Published in final edited form as:

J Acquir Immune Defic Syndr. 2012 October 1; 61(2): 194–202. doi:10.1097/QAI.0b013e31826867ae.

Changes in the Soluble Mucosal Immune Environment during Genital Herpes Outbreaks

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Abstract

Background—Genital tract secretions provide variable inhibitory activity against herpes simplex virus (HSV) *ex vivo*. We hypothesize that the anti-HSV activity may prevent the spread of virus from the more commonly affected sites, such as the external genitalia, to the upper genital tract.

Methods—The antimicrobial activity of cervicovaginal lavage (CVL) and concentrations of mucosal immune mediators were measured in ten HIV-seronegative women with an active external herpetic lesion and compared to ten HIV-seronegative women who were HSV-1 and HSV-2 seronegative. Samples were obtained at the time of a symptomatic external lesion (day 0), following one week of oral acyclovir (ACV) (day 7), and one week after completing treatment (day 14). Controls were evaluated at parallel intervals.

Results—The anti-HSV activity was higher in CVL obtained from cases compared to controls at presentation (Day 0) (54.3% vs. 28%), fell to similar levels on Day 7, and then rebounded on Day 14 (69% vs. 25%). The anti-HSV activity correlated positively and significantly with the concentrations of several inflammatory proteins; the concentrations of these proteins tended to be higher in cases compared to controls and followed a similar temporal pattern.

Conclusions—Increases in inflammatory immune mediators and anti-HSV activity were detected in CVL at the time of clinical outbreaks and following completion of a short course of

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ACV. These mucosal responses may protect against HSV spread, but could facilitate HIV infection and contribute to the clinical observation that, independent of clinical lesions, HSV-2 is a risk factor for HIV acquisition.

Keywords

herpes simplex virus; soluble mucosal immunity; human immunodeficiency virus

Introduction

Genital herpes simplex virus (HSV) infections pose a global public health threat. In the US, ~17% of the population is seropositive for HSV-2 and 58% for HSV-1, which has emerged as a major cause of genital herpes in developed countries¹⁻³. HSV-2 seroprevalence rates approach 90-95% among HIV-infected individuals and among female sex workers in developing countries, where HSV-2 remains the dominant cause of genital ulcerative disease⁴. Incident or prevalent HSV-2 infection increases the risk of acquiring and transmitting HIV and contributes more to the spread of HIV-1 than the number of sex partners or other sexually transmitted infections (STI)^{5,6}.

Overt genital ulcers likely promote HIV acquisition by disrupting the epithelium and recruiting activated CD4+ T cells. This is supported by the finding of increased numbers of activated T cells found at the dermal-epidermal junction in sequential biopsies of genital skin obtained during and after healing of clinically evident HSV ulcers⁷. Efforts to reduce HSV and its impact on HIV include suppressive therapy with oral acyclovir (ACV), topically delivered antivirals, and prophylactic vaccines. Trials to test whether suppressive ACV could reduce HIV acquisition or transmission yielded disappointing results. Oral suppressive ACV had no impact on HIV acquisition⁸ nor did it affect the rate of HIV transmission from HIV-1/HSV-2 co-infected persons to their HIV-uninfected partners⁹, but ACV did reduce the frequency of clinical HSV recurrences^{8,9}.

The importance of soluble mucosal immunity is supported by the observation that genital secretions from healthy women exhibit *ex vivo* activity against HIV and HSV, independent of serostatus¹⁰⁻¹². Yet the precise molecules that contribute to the activity against each pathogen and the biological implications are unknown. In prior studies, we found that cervicovaginal lavage (CVL) blocked HSV entry and the antiviral activity correlated with concentrations of soluble immune mediators including interleukin (IL)-8, human neutrophil peptides (HNP1-3), lactoferrin, lysozyme, and immunoglobulins^{10,11}. HNP1-3 are α -defensins secreted primarily by immune cells and exhibit anti-HSV and anti-HIV activity *in vitro*^{13,14}. The concentrations of these peptides are increased in the setting of inflammation and, despite their *in vitro* antiviral activity, CVL concentrations of HNP1-3 have been independently associated with increased HIV acquisition¹⁵. This observation led to speculation that any protective effects of the antimicrobial peptides were overcome by the detrimental effects of inflammation, which may disrupt the epithelial barrier, recruit and activate HIV target cells, and directly augment HIV replication through activation of the long terminal repeat (LTR).

