Cervicovaginal Secretions Contribute to Innate Resistance to Herpes Simplex Virus Infection

Minnie John,[1,a] Marla J. Keller,[2,a] Ehsan H. Fam,[1] Natalia Cheshenko,[1] Kathleen Hogarty,[2] Andrea Kasowitz,[2] Sylvan Wallenstein,[3] Maria J. Carlucci,[1] Ana C. Tuyama,[1] Wuyuan Lu,[4] Mary E. Klotman,[2] Robert I. Lehrer,[5] and Betsy C. Herold[1]

Departments of [1]Pediatrics, [2]Medicine, and [3]Community Medicine, Mount Sinai School of Medicine, New York, New York; [4]Institute of Human Virology, University of Maryland Biotechnology Institute, Baltimore; [5]Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles

Defining and preserving the innate antiviral activity found in cervicovaginal secretions is critical. Cervicovaginal lavage (CVL) samples were obtained from 20 healthy women and evaluated for anti–herpes simplex virus (HSV) activity. CVL samples reduced HSV-2 yields by 23-fold (median), and the anti-HSV activity of CVL samples correlated with the concentration of human neutrophil peptides (HNP)–1–3. Both CVL samples and HNP-1–3 interacted with virus and prevented entry after binding. Substantially less protective activity was observed in CVL samples obtained from 20 human immunodeficiency virus—infected subjects, but the addition of CVL samples from healthy subjects enhanced the antiviral activity. The significance of the innate activity was further demonstrated by showing that CVL samples prevented murine genital herpes. Fourteen of 15 mice were protected from genital herpes if they were challenged with HSV-2 pretreated with CVL samples from healthy subjects. In contrast, all 15 mice challenged with untreated HSV-2 died. These findings are evidence that cervicovaginal secretions contribute to innate resistance to HSV-2 and identify defensins as contributors to this activity.

Herpes simplex virus (HSV)–2 is the most frequent cause of genital ulcer disease globally [1–4]. Considerable attention is being directed to developing topical microbicides that can reduce transmission of HSV and other sexually transmitted infections. Although the cervicovaginal environment is believed to act as a barrier to sexually transmitted infections, this has not been systematically evaluated. Identifying the components of cervicovaginal secretions that contribute to innate defenses is important, both for understanding the pathogenesis of sexually transmitted infections and to facilitate the design of microbicides that augment these immune defenses.

Among cervicovaginal proteins that may protect against infections are defensins and secretory leukocyte protease inhibitor (SLPI). Human defensins include neutrophil peptides (HNP-1–4), epithelial-cell α-defensins HD-5 and HD-6, and ~30 β-defensins [5]. Although defensins are present in cervicovaginal secretions and in vitro studies have indicated that HNP-1–3 inhibit HSV infection at an early step after binding [6, 7], the anti-HSV activity of cervicovaginal secretions has not been reported. SLPI, which is secreted by mucosal epithelial cells, inhibits HIV-1 infection in vitro, but its activity against HSV is not known [8]. To determine the ability of cervicovaginal secretions to protect against HSV infection, we tested the activity of cervicovaginal lavage (CVL) samples from 20 healthy women against HSV-2 and explored the contributions of HNP-1–3 and SLPI to the observed anti-HSV activity. Given the epidemiological link between HIV-1 and frequent genital HSV recurrences, we also assessed the

Received 29 April 2005; accepted 9 June 2005; electronically published 13 October 2005.

Presented in part: 42nd Annual Meeting of the Infectious Diseases Society of America, 30 September–3 October 2004, Boston (abstract 817).

Financial support: National Institutes of Health (grants AI-37940, HD-43733, HD-41763, and RR-00071 and training grant AI-07623 to M.J.).

Potential conflicts of interest: none reported.

[a] M.J. and M.J.K. contributed equally to the work.

Reprints or correspondence: Dr. Betsy C. Herold, Mount Sinai School of Medicine, 1 Gustave L. Levy Pl., Box 1657, New York, NY 10029 (betsy.herold@mssm.edu).

The Journal of Infectious Diseases 2005; 192:1731–40

0022-1899/2005/19210-0010$15.00

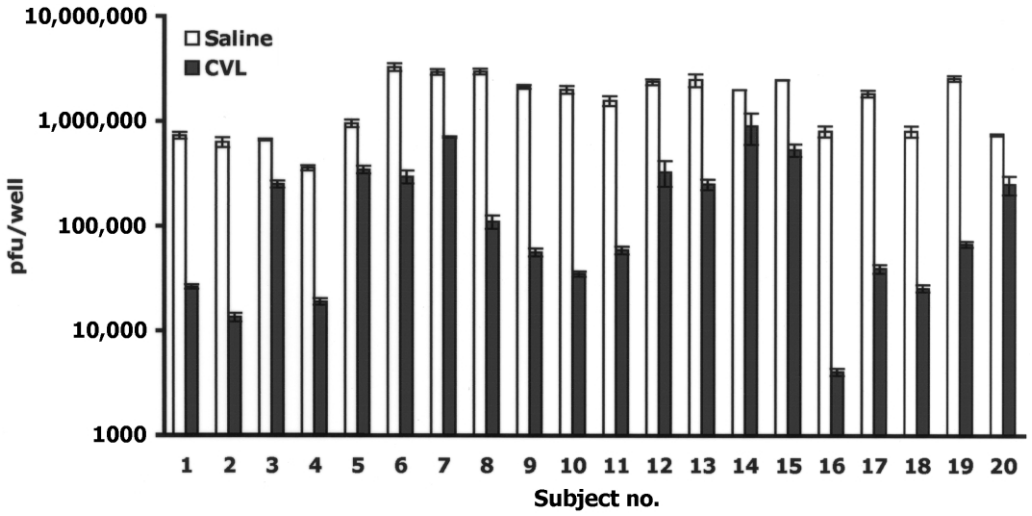

Figure 1. Virus yields in the presence of cervicovaginal lavage (CVL) samples. CaSki cells were exposed to CVL samples or control buffer (saline [pH 5.0] that contained 200 μg/mL bovine serum albumin) for 1 h at 37°C and then challenged with serial 10-fold dilutions of herpes simplex virus (0.01–1000 pfu/cell). The virus titer (in plaque-forming units per well) in the presence of CVL samples or control buffer was determined by counting plaques 48 h after infection after immunostaining by black plaque assay. Results are means ± SDs from triplicate wells.

anti-HSV activity of CVL samples collected from 20 HIV-1–infected women who were being evaluated for participation in an unrelated study.

