovering vaginal secretions. Importantly, we demonstrate that vaginal secretions collected by the menstrual cup contain antiviral proteins whose concentration profile is unique compared to that observed with other recovery techniques. The concentration of the antiviral proteins varies dramatically, both between volunteers, but also between samples recovered from the same volunteer taken at the same stage of different menstrual cycles. Reflecting this, the secretions also possessed variable antiviral activity against four specific strains of HIV, and neither antiviral protein concentration nor antiviral activity consistently changed with the menstrual cycle suggesting that factors in addition to sex hormones may be important regulators of innate immune protection in the lower FRT. Further studies are essential to fully understand how the endocrine system modulates the immune system and thus affects innate immune protection in the lower FRT.

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Figure 1. Human vaginal secretions

A representative freshly recovered menstrual cup from a volunteer after a 1 hr insertion. (A) Top view. (B) Side view.

Figure 2. Profile of vaginal secretions from individual volunteers

Vaginal secretions from four volunteers (101–104) were obtained across two consecutive menstrual cycles and analyzed by weight (A), total protein (B), CCL20 (C), RANTES (D), elafin (E), HBD2 (F), SDF-1 α (G) and IL-8 (H). Data are shown as mean $\pm$ SEM. In panel C, D and G a value of 1 is assigned to samples where protein levels were below the level of detection for the assay.

Figure 3. Anti-HIV activity in vaginal secretions

Vaginal secretions were recovered and diluted 1:20 in complete media and pre-incubated with BaL (n=29) (A), IIIB (n=29) (B), CH077.t (n=20) (C) and CH058.c (n=20) (D) at a MOI of 1 for 1 hour prior to a 48 hour incubation on TZM-bl cells after which infection levels were determined by relative luminescence (RLU). Data are shown as mean RLU $\pm$ SEM. (VS = vaginal secretions). * $P < 0.05$; ** $P < 0.01$ with respect to virus alone. Each symbol represents a single menstrual cup.

Figure 4. Anti-HIV activity in vaginal secretions across the menstrual cycle

Vaginal secretions were sorted by cycle stage (proliferative, mid-cycle and secretory), diluted 1:20 in complete media and pre-incubated with BaL (A), IIIB (B), CH077.t (C) and CH058.c (D) at a MOI of 1 for 1 hour prior to a 48 hour incubation on TZM-bl cells after which infection levels were determined by relative luminescence (RLU). The number of samples tested for CH058 and CH077 were lower than those for BaL and IIIB as sample quantity was limited. * $P < 0.05$; ** $P < 0.01$ with respect to virus alone.

Figure 5. Individual profile of anti-HIV activity in vaginal secretions

Vaginal secretions from two consecutive menstrual cycles from four volunteers (101–104) were recovered and diluted 1:20 in complete media and pre-incubated with BaL (A), IIIB (B), CH077.t (C) and CH058.c (D) at a MOI of 1 for 1 hour prior to a 48 hour incubation on TZM-bl cells after which infection levels were determined by relative luminescence (RLU). Dashed line indicates the RLU value for virus alone. Data are shown as mean RLU $\pm$ SEM. Open symbols represent samples that are significantly different from virus alone ($P < 0.05$). Closed symbols represent samples that are not significantly different from virus alone.

Table 1
Profile of the vaginal secretions

Vaginal secretions were recovered from the proliferative (d6–8), mid-cycle (d13–15) and secretory (d21–23) stages of the menstrual cycle. Data are shown as median average and *range*.

	Proliferative	Mid-Cycle	Secretory
Weight (g)	1.00 0.58–1.69	1.17 0.63–2.1	1.07 0.64–1.64
Total Protein (µg/ml)	3624 946–6205	2998 1977–8680	3123 1060–4780
CCL20 (pg/ml)	2543 0–12664	3699 30–12411	713.3 0–13290
Elafin (pg/ml)	13235 6210–26287	11793 737–26598	19242 1655–39225
HBD2 (pg/ml)	2955 834–16615	1740 624–17457	2244 635–17993
RANTES (pg/ml)	124 0–329	34 4–123	81 21–247
SDF-1α (pg/ml)	257 0–4622	431 0–9723	766 0–3470
IL-8 (pg/ml)	5662 831–26522	10248 162–27470	3879 622–41452