No studies have examined the endogenous anti-HSV activity or changes in concentrations of soluble mucosal immune mediators in the female genital tract in the setting of active genital herpes. We hypothesize that CVL antimicrobial activity may be increased during episodes of external herpes lesions, perhaps as a host protective response to prevent the spread of virus locally and from the more commonly involved external sites (vulva, labia, and introitus) to the upper genital tract. HSV is isolated less frequently from cervical than from vulval, perineal, perianal or vaginal swabs¹⁶. However this increase in anti-HSV activity and its

associated inflammatory mediators may, paradoxically, facilitate HIV infection through immune activation and recruitment of HIV target cells.

To explore this notion, we compared the antimicrobial activity and concentrations of selected immune mediators in HIV-seronegative women with an external herpetic lesion to women who were seronegative for HIV, HSV-1, and HSV-2. Samples were obtained at the time of a symptomatic lesion (day 0), following oral ACV treatment (day 7), and one week after completing treatment (day 14). Controls were evaluated at parallel intervals.

Methods

Participants

Women between the ages of 18 and 50 years were recruited from the New York metropolitan area between July 2006 and October 2009. Albert Einstein College of Medicine and Mount Sinai School of Medicine Institutional Review Boards and the NIAID Division of AIDS Prevention Science Review Committee approved the study. All participants provided written informed consent. Inclusion criteria for cases included an ulcerative or vesicular eruption located on the vulva, labia, or perineum, positive HSV culture or direct fluorescent antibody (DFA) test, willingness to receive anti-HSV treatment, and to abstain from sex and vaginal product use for the study duration. Control subjects had no history of genital HSV lesions, were HSV-1 and HSV-2 seronegative, and frequency matched to cases by age (within 5 years), race (Black, White, Asian or Mixed) and hormonal contraceptive use. Participants were excluded for pregnancy, breastfeeding, menopause, HIV, genitourinary infection, bacterial vaginosis (BV), abnormal Pap test, positive semen test, and anti-HSV treatment for >2 days prior to screening.

At the initial visit, participants had urine collected for microscopy, culture and pregnancy. A gynecological examination was performed at each visit for detection of BV (wet prep with Amsel clinical criteria), *Trichomonas vaginalis* (wet prep), and *Candida* species (KOH prep). Genital secretions were evaluated for the presence of semen with an immunoassay that detects p30 (Abacus Diagnostics, West Hills, CA). Vaginal pH was measured from a swab of the lateral vaginal wall (Whatman pH paper, pH 3.8-5.5). Suspected lesions were swabbed for DFA testing (Millipore, Temecula, CA) and culture. The lesion and vagina were sampled with a single swab for HSV DNA by PCR. Genital secretions were collected by lavage with 10 ml of normal saline (pH ~5.0). At the initial visit, a Pap test was collected, and *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection were determined by nucleic acid amplification testing of endocervical swabs (Gen-Probe, Inc., San Diego, CA). Blood was collected for HIV ELISA, syphilis (rapid plasma reagin test), serotype-specific antibodies for HSV-1 and HSV-2 (HerpeSelect, Focus Diagnostics, Cypress, CA). Controls underwent similar procedures but did not have swabs collected for HSV DFA and culture.

Cases were provided with a seven-day course of oral ACV (400 mg three times a day), and cases and controls were asked to return 7 and 14 days later for pelvic exam, collection of a vaginal swab for HSV PCR, and CVL for mucosal studies.

Cervicovaginal lavage

CVL were transported to the laboratory on ice and were clarified by centrifugation at 700 g for 10 minutes at 4°C. Supernatants were divided into aliquots and stored at -80°C. An aliquot of unspun CVL was stored for HSV PCR testing.

HSV PCR

Vaginal swabs from cases and controls (Days 0, 7, and 14) and unspun CVL from cases (Day 0) were evaluated for HSV DNA by quantitative, real-time, fluorescence-based PCR as described ¹⁷.