SUBJECTS AND METHODS

Subjects. With informed consent, and in accordance with human experimentation guidelines of Mount Sinai School of Medicine, CVL samples were obtained from 20 healthy and 20 HIV-1–infected subjects. The healthy women had 2 visits, 14 days apart, to ascertain whether anti-HSV activity in CVL samples varies over time. All subjects were asked to abstain from sex and the use of intravaginal products for 48 h before each visit.

Assessment for sexually transmitted infections and CVL. Healthy subjects had HIV-1 (ELISA) and HSV serological tests (type common and type specific; Herpes Select; Focus Technologies). All participants had a urinalysis and culture, pregnancy test, and gynecological examination for bacterial vaginosis, *Trichomonas vaginalis,* and *Candida* species. *Neisseria gonorrhoeae* and *Chlamydia trachomatis* were tested by DNA probe. All healthy participants had a Pap smear done at the initial visit; HIV-1–infected subjects had a documented normal or atypical squamous cells of undetermined significance Pap smear result within 6 months of participation. HIV-1 plasma RNA was determined by reverse-transcription polymerase chain reaction (Amplicor HIV-1 Monitor, version 1.5; Roche) within 2 months of participation. CVL was done by washing the cervicovaginal area with ~10 mL of sterile normal saline (pH ~5.0); CVL samples were transported on ice and centrifuged at ~1000 *g* for 10–15 min at 4°C. A mixture of antibiotics (final concentrations: penicillin, 500 U/mL; streptomycin, 50 μg/mL; amphotericin, 0.5 μg/mL) was added to the supernatants, which were subdivided and stored at −80°C. The protein concentration (BCA Protein Kit; Pierce) and pH of each sample were determined. For studies involving antiviral mechanisms and for murine studies, CVL samples from 3–6 subjects were pooled.

Cells and viruses. CaSki (human cervical) and Vero (monkey kidney) epithelial cell lines were obtained from the American Type Culture Collection [9]. The HSV-2 strains were HSV-2(G), a well-characterized laboratory strain; WTW1A, a clinical isolate (obtained from P. Spear, Northwestern University, Evanston, IL) [10]; and C1 and C3, clinical isolates from the Mount Sinai Clinical Microbiology Laboratory. Clinical isolates were passed ⩽3 times in Vero cells before study.

Reagents and cytotoxicity assays. HNP-1–3 was purified from normal human neutrophils [11]. Alternatively, HNP-1, -2, and -3 were individually synthesized [12]. Recombinant SLPI (R&D Systems), heparin (Sigma), and PRO 2000 (Indevus Pharmaceuticals) were included as controls in selected experiments. Concentrations in CVL samples were determined by ELISA for HNP-1–3 (Cell Sciences) and SLPI (R&D Systems). To determine whether CVL samples interfered with the ELISA, recombinant standards were diluted in pooled CVL samples, and results were compared with those obtained with standards diluted in the kit diluent. The impact of recombinant SLPI or HNP-1–3 on cell proliferation and viability was determined by the CellTiter 96 AQ_{ueous} cell proliferation assay (Promega).

Plaque assays. CaSki cells, plated in triplicate in 96-well

Table 1. Characteristics of healthy subjects in a study of the activity against herpes simplex virus (HSV) of cervicovaginal secretions obtained by cervicovaginal lavage (CVL).

Subject no.	Age, years	Race/ethnicity	Vaginitis	HSV serum antibody	Birth control	Fold reduction[a]		HNP-1–3, ng/mL (day)	SLPI, ng/mL (day)
						Day 1	Day 14		
1	30	Black	…	Negative	Condom	28	5	13 ± 6 (14)	86.5 ± 7 (14)
2	24	Asian	*Candida*[b]	Negative	OCP	47	8	16 ± 1 (14)	168 ± 26 (14)
3	24	White	…	Negative	OCP	3	5	11 ± 1 (1)	130.6 ± 18 (1)
4	34	White	*Candida*[b]	Negative	OCP	19	37	82 ± 13 (1)	150.5 ± 5 (1)
5	31	Black	*Candida*[b]	Negative	BTL	3	6	2 ±1 (14)	30 ± 6 (14)
6	31	Asian	…	Negative	OCP	11	10	11 ± 1 (14)	51 ± 10 (14)
7	25	Black	…	Negative	Condom	4	17	5 ± 0.4 (1)	22 ± 0.5 (1)
8	28	Black/Native American	*Candida*[c]	Negative	OCP	27	19	41 ± 8 (1)	77 ± 1 (1)
9	23	Hispanic	…	HSV-1	OCP	38	34	101 ± 5 (1)	90 ± 17 (1)
10	30	White	…	HSV-1	OCP	57	50	89 ± 9 (1)	160 ± 8 (1)
11	22	White	…	Negative	OCP	27	8	38 ± 11 (1)	99 ± 12 (1)
12	39	Black	Bacterial vaginosis[c]	HSV-1 and -2	None	7	4	61 ± 7 (14)	32 ± 0.25 (14)
13	26	White	…	Negative	OCP	10	10	50 ± 6 (14)	24 ± 2.4 (14)
14	31	White	…	HSV-1 and -2	Condom	2	9	3 ± 0.6 (1)	284 ± 52 (1)
15	23	White	…	Negative	OCP	5	4	85 ± 9 (1)	92 ± 0.2 (1)
16	25	White	…	HSV-1	Condom	199	117	2162 ± 23 (1)	107 ± 0.7 (1)
17	24	White	*Candida*[c]	Negative	Condom	47	2	6 ± 2 (14)	185 ± 2 (14)
18	22	White	Bacterial vaginosis[c]	HSV-1	OCP	32	98	39 ± 6 (14)	184 ± 0.7 (14)
19	29	White	…	HSV-1	OCP	38	11	305 ± 15 (14)	392 ± 54 (14)
20	25	White	…	HSV-1	OCP	3	10	14 ± 0.2 (1)	67 ± .04 (1)

NOTE. Concentrations of human neutrophil peptide (HNP) correlated significantly with fold reduction in HSV-2 titer ($r = 0.67$; $P = .011$, Spearman's correlation coefficient). In contrast, the concentration of secretory leukocyte protease inhibitor (SLPI) did not correlate with anti-HSV activity ($r = 0.1$; $P = .67$, Spearman's correlation coefficient). BTL, bilateral tubal ligation; OCP, oral contraceptive pills.

[a] HSV titer in the presence of control buffer/titer in presence of CVL samples. Cells were pretreated with CVL samples or control buffer for 1 h before infection with serial dilutions of HSV-2 in triplicate; plaques were counted 48 h after infection, and the virus titer was determined.

[b] Observed at the first visit only.

[c] Organisms consistent with bacterial vaginosis; *Candida* noted on the Pap smear, from the first visit only.

dishes, were exposed to CVL samples or control fluid (saline [pH 5.0] that contained 200 μg/mL bovine serum adjuvant), 60 μL/well, for 1 h at 37°C and were then challenged with serial 10-fold dilutions of HSV-2 (0.01–1000 pfu/cell) (60 μL/well). After 2 h, the inoculum and CVL samples or control fluid were removed and replaced by medium 199 supplemented with 1% fetal bovine serum and 0.1% pooled human γ-globulin (199-O). Virus titers were determined by counting plaques 48 h after infection by immunoassay using an anti–human IgG antibody peroxidase conjugate ("black plaque assay"; Calbiochem) [9]. Only data from wells with 25–100 plaques were used to calculate the titer. The ratio of the titer of HSV-2(G) in control fluid to the titer in CVL samples was defined as the fold reduction. To examine the anti-HSV activity of synthetic defensins and SLPI, CaSki cells (6-well dishes) were pretreated with various concentrations of each for 1 h before challenge with 200–300 pfu/well HSV-2(G).

Preincubation of virus or cells with CVL samples. To examine whether CVL fluid targets the viral envelope, cells, or both, 10^4 pfu/mL HSV-2(G) was preincubated with pooled CVL samples, HNP-1–3, SLPI, or control fluid for 1 h at 37°C. Each mixture was then diluted 50-fold to yield ~200 pfu/well on control plates and inoculated onto CaSki cells [13]. For comparison, diluted virus (~200 pfu) was preincubated for 1 h with the pooled CVL samples, HNP-1–3, SLPI, or control fluid, and the mixture was plated (without dilution) on cells. Alternatively, pooled CVL samples, HNP-1–3, SLPI, or control buffer was preincubated with cells for 1 h at 37°C and then either washed extensively or left intact before infection with HSV-2(G).

Binding. CaSki cells were grown in 6-well dishes and pretreated with CVL samples, HNP-1–3, heparin, or control fluid for 1 h at 37°C, washed, cooled to 4°C for 30 min, and then exposed to dextran gradient–purified HSV-2(G) (MOI, 1 pfu/cell) for ~5 h at 4°C [13–15]. The unbound virus was removed by washing 3 times with cold PBS. The cell-bound virus was analyzed by preparing Western blots of cell lysates and probing with anti–glycoprotein D monoclonal antibody (MAb) 1103 (Goodwin Institute) and anti–β-actin MAb (A5441; Sigma-Aldrich), to control for protein loading. Alternatively, CaSki cells pretreated as described above were exposed to recombinant glycoprotein B-2 for 1 h at 37°C [13]. Cells were washed, and the cell-bound glycoprotein was analyzed by probing Western

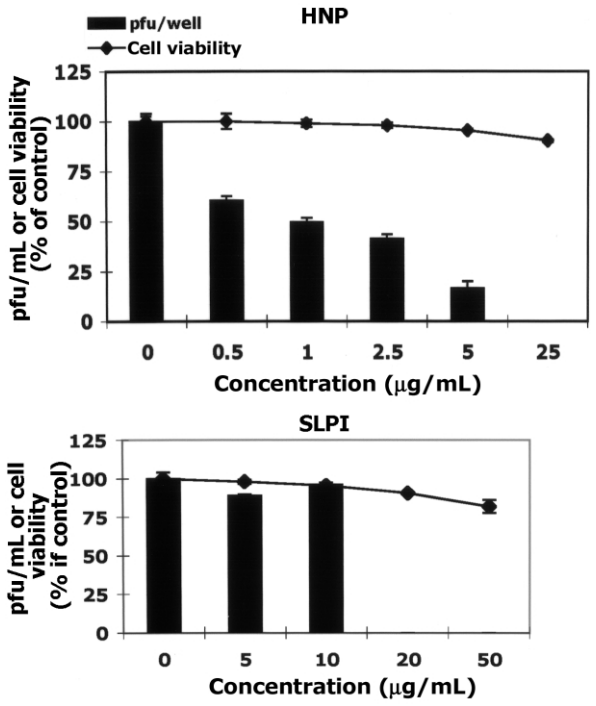

Figure 2. Impact of human neutrophil peptides (HNP)–1–3 and secretory leukocyte protease inhibitor (SLPI) on herpes simplex virus (HSV)–2 infection and cell proliferation. CaSki cells were exposed to a mixture of synthetic HNP-1, -2, and -3 (each added at the indicated concentrations, *top*) or SLPI *(bottom)* for 1 h and then challenged with HSV-2(G) (200–300 pfu/well). Plaques were quantified 48 h after infection, and results are presented as plaque-forming units formed in the presence of protein as a percentage of plaque-forming units formed in the presence of control medium. Each value is the mean ± SD of at least 3 independent experiments conducted in duplicate. In parallel plates, the impact of these mediators on cell proliferation and viability was determined by the CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay; results are presented as optical density units obtained from cells treated with the indicated concentration of each reagent as a percentage of optical density units obtained from cells incubated in control buffer. Each value is the mean ± SD of at least 3 independent experiments conducted in triplicate.

blots of cell lysates with anti–glycoprotein B MAb 1123 (Goodwin Institute). Blots were scanned and analyzed with the Bio-Rad GelDoc 2000 system.