Measurement of immune mediators

The concentration of protein recovered in CVL supernatants was measured with the Micro BCA Protein Assay kit (Thermo Scientific Pierce). IL-1 α , IL-1 β , IL-6, IL-8, Interferon (IFN)- γ , IFN- α 2, IL-1 receptor antagonist (IL-1ra), macrophage inflammatory protein (MIP)-1 α , MIP-1 β , and regulated upon activation, normal T-cell expressed and secreted (RANTES) were quantified in each CVL sample using a multiplex proteome array with beads from Chemicon International (Billerica, MA), measured using Luminex100 (Austin, TX) and analyzed using StarStation (Applied Cytometry Systems, Sacramento, CA). The levels of all other mediators were determined using commercial ELISA kits: secretory leukocyte protease inhibitor (SLPI) (R & D Systems, Minneapolis, MN), lactoferrin (Calbiochem, San Diego, CA), lysozyme (Alpco Diagnostics, Salem, NH), human beta defensins (HBD) 1, 2 and 3 (Alpha Diagnostic International, San Antonio, TX), HNP1-3 (the ELISA does not differentiate between these three related peptides; HyCult Biotechnology, Uden, The Netherlands), and IgG and IgA (Cygnus Technologies, Southport, NC).

Measurement of endogenous antimicrobial activity

The antimicrobial activity against HSV-2, HIV-1, and *Escherichia coli* (*E. coli*) in CVL was measured as previously described ¹⁸. For anti-HSV activity, Vero cells were infected in duplicate in two independent experiments with ~ 50-200 pfu of HSV-2(G) mixed 1:1 with each CVL or control buffer (normal saline containing 200 μ g/mL bovine serum albumin) and plaques were counted after 48 hours. For anti-HIV activity, TZM-bl cells were cultured in 96 well dishes overnight and infected in triplicate in two independent experiments with HIV-1_{BaL} (approximately 10³ TCID₅₀) mixed 1:1 with CVL or control buffer. After 48-hour incubation at 37°C, the inoculum was removed by washing, cells were lysed with addition of luciferase cell culture lysis reagent (Promega) and the plates were stored at -80°C until assessed for luciferase activity. To assess the bactericidal activity, *E. coli* (ATCC strain 4382627) was grown overnight to stationary phase and then mixed with CVL or control buffer (20 mmol/L potassium phosphate, 60 mmol/L sodium chloride, 0.2 mg/ml albumin, pH 4.5) and incubated at 37°C for two hours. The mixtures were further diluted in control buffer (to yield 800-1000 colonies on control plates) and plated on agar enriched with trypticase soy broth. Colonies were counted using ImageQuant TL v2005 after an overnight incubation at 37°C. All samples were tested in duplicate and the percentage inhibition determined relative to the colonies formed on control plates.

Statistical analysis

Sample values for mediators below the limit of detection (LOD) were set at the midpoint between zero and the LOD. Mediator concentrations were log-transformed to reduce skewness in the distribution. Categorical variables were compared between groups by Fisher's exact test. Continuous variables at each time point were compared between cases and controls by either the Student's t test or the Wilcoxon rank sum test. Spearman rank-order correlation coefficients were estimated to assess associations between mediators from Day 0 samples. Analyses were performed with GraphPad Prism (version 4; GraphPad Software, La Jolla, CA), and a two-sided p value <0.05 was considered significant. P-values were not adjusted for multiple comparisons given the limited sample sizes and descriptive nature of the analyses.

Results

Study participants

Thirty-four women with presumed herpes lesions were assessed for eligibility and ten were enrolled. The majority of exclusions were due to the absence of a positive DFA or culture (n=14) or the presence of BV (n=6). Eighteen women without a history of oral or genital herpes were assessed for eligibility as controls. Eight were excluded; one had BV, four were HSV-1 seropositive, one was HSV-2 seropositive, and two were seropositive for HSV-1 and HSV-2. The median age of all participants was 30 years; one case and one control were using hormonal contraception at enrollment. There were no significant differences in demographics or other characteristics between cases and controls (Table 1).

Detection of virus from lesions and CVL

All ten cases had external lesions (nine labial and one mons pubis), which were confirmed by culture. HSV-2 was isolated from eight cases and HSV-1 from two (Table 1). Two subjects enrolled during their first clinical occurrence. HSV was also detected by quantitative PCR in the lesional/vaginal swab collected at presentation in all subjects tested (one did not have a swab available for HSV PCR) (Table 1). However, HSV DNA was not detected in any of the vaginal swabs collected from the cases on Days 7 or 14 nor from any swabs collected from controls.

To assess whether virus was also present within the genital secretions at clinical presentation, unspun CVL obtained on Day 0 were analyzed for the presence of HSV DNA. No HSV DNA was detected in five samples, four had low levels (2×10^2 – 6×10^3 DNA copies/ml) and one subject (J37), who was experiencing her first clinical episode, had 5×10^5 copies/ml (Table 1). None of the CVL had infectious virus recovered by plaque assay.

HSV inhibitory activity is increased during outbreaks, falls during treatment and then rebounds following cessation of ACV

The median percent inhibition of HSV was higher in CVL obtained from cases compared to controls at presentation (Day 0) (54.3 vs. 28; $p=0.20$), declined and was similar to controls on Day 7 (following ACV treatment) (25.5 vs. 29.6, $p=0.68$) and rebounded on Day 14 (69 vs. 25, $p=0.27$) (Fig. 1a). The percent inhibition correlated positively and significantly among cases and controls with HNP1-3 ($r=0.63$ and 0.64 , respectively), lactoferrin (0.63 and 0.67), IL-1 β (0.86 and 0.82) and IgG (0.67 and 0.70) (Table 2). The inhibitory activity also correlated significantly with MIP-1 α (0.78) among cases and with lysozyme (0.83), IL-6 (0.77), and IL-8 (0.82) among controls. Conversely, the percent inhibition correlated negatively (but not significantly) with the anti-inflammatory protein, SLPI (-0.30 and -0.28). These findings suggest that inflammatory responses are associated with endogenous anti-HSV activity.

Changes in soluble immune mediator concentrations during herpes outbreaks

We next explored whether the concentrations of soluble mediators differed between cases and controls at each visit. There was a trend towards higher levels of inflammatory proteins and peptides in CVL from cases compared to controls (Table 3). Specifically, cases had significantly higher levels of IL-1 α and IL-1 β relative to controls on Day 14, a time associated with a rebound in anti-HSV activity in cases (IL-1 α , $p=0.004$ and IL-1 β , $p=0.03$; (Fig. 1b, c). In contrast, significantly lower levels of SLPI were detected in cases on Day 0 ($p=0.02$) (Fig. 1d).

IFN- α was below the LOD cases and controls, whereas there was a trend towards increased IFN- γ for cases compared to controls ($p=0.23$, $p=0.16$, and $p=0.04$ on Days 0, 7 and 14,

respectively) (Table 3). These findings are consistent with a prior study demonstrating persistent expression of IFN- γ , but not Type 1 IFNs (e.g. IFN- α) in transcriptional analyses of sequential biopsy specimens of recurrent mucocutaneous HSV-2 lesions¹⁹.

Differences in activity against HIV and *E. coli* in CVL from cases compared to controls

Prior studies have shown that CVL are frequently bactericidal for *E. coli*^{12,20-22} and display variable activity against HIV^{12,20,21}. CVL from controls exhibited variable activity against HIV with the majority (17/30) enhancing HIV infection *ex vivo* (Fig. 2a). The enhancing activity correlated significantly with IL-1 α (0.68), MIP-1 α (0.71) and RANTES (0.72). In contrast, CVL from cases showed less variable activity and was modestly inhibitory against HIV (median 27.5 vs. -54.5 on Day 0, cases vs. controls, $p=0.002$; 36.5 vs. -7 on Day 7, $p=0.001$; and 31 vs. 11, $p=0.07$ on Day 14) (Fig. 2b). The inhibitory activity in the cases correlated positively and significantly with HBD-2 (0.75, $p=0.013$).

Conversely, the median bactericidal activity against *E. coli* was lower in cases compared to controls on Day 0, increased to a level similar to that observed in controls on Day 7, and decreased on Day 14 (47.4 vs. 78.5, $p=0.07$ on Day 0, 64.4 vs. 67.6, $p=0.51$ on Day 7 and 52.7 vs. 67.1, $p=0.19$ on Day 14) (Fig. 1e). Among cases, the bactericidal activity correlated negatively with IL-8 (-0.6, $p=0.12$), MIP-1 β (-0.66, $p=0.07$), RANTES (-0.54, $p=0.16$), and IL-1 α (-0.755, $p=0.03$), but not with any mediators among controls. These findings suggest that the mucosal responses that promote higher anti-HSV activity in cases are associated with reduced *E. coli* bactericidal activity.