Nuclear transport of viral tegument protein VP16. Cells were treated with CVL samples, HNP-1–3, heparin, or control fluid for 1 h at 37°C and then inoculated with HSV-2 (MOI, 1 pfu/cell). After 1 h, cells were washed with citrate buffer (pH 3.0) for 2 min, to inactivate nonpenetrated virus. The cells were then washed and overlaid with medium, and nuclear extracts were prepared 1 h after citrate treatment [16]. Western blots were prepared and probed with mouse anti-VP16 (1:500; Santa Cruz Biotechnology) for 1 h and then incubated with horseradish peroxidase–conjugated goat anti–mouse IgG (1:1000; Calbiochem) for 1 h. Blots were stripped, probed with MAb to nuclear pore complex protein (Mab414; Covance) to control for nuclear extraction and protein loading, scanned, and analyzed.

Detection of focal adhesion kinase (FAK) phosphorylation. CaSki cells (12-well plates) were preincubated with serum-free medium for 24 h before infection and then treated for 1 h with pooled CVL samples from healthy or HIV-1–infected subjects, control fluid, HNP-1, or PRO 2000 and then challenged with HSV-2(G) (MOI, 1 pfu/cell). Mock-infected cells were included as a control. Cell lysates were prepared 5 min after infection. Immunoblots were prepared, and membranes were incubated with a 1:1000 dilution of rabbit anti-FAK[pY^{397}], which recognizes the tyrosine autophosphorylation site 397 of FAK (44-624G; Biosource International). The membranes were then stripped and reincubated with a 1:1000 dilution of mouse anti-FAK MAb (F-15020; Transduction Laboratories), which recognizes total FAK.

Serum sensitivity of CVL samples. To determine whether the antiviral activity was sensitive to serum, 10% human serum from an HSV-seronegative donor was added to the CVL samples, HNP, SLPI, heparin, PRO 2000, or control fluid before plaque-reduction assays were conducted.

Mouse model of genital herpes. Murine studies were conducted with the approval of the Mount Sinai School of Medicine Institutional Animal Care and Use Committee. Female BALB/c mice (weight, 18–21 g; age, 8–10 weeks) were pretreated with 2 mg/mL medroxyprogesterone acetate subcutaneously 5 days be-

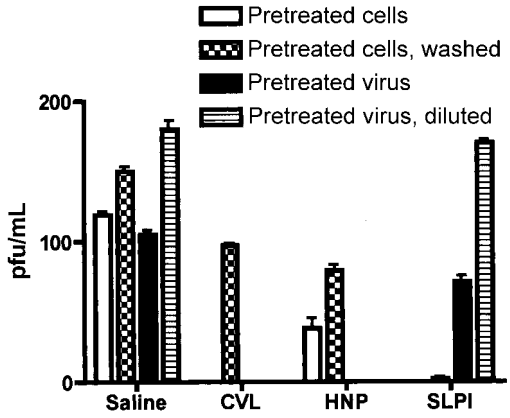

Figure 3. Irreversible inactivation of herpes simplex virus–2 by cervicovaginal secretions. CaSki cells were pretreated with control buffer, pooled cervicovaginal lavage (CVL) samples, human neutrophil peptide (HNP)–1–3 (25 μg/mL), or secretory leukocyte protease inhibitor (SLPI; 20 μg/mL) for 1 h at 37°C and then either washed extensively or left intact before infection with 200–300 pfu/well HSV-2(G). Alternatively, ~10^4 pfu/mL HSV-2(G) was incubated with each of the reagents for 1 h at 37°C and the mixture diluted 50-fold to yield ~200 pfu/well on control plates and inoculated onto monolayers of CaSki cells. For comparison, diluted virus (~200 pfu) was preincubated for 1 h with the reagents, and the mixture was plated (without dilution) on cells. Results are means ± SDs from triplicate wells and are representative of 4 independent experiments.

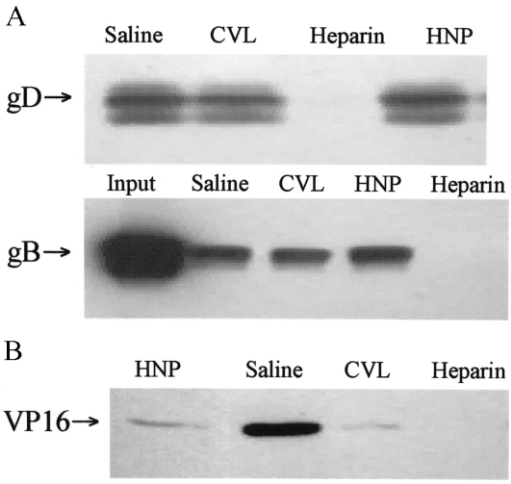

Figure 4. Effects of cervicovaginal lavage (CVL) samples and human neutrophil peptide (HNP)–1–3 on binding of herpes simplex virus (HSV)-2 *(A)* or nuclear transport of viral tegument protein VP16 *(B). A,* CaSki cells were exposed to saline, pooled CVL samples, HNP-1–3 (25 μg/mL), or heparin (100 μg/mL) for 1 h at 37°C and then shifted to 4°C and exposed to purified HSV-2(G) (1 pfu/cell) for 5 h. Bound virus was detected by analyzing Western blots of cell lysates for glycoprotein D (gD; *top*). Alternatively, cells were treated with control saline, pooled CVL samples, HNP-1–3, or heparin for 1 h at 37°C and then exposed to recombinant glycoprotein B-2 (gB; 10^{-5} μg/cell). Bound gB was detected by analyzing Western blots of cell lysates *(bottom).* Blots are representative of 3 independent experiments. *B,* CaSki cells were pretreated with saline, CVL samples, HNP-1–3, or heparin for 1 h and then exposed to HSV-2(G), and nuclear extracts were prepared as described in Subjects and Methods. Shown is a representative blot probed for VP16, which is representative of 3 independent experiments.