Discussion

This is the first study to examine the soluble mucosal immune environment during symptomatic genital herpes outbreaks and following ACV treatment. The findings suggest that the *ex vivo* anti-HSV activity of CVL is increased at the time of an outbreak compared to HSV-seronegative controls, although the difference did not reach statistical significance. The anti-HSV activity measured likely underestimates the local inhibitory activity, as CVL represents an ~50-fold dilution of genital secretions^{23,24}. Possibly, the increased activity observed in cases reflects a local host response to prevent virus spreading to the upper genital tract. Indeed, although virus may be detected in the cervix by PCR, HSV DNA is more frequently isolated from the lower genital tract, and cervical disease is uncommon^{16,25}. In this study, only five of 10 participants had detectable CVL HSV DNA when presenting with external genital lesions.

The anti-HSV activity fell in cases during ACV treatment (Day 7) to levels comparable to that observed in HSV-seronegative controls, and then rebounded on Day 14. Concentrations of pro-inflammatory mediators followed a similar temporal pattern (Fig. 1 b-d). To further explore the rebound in HSV inhibitory activity on Day 14, we analyzed data from asymptomatic, HSV-2 seropositive subjects at a single time point (baseline) who were not being treated with antivirals ($n=16$). These women had participated in IRB-approved microbicide safety studies. The CVL anti-HSV activity in this asymptomatic cohort was similar to that observed on Day 14 in the cases (median 47% vs. 69%, respectively; $p=0.38$) and higher than that observed in HSV-2-seronegative controls (47% vs. 25%, respectively; $p=0.08$). This suggests that the rebound observed on Day 14 may reflect a return to the steady state in HSV-seropositive women, independent of recent lesions. The higher anti-HSV activity in the cases and asymptomatic HSV-2 seropositive women may represent a mucosal response to subclinical viral shedding, which was likely suppressed by ACV treatment. Larger studies with more frequent sampling in symptomatic and asymptomatic women are needed to determine whether there is an association between subclinical shedding and CVL inhibitory activity.

The anti-HSV activity correlated with several pro-inflammatory, antimicrobial proteins and IgG, which is consistent with prior studies^{10,11,27}. Whether these mediators directly contribute to the CVL anti-HSV activity or reflect a biomarker of other unidentified antiviral factors will require fractionation and depletion studies. Possibly, HSV-specific antibodies, which were not evaluated previously reflecting the lack of established assays, contribute to the CVL inhibitory activity in cases. Efforts to specifically deplete CVL of immunoglobulin with Protein A were unsuccessful, as other proteins including HNP1-3 were also lost. In ongoing studies, we are adapting a commercial ELISA (GenWay Biotech, San Diego, CA) designed to measure HSV-specific IgG in blood to measure antibodies in CVL. HSV-specific IgG was detected from three of 10 cases (Day 0 samples), three of 14 asymptomatic HSV-2 seropositive subjects, and none of the controls. There was no significant difference in anti-HSV activity when comparing subjects with detectable to those without detectable CVL HSV-specific IgG ($p=0.67$), suggesting that HSV-specific IgG contributes little to CVL inhibitory activity. Larger studies including measurements of mucosal HSV-specific IgG and IgA are needed.

Paradoxically, we observed that, compared to controls, CVL from cases were more likely to exhibit HIV inhibitory activity, although HSV recurrences have been associated with increased HIV shedding and an increased risk of HIV infection^{4,28,29}. Perhaps, the inflammatory response to HSV overcomes the modest protective effect of CVL against HIV. The inflammatory response may recruit T cells to the dermal-epidermal junction, which could serve as HIV targets if the virus traverses the keratinized skin barrier, perhaps weakened by HSV-induced disruption of the epithelial barrier.

Protease inhibitors may also provide antiviral and anti-inflammatory activity. SLPI was present at significantly lower concentrations in CVL from cases compared to controls on Day 0. This observation is consistent with the *in vitro* finding that HSV immediate early proteins down-regulate SLPI expression in epithelial cells and keratinocytes³⁰. Whether the concentrations of other antiproteases were altered in CVL and how this contributes to CVL anti-HIV activity, and ultimately, to the risk of HIV infection, requires additional studies. A proteomic study found that antiproteases, including serpins and cystatins, were present at higher concentrations in CVL from HIV-highly exposed seronegative women compared to uninfected controls and correlated positively and significantly with pro-inflammatory cytokines³¹. The authors speculated that antiproteases might control inflammatory responses and provide a more potent antiviral mucosal environment, although they did not measure anti-HIV activity. In this study, HBD-2 correlated positively and significantly with CVL anti-HIV activity, but only among cases. A recent study also found that HBD-2 correlated with CVL anti-HIV activity of CVL, but only among HIV-infected women, not among healthy controls¹².