fore intravaginal challenge with HSV-2(C3) (5 $\log_{10}$ pfu/mouse). The virus was preincubated for 1 h with pooled CVL samples, pooled CVL samples to which 10% human serum from an HSV-seronegative donor was added, or untreated virus. Inoculated mice were evaluated for 21 days for evidence of infection, including genital ulcers, perineal erythema, hair loss, and hind-limb paralysis. Mice were killed if severe ulceration or hind-limb paralysis developed. Vaginal washes were obtained on day 3 after infection to determine virus titers by plaque assay.

Statistical analysis. The geometric mean, obtained by computing the mean and confidence intervals for log-transformed data, was used to describe the fold reduction in virus titer in CVL samples, compared with that in the control buffer. The Wilcoxon nonparametric test with exact *P* values was used to determine whether anti-HSV activity in CVL samples varied significantly between the 2 visits of healthy subjects; if it varied significantly with contraceptive use, the presence of serum antibodies to HSV, or vaginal candidiasis; and whether the antiviral activity in CVL samples obtained from healthy subjects differed from that obtained from HIV-1–infected subjects. Spearman's correlation coefficients were used to determine whether antiviral activity in CVL samples correlated with concentrations of SLPI or HNP-1–3. Calculations were done with SAS software (version 9; SAS Institute).

RESULTS

Inhibition of HSV infection by CVL fluid from healthy subjects. HSV-2(G) infection was significantly reduced if cells had been cultured in the presence of CVL samples obtained from all 20 healthy subjects, compared with control buffer that had been matched for pH and protein concentration ($P < .0001$) (figure 1). The geometric mean fold reduction in HSV-2 yields in the presence of CVL samples relative to that in control samples was 15.1 (95% confidence interval [CI], 9–26-fold). The CVL samples also inhibited >90% of infection by 3 different clinical isolates of HSV-2 (WTW1A, C1, and C3; data not shown). To assess whether the antiviral activity fluctuated over time, a second CVL sample was obtained from each healthy subject 14 days later. The mean reduction in HSV-2 yields in the presence of the second CVL samples was 12.4-fold (95% CI, 8–12-fold). Although there was considerable variability for some subjects, overall, the day 1 reading was neither significantly higher nor lower than the day 14 value ($P = .23$) (table 1). The reasons for this variability and identifying what modifies the anti-HSV activity in CVL samples warrant further study.

The anti-HSV activity of CVL samples was independent of age, vaginal pH, the presence of *Candida* or bacterial vaginosis, and the presence or absence of serum antibodies to HSV (table 1). Although CVL samples from 40% (8/20) of subjects could

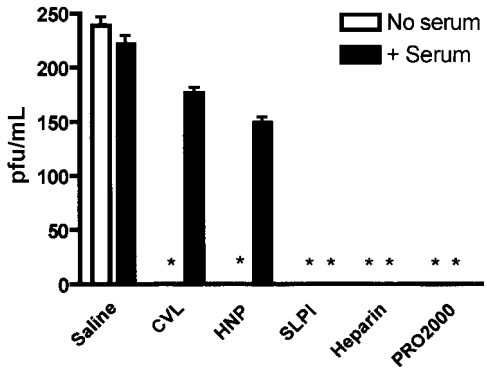

Figure 5. Reduced anti–herpes simplex virus (HSV) activity of cervicovaginal lavage (CVL) samples and human neutrophil protein (HNP), but not secretory leukocyte protease inhibitor (SLPI), heparin, or PRO 2000, in the presence of serum. CaSki cells were treated with pooled CVL samples, HNP-1–3 (25 μg/mL), SLPI (20 μg/mL), PRO 2000 (100 μg/mL), heparin (100 μg/mL), or control buffer that had been incubated for 1 h with 10% serum or saline and then challenged with 200–300 pfu/well HSV-2(G). Results are means ± SDs from triplicate wells and are representative of 3 independent experiments. *No visible plaques.

Table 2. Impact of cervicovaginal lavage (CVL) samples on herpes simplex virus (HSV)–2 infection and disease in mice.

Treatment of HSV-2 before infection	Disease free (mortality, %)	HSV-2 titer, mean ± SD, pfu/mL
Control (n = 15)	0 (100)	$5.5 \pm 3.9 \times 10^4$
Pooled CVL samples (n = 15)	14 (7)	0
CVL samples + 10% serum (n = 10)	0 (90)	$2.3 \pm 0.5 \times 10^5$

NOTE. Vaginal washes were obtained on day 3 after infection in 2 of 3 experiments (10 control mice and mice challenged with HSV-2 pretreated with pooled CVL samples and 5 mice challenged with HSV-2 pretreated with CVL samples + 10% serum). All control mice developed symptomatic disease and were killed between days 6 and 8 after infection. Only 1 mouse challenged with HSV-2 pretreated with pooled CVL samples developed symptomatic disease; vaginal washes were not obtained from that mouse. However, no virus was detected in any mouse in that group from which vaginal washes were obtained (n = 5). All mice challenged with HSV-2 pretreated with CVL samples + 10% serum developed moderate genital-tract symptoms 6 days after infection. The symptoms did not progress in 1 mouse, but the others developed severe genital-tract swelling, ulceration, and hair loss on days 7–9 and were killed.

have contained HSV-specific antibodies, it is unlikely that antibody contributed substantially to the anti-HSV activity. There were no differences in anti-HSV activity in CVL samples in women with or without serum HSV antibodies (P = .49). Furthermore, size fractionation of CVL samples demonstrated that the anti-HSV activity resides within the <50-kDa fraction (data not shown). Because many of the women were receiving oral contraceptive pills, the effects of the menstrual cycle on anti-HSV activity could not be addressed.

Candidate components for anti-HSV activity in CVL samples. Mucosal components that might contribute to anti-HSV activity include defensins and SLPI. To examine their ability to inhibit HSV infection in vitro, we pretreated CaSki cells with SLPI or a mixture of synthetic HNP-1, -2, and -3 for 1 h and then challenged the cells with 200–300 pfu of HSV-2(G)/well and counted plaques 48 h after challenge. Both SLPI and the α-defensins inhibited HSV-2 infection without inducing cytotoxicity at the concentrations tested (figure 2). SLPI

Table 3. Characteristics of HIV-infected subjects and anti-viral activity in their cervicovaginal lavage (CVL) samples.

Patient no.	Age, years	Race/ethnicity	Birth control	HIV-1 RNA, $\log_{10}$ copies/mL	$CD4^+$ cell count, cells/mm^3	Fold reduction[a]	HNP-1–3, ng/mL	SLPI, ng/mL
1	40	Hispanic	Condom	5.8	94	15	94 ± 6	185 ± 2
2	44	Black	Condom	4.7	125	1	1.4 ± 0	180 ± 3
3	34	Black	Condom	4.6	366	18	32 ± 2	134 ± 8
4	43	Hispanic	None	4.6	675	0.8	1 ± 0.02	15 ± 2
5	46	Hispanic	Condom	4.7	407	5	7.7 ± 1	150 ± 19
6	43	Hispanic	Condom	4.9	NA	4	23 ± 4	98 ± 7
7	35	Black	None	5.1	62	1.2	1.4 ± 0.06	25 ± 2
8	40	Black	Condom	5.1	151	1.5	15 ± 1	31 ± 5
9	39	Hispanic	BTL	5.1	122	1.8	1.9 ± 0.05	124 ± 15
10	37	Hispanic	BTL	4.4	264	1.5	2.6 ± 0.02	232 ± 24
11	47	Hispanic	Condom	5.0	264	0.7	2.85 ± 0.2	328 ± 19
12	42	Black	BTL	4.6	283	1.58	315 ± 25	593 ± 73
13	35	Hispanic	Condom	5.8	686	24.7	406 ± 9	131 ± 15
14	41	Black	BTL	5.9	8	1.3	173 ± 17	394.5 ± 5
15	36	Black	Condom	5.0	353	2.04	53.5 ± 0.44	360 ± 16
16	47	Black	Condom	5.0	188	2.3	16 ± 0.76	129 ± 10
17	31	Hispanic	None	5.1	183	3.5	601 ± 9	22 ± 4
18	34	Asian	None	5.3	525	4.7	31.8 ± 2.3	123 ± 2
19	29	Black	None	4.6	616	1.38	3.2 ± 1.2	675 ± 48
20	31	Black	Condom	4.2	375	8.8	4.5 ± 0.007	163 ± 9

NOTE. Concentrations of human neutrophil peptide (HNP) correlated significantly with fold reductions in herpes simplex virus (HSV)–2 titer (r = 0.59; P = .006, Spearman's correlation coefficient). In contrast, the concentration of secretory leukocyte protease inhibitor (SLPI) did not correlate with anti-HSV activity (r = −0.115; P = .63, Spearman's correlation coefficient). BTL, bilateral tubal ligation.

[a] Virus titer in presence of control buffer/titer in presence of CVL samples. Cells were pretreated with CVL samples or control buffer for 1 h before being inoculated with serial dilutions of HSV-2 in triplicate; plaques were counted 48 h after infection, and the virus titer was determined.

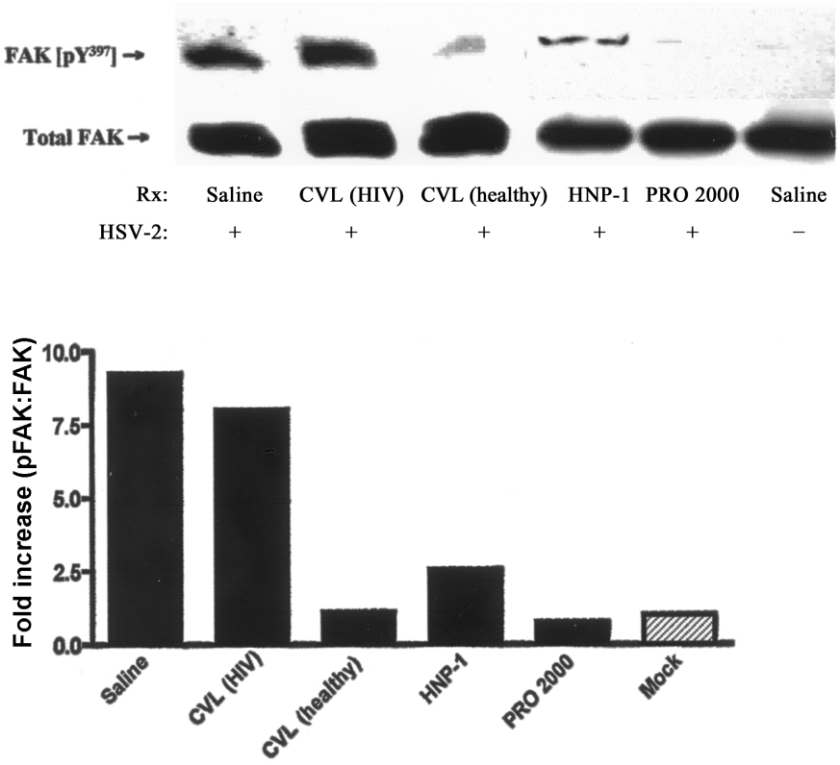

Figure 6. Blocking of herpes simplex virus (HSV)–induced focal adhesion kinase (FAK) phosphorylation by cervicovaginal lavage (CVL) fluid obtained from healthy subjects, but not that from HIV-1–infected subjects. CaSki cells were preincubated overnight with serum-free medium and then pretreated with saline or CVL samples pooled from HIV-1–infected or healthy subjects for 1 h and then challenged with HSV-2(G) (1 pfu/cell); 5 min after exposure, cell lysates were prepared, and proteins were separated, transferred by Western blotting, and incubated with anti–FAK pY397 antibody. Blots were then stripped and reprobed with monoclonal antibody to total FAK. The blots were scanned, and the results are expressed as the fold increase in levels of phosphorylated FAK as a percentage of total FAK, compared with mock-infected cells (Mock). Results are representative of 2 independent experiments. HNP-1, human neutrophil peptide–1.