Conversely, while CVL anti-HSV and anti-HIV activity was increased in cases, the bactericidal activity against *E. coli* was reduced. These findings indicate that different molecules contribute to the activity against different pathogens. Moreover, the reduction in *E. coli* bactericidal activity during an outbreak is consistent with the epidemiological link between BV and HSV^{32,33}. Vaginal fluid from women with BV exhibited reduced inhibitory activity against *E. coli*, which was partially restored after metronidazole treatment³⁴. A substantial percentage of women with microbiota changes consistent with BV are asymptomatic by Amsel clinical criteria^{35,36} and we did not assess vaginal microbiota populations in this study. We speculate that changes in vaginal bacteria including an increase in *E. coli* colonization, loss of protective lactobacilli and/or an increase in anaerobic species may have characterized the microenvironment in the cases^{35,37}.

In summary, despite several limitations, including small sample size, lack of parallel samples from asymptomatic HSV-2 seropositive women, infrequent sampling for silent HSV reactivation, and lack of specimens just prior to lesion appearance (when HSV replication may be highest), the findings obtained indicate that genital outbreaks are associated with increases in pro-inflammatory mediators and greater CVL anti-HSV activity compared to HSV seronegative controls. We speculate that these mucosal responses may limit HSV spread and prevent viral ascension into the upper genital tract, but may also paradoxically increase HIV acquisition risk. While the concentrations of inflammatory proteins and anti-HSV activity fell during treatment, they returned to pretreatment levels following ACV cessation. The rebound to a more inflammatory mucosal environment may contribute to the clinical observation that HSV-2 seropositivity is a risk factor for HIV acquisition, independent of overt herpetic lesions^{6,38-44}. These findings underscore the need to further investigate subclinical shedding and its impact on mucosal immunity and HIV risk and to optimize strategies to suppress HSV reactivation.

Acknowledgments

We thank Grace Chow for clinical operations support, Lydia Soto-Torres and James Turpin for advice, Erin Diamant, Anna Lee, Julie Petrie, and Tara Ford for assistance in recruiting subjects and Richard Henry, Momka Narliev and Amy Fox in the Clinical Virology Lab at Montefiore Medical Center.

This work was supported by R01AI065309, K23AI089271, UL1RR02570, P30AI051519, and T32AI070117. The contents of this publication are solely the responsibility of the authors and do

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Figure 1. HSV recurrences are associated with an increase in HSV inhibitory activity, increased pro-inflammatory cytokines, decreased anti-inflammatory mediators and reduced *E. coli* bactericidal activity in genital tract secretions collected by lavage

Box-and-whisker plots showing the percent inhibition of HSV-2 plaque formation (a); the log-transformed concentrations of IL-1 α (b), IL-1 β (c), and SLPI (d) and percentage inhibition of *E. coli* (e) in CVL samples for controls (white) and cases (grey) at enrollment (Day 0), Day 7 and Day 14. The line indicates the median values and the circles are outliers. The asterisks denote significant differences between cases and controls.

Figure 2. CVL exhibit variable HIV inhibitory or enhancing activity

Individual CVL samples or control buffer were mixed with HIV-1_{BaL} prior to infecting TZM-bl cells and infection monitored by luciferase activity. Results are presented as histograms of the percentage inhibition (positive values) or enhancement (negative values) of HIV infection relative to control buffer.

Table 1
Demographic and medical history of all participants (upper panel) and current presentation and HSV history for cases (lower panel)

	Controls n=10	Cases n=10	p value
Age (mean + SD years)	28.6 + 5.3	29.7 + 7.4	0.64
Race (number, %)			0.96
Black	4 (40%)	3 (30%)	
White	2 (20%)	2 (20%)	
Asian	1 (10%)	1 (10%)	
Mixed	3 (30%)	4 (40%)	
Ethnicity			
Hispanic	2 (20%)	4 (40%)	
Non-Hispanic	8 (80%)	6 (60%)	
Level of Education			0.46
High school/GED	0	1 (10%)	
Some college	4 (40%)	5 (50%)	
College	2 (20%)	3 (30%)	
Graduate/Professional degree	2 (20%)	1 (10%)	
Some post-college	2 (20%)	0	
Number lifetime sex partners			
Median (range)	6.5 (1-12)	4.5 (1-20)	0.9
Reported history of anal sex	4 (40%)	4 (40%)	1.0
Reported history of receptive oral sex	8 (80%)	10 (100%)	0.47
Current cigarette smoker	0	1 (10%)	1.0
Tampon use	8 (80%)	6 (60%)	0.63
History of douching	0	2 (20%)	0.47
Current contraceptive method*			0.57