and HNP-1–3 concentrations in 1 CVL sample from each participant were measured by ELISA (table 1). Notably, diluting the protein standards for each of the ELISAs in CVL samples did not interfere with protein recovery (data not shown). The HNP-1–3 concentrations correlated with anti-HSV activity ($r = 0.66$; $P = .0015$, Spearman's correlation coefficient), but SLPI concentrations did not. The correlation persisted when subject 16, who had the greatest anti-HSV activity and the highest concentration of HNP-1–3 in her CVL samples, was excluded from the analysis ($r = 0.53$; $P = .019$, Spearman's correlation coefficient).

Mechanism of anti-HSV activity. The anti-HSV activity persisted if virus was pretreated with CVL samples or HNP-1–3, followed by dilution, before cells were infected. In contrast, SLPI-mediated anti-HSV activity was retained when cells were exposed to SLPI and extensively washed but not when the virus was preincubated with SLPI followed by dilution of the mixture (figure 3). These findings suggest that CVL samples and HNP-1–3 preferentially target the virus to irreversibly inhibit HSV infection, whereas SLPI targets the epithelial cell.

We further examined how CVL samples and HNP-1–3 inhibit HSV infection, binding, and entry. Heparin, which was included as a positive control, is a competitive inhibitor of HSV binding. Its presence completely abolished virus binding at 4°C and recombinant glycoprotein B-2 binding at 37°C (figure 4*A*, *top* and *bottom*, respectively) [15]. In contrast, HNP-1–3 and CVL samples showed little or no ability to reduce binding in 3 independent experiments. In contrast, nuclear transport of the viral tegument protein VP16, a surrogate marker of virus entry, was substantially reduced by CVL samples or HNP-1–3 and (as was anticipated) by heparin (figure 4*B*). Together, these results suggest that CVL samples and HNP-1–3 inactivate HSV by preventing virus entry at a postbinding step.

HNP-1–3 bind to and are inactivated by serum proteins. To examine whether the anti-HSV activity of HNP-1–3 and/or CVL samples is inhibited by serum, virus was pretreated with CVL samples and HNP-1–3 with or without 10% serum, and viral infection was monitored by plaque assay. SLPI, heparin, and PRO 2000 were included for comparison. Results demonstrate that the anti-HSV activity of HNP-1–3 and CVL samples is substantially reduced in the presence of 10% serum but that the activity of SLPI, PRO 2000, or heparin is not (figure 5).

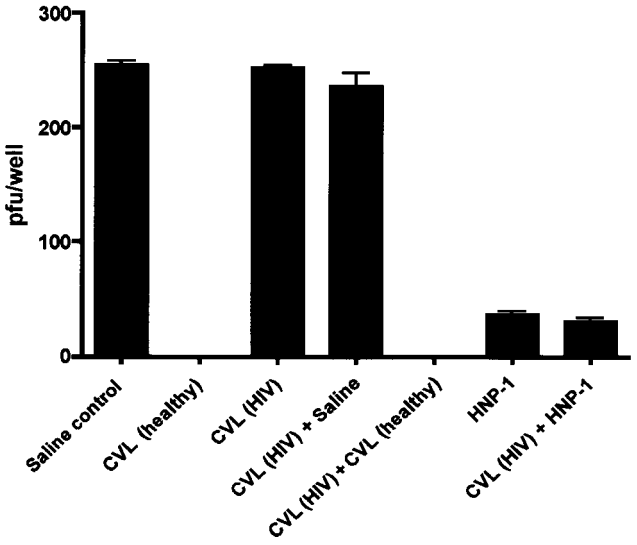

Figure 7. Mixing of cervicovaginal lavage (CVL) samples obtained from healthy participants with CVL samples obtained from HIV-1–infected subjects or addition of exogenous human neutrophil peptide (HNP)–1 caused an enhancement of anti–herpes simplex virus activity. CaSki cells were treated for 1 h with saline, CVL samples pooled from 3 healthy subjects, CVL samples pooled from 3 HIV-1–infected subjects, or the pooled CVL samples from HIV-1–infected subjects mixed 1:1 with saline or with pooled CVL samples from healthy subjects. Alternatively, cells were treated for 1 h with synthetic HNP-1 (50 μg/mL) diluted in control buffer or in CVL samples pooled from HIV-1–infected subjects. Cells were then challenged with 200–300 pfu/well, and infection was monitored by counting plaques 48 h after infection. Results are means ± SDs from duplicate wells and are representative of 3 independent experiments.

Protection of mice from genital herpesvirus infection by CVL fluid. A murine genital herpes model was adopted to determine whether the antiviral properties of CVL fluid could reduce in vivo viral pathogenicity. Mice inoculated intravaginally with HSV-2 that had been pretreated with pooled CVL samples were protected from infection (i.e., no virus detected in vaginal wash) and fatal disease (table 2). No protection was observed if the mice were inoculated with virus that was pretreated with CVL samples inactivated by the addition of 10% serum. These results support the in vitro findings that CVL fluid irreversibly inactivates HSV-2 and that the antiviral effect is neutralized in the presence of 10% serum.

Anti-HSV activity of CVL samples from HIV-1–infected subjects. Epidemiological evidence has demonstrated that HIV-1–infected persons coinfected with HSV have frequent recurrences of genital herpes [17]. Accordingly, we examined the anti-HSV activity in CVL samples obtained from a cohort of 20 subjects with HIV-1 infection (table 3). These women were being screened for participation in an unrelated study and differed from the healthy cohort with respect to age, race, and contraceptive methods (tables 1 and 3). In the HIV-1–infected subjects, the geometric mean of the fold reduction of HSV-2 was 5.04 (95% CI, 1.9–8.1-fold), which is significantly lower than that observed with CVL samples obtained from healthy women ($P < .001$).