	Controls n=10	Cases n=10	p value
Male condoms	4	4	
Female condoms			
Tubal ligation	1	0	
Diaphragm cap	0	1	
withdrawal	0	1	
Hormonal	1	1	
Prior history of vaginitis			0.21
Candida vaginitis	7	8	
Bacterial vaginosis	0	2	
Prior history of STI			0.3
Trichomonas	0	1	
Chlamydia	1	2	
Gonorrhea	0	1	

*More than 1 method may have been reported

Cases	Serostatus		History			Current Presentation			
	HSV-1	HSV-2	Oral HSV	Duration of genital HSV (months)	# annual recurrences	Lesion Site	Culture Results	DNA Copy/Swab	DNAcopies/ml (CVL)
J3	-	+	No	24	4	Labia	HSV-2	4,173	220
J15	+	+	Yes	18	12	Labia	HSV-2	515,173	0
J17	-	+	No	26	24	Labia	HSV-2	12,340	0
J18	-	+	No	2	3 (total)	Labia	HSV-2	1,241,708	6,440
J19	-	+	Yes	10	10	Labia	HSV-2	946,069	3,212
J24*	+	+	No	1st episode		Labia	HSV-2	83,649	3,419
J29	+	+	Yes	216	24	Labia	HSV-2	NA	0
J34	+	+	No	16	4	Labia	HSV-2	7,424	0
J37	+	-	No	1st episode		Labia	HSV-1	76,970	551,410
J38	+	+	No	24	6	Mons	HSV-1	21,169,294	0

* All participants were naturally cycling except J24 who was receiving hormonal contraception

NA: not available; no swab collected for PCR at enrollment.

Table 2
Spearman's correlation coefficients (SCC) for anti-HSV activity and mediators

Mediator	Control subjects		Case subjects	
	σ	P value	σ	P value
Protein	0.16	0.66	0.16	0.68
HNP1-3	0.64	0.05	0.63	0.05
Lysozyme	0.83	0.01	0.56	0.10
Lactoferrin	0.67	0.04	0.63	0.05
IL-1 α	0.1	0.81	0.70	0.05
IL-1 β	0.82	0.01	0.86	0.001
IL-6	0.77	0.01	0.46	0.19
IL-8	0.82	0.01	0.15	0.68
MIP-1 α	0.58	0.08	0.72	0.02
MIP-1 β	0.63	0.06	0.17	0.64
RANTES	0.41	0.23	0.19	0.60
SLPI	-0.28	0.43	-0.30	0.40
IL-1ra	0	1	-0.08	0.83
IgA	0.28	0.46	0.48	0.16
IgG	0.70	0.03	0.67	0.04
HBD-2	-0.27	0.45	0.22	0.54
HBD-3	-0.58	0.09	-0.11	0.76
IFN δ	0.20	0.58	0.36	0.31

σ and respective p values refer to the SCC associating anti-HSV activity with each mediator on Day 0 for cases and controls

Table 3
Median concentrations and interquartile ranges of mucosal immune mediators*

Mediator	Controls D0	Cases D0	Controls D7	Cases D7	Controls D14	Cases D14
Protein (ng/ml)	233 (146.8-300.3)	271 (139.5-574.3) p=0.69	192.5 (81.3-425.3)	347 (196-493.4) p=0.20	158 (130-245.8)	275 (145.5-631) p=0.25
IL-1 α (pg/ml)	20.6 (16.9-60.7)	153.5 (27.8-176) p=0.13	32 (22.7-49.3)	117 (35.3-1108) p=0.16	37.5 (15.9-44.7)	151 (65.5-578.5) p=0.004
IL-1 β (pg/ml)	1.5 (0.2-11.2)	2.5 (1.2-26.1) p=0.43	1.6 (0.4-3.4)	3.0 (0.2-55) p=0.28	1.2 (0.2-16.8)	5.4 (3.4-66) p=0.03
IL-6 (pg/ml)	4.1 (1.5-34.8)	12.5 (0.7-26.5) p=0.85	4.7 (2.1-14)	11.5 (1-23.8) p=0.82	8.6 (4.1-55.6)	17.0 (3-26.5) p=0.85
IL-8 (pg/ml)	188.1 (78.9-826.5)	348.7 (24.5-1283) p=0.76	184.7 (139.4-294)	580 (80.5-1955) p=0.16	223.1 (95.9-383)	663 (234.5-2445) p=0.07
MIP-1 α (pg/ml)	2.9 (1.8-12.8)	6.0 (1.8-19.5) p=0.65	8.1 (1.8-16.6)	3.9 (1.8-21.3) p=0.80	4.3 (1.8-8.8)	7.3 (4.5-27.5) p=0.10
MIP-1 β (pg/ml)	2.3 (2.3-23.3)	2.3 (2.3-17) p=0.73	6.9 (2.3-11.4)	5.3 (2.3-16.3) p=0.90	5.2 (2.3-9.4)	5.3 (2.3-24.5) p=0.43
RANTES (pg/ml)	2.3 (0.5-9.4)	3.0 (1.5-8) p=0.65	2.9 (1.9-5.3)	3.4 (1.6-17) p=0.54	4.2 (1.8-40.5)	4.0 (2.8-13.5) p=0.90
IFN- γ (pg/ml)	0.6 (0.2-2.1)	1.5 (0.5-3.5) p=0.23	0.5 (0.1-1.8)	0.8 (0.5-2.8) p=0.16	0.5 (0.1-0.9)	0.9 (0.5-3.5) p=0.04
IL-1 α (pg/ml)	541.5 (2131-7550)	9571 (413.6-14091) p=0.76	6325 (1704-9177)	8548 (4599-13532) p=0.72	6123 (1671-6988)	13879 (11484-16340) p=0.07
SLPI (ng/ml)	753 (384-2100)	179 (36-298) p=0.02	132 (78-221)	140 (60-196) p=0.43	119 (40-299)	129 (77-178) p=0.29
HNPI-3 (ng/ml)	57.4 (17-941)	97 (26-531)	59.6 (20-350)	99 (14-488)	84.7 (33-481)	94.4 (19-365)
HBD-1 (pg/ml)	3016 (940.5-4884)	881.9 (334.8-1596) p=0.14	1217 (737.2-2252)	873.5 (386-2879) p=0.73	1455 (644.8-2690)	528.7 (331.8-1459) p=0.41
HBD-2 (pg/ml)	1918 (225.3-7707)	3466 (414.7-9033) p=0.92	912.5 (164.3-6448)	2543 (172.2-5569) p=0.98	516.9 (48.6-5357)	1543 (192-2032) p=0.47
HBD-3 (pg/ml)	195.6 (125-1547)	1029 (296.2-2400) p=0.49	1268 (125-4329)	1542 (150.2-3185) p=0.07	125 (125-5686)	315.4 (30-1902) p=0.66
Lactoferrin (ng/ml)	376 (87.3-1751)	540.5 (173.3-1501) p=0.71	197.5 (91-365.8)	347 (147.3-856.5) p=0.11	218.5 (102.3-471.3)	296 (115-1347) p=0.69
Lysozyme (ng/ml)	95.5 (72.5-298.8)	166.5 (82-653.3) p=0.40	88 (79.3-442.5)	106.5 (85.3-420) p=0.71	118.5 (69.8-261)	212 (95.5-925) p=0.19
IgA (ng/ml)	1806 (593.9-3300)	2843 (710.8-13380) p=0.28	834.9 (331.4-1305)	3702 (897.6-12409) p=0.01	2485 (659.3-4657)	7297 (1261-20849) p=0.09
IgG (ng/ml)	3864 (1921-18168)	7226 (4465-16850) p=0.71	3343 (1493-5139)	5650 (2863-11870) p=0.78	4196 (1867-8972)	9540 (3657-28799) p=0.22
Mean vaginal pH (SD)	4.62 (0.2)	4.70 (0.5) p=0.64	4.79 (0.3)	4.70 (0.3) p=0.59	4.71 (0.4)	4.89 (0.4) p=0.08

* P values reflect comparison of log10-transformed variables by Student's t test or Wilcoxon rank sum test