To further explore the observed differences, we compared the effects of CVL samples from healthy or HIV-1–infected subjects on the virus-induced phosphorylation of FAK. HSV induces FAK phosphorylation within 5 min after exposure to penetration-competent virus, which renders this one of the earliest detectable markers of successful virus entry [18]. HSV induces a 9-fold increase in FAK phosphorylation, compared with mock-infected cells (figure 6). Pretreatment of the virus with pooled CVL samples from healthy subjects or HNP-1 reduced the virus-induced FAK phosphorylation substantially. In contrast, CVL samples obtained from HIV-1–infected subjects failed to block the virus-induced phosphorylation of FAK. As anticipated, PRO 2000 also prevented the virus-induced FAK phosphorylation. These results suggest that the CVL samples obtained from the healthy cohort, but not the HIV-1–infected cohort, prevented virus entry and virus-triggered FAK phosphorylation.

To determine whether the reduction in anti-HSV activity in CVL samples obtained from the HIV-1–infected subjects reflects a loss in protective factors and/or the presence of inhibitors, several strategies were adopted. First, the concentration of HNP and SLPI in CVL samples from the HIV-1–infected cohort was measured (table 3). The concentrations of HNP-1–3 (but not of SLPI) were lower in the HIV-1–infected cohort. Despite the lower levels, a significant correlation between HNP-1–3 levels and anti-HSV activity persisted ($r = 0.59$ $P = .006$, Spearman's correlation coefficient). Mixing pooled CVL samples from HIV-1–infected subjects with pooled CVL samples from healthy subjects completely restored the anti-HSV activity, and the addition of synthetic HNP-1 to the pooled HIV-1–infected CVL samples partially restored the anti-HSV activity (figure 7). These observations suggest that reduced anti-HSV activity in CVL samples obtained from the HIV-1–infected cohort reflects a loss in mucosal protective mediators.

DISCUSSION

To our knowledge, the present studies provide the first demonstration that cervicovaginal secretions from healthy women significantly inhibit HSV infection in vitro. These results were confirmed by showing that CVL samples prevented infection in a murine genital herpes model, which thus suggests an important role of cervicovaginal secretions in providing innate protection against HSV. The protective activity in vivo may be considerably higher because of the dilution imparted by the procedure used to collect the CVL samples. Precisely what regulates the innate anti-HSV activity in cervical secretions and how HSV overcomes this activity is not yet understood. Possibilities include exposure to a virus inoculum sufficient to

overcome innate defenses or a loss in protective factors, as was observed in the HIV-1–infected cohort.

Notably, the anti-HSV activity of CVL samples is independent of the presence of serum HSV antibodies and correlates with the concentration of HNP-1–3. CVL fluid and HNP-1–3 act in a similar manner: both irreversibly inactivate HSV and act after binding to block entry, as indicated by inhibition of virus-induced FAK phosphorylation and nuclear transport of VP16. Moreover, the antiviral activity of both is neutralized by serum, which contains several defensin-binding proteins [19]. However, the presence of HNP-1–3 alone does not fully account for the observed activity, as evidenced by the findings that the concentration of synthetic HNP-1, -2, and -3 required to inhibit HSV infection was greater than the concentrations measured in CVL samples and that the addition of synthetic peptides to CVL samples obtained from HIV-1–infected women partially restored antiviral activity, whereas the addition of CVL samples from healthy subjects completely restored the activity. Thus, other CVL fluid components may act additively or synergistically with HNP-1–3 to provide protection.

The observation that HNP-1–3 contribute to the intrinsic anti-HSV activity in cervicovaginal secretions is consistent with previous in vitro findings [6, 7, 12] that identified carbohydrate moieties on envelope glycoproteins as binding sites for HNP-1–3 [7]. The enzymatic removal of O- or N-linked glycans from HSV-2 glycoprotein B-2 has been shown to significantly reduce the affinity of HNP-1–3 for glycoprotein B-2 [7]. HSV-2 glycoprotein B-2 plays a key role in HSV-2 binding and is also required for virus penetration. Because HNP-1–3 (and CVL samples) block virus entry after binding, these findings suggest that the carbohydrate moieties on glycoprotein B-2 contribute to penetration after binding [14].

These studies suggest that SLPI is not a major contributor to the observed anti-HSV activity in CVL samples. The SLPI concentration in CVL samples did not correlate with anti-HSV activity, although we did find that recombinant SLPI inhibits HSV infection in vitro. SLPI's mechanism of anti-HSV activity appears to be different from that of HNP-1–3, because it seems to act on the target cell rather than on the virus. Cells pretreated with SLPI remain resistant to infection by HSV-2, even after extensive washing. Cells pretreated with CVL samples are also unable to be infected by HSV; however, the effect is not sustained after washing (figure 3). The addition of exogenous serum did not interfere with SLPI, which further distinguishes its mechanism of anti-HSV activity from that observed for HNP-1–3. Because SLPI and HNP-1–3 evidently prevent HSV-2 infections via different mechanisms, the possibility that they act synergistically deserves consideration.

We found considerably less anti-HSV activity in CVL samples from a small cohort of HIV-1–infected subjects. We can only speculate about the relationship between these lower levels of anti-HSV activity in their CVL samples and the recognized correlation of susceptibility between HIV-1 and HSV infections. Because adding CVL samples from healthy subjects enhanced the anti-HSV activity of CVL samples from HIV-1–infected subjects, the loss in activity is most likely attributable to a relative lack of protective factors rather than to the presence of inhibitory ones. That we found lower concentrations of HNP-1–3 in CVL samples from the HIV-1–infected subjects is consistent with this interpretation.

The present results demonstrate that cervicovaginal secretions obtained from healthy subjects exhibit substantial intrinsic anti-HSV activity and that this activity is mediated, in part, by HNP-1–3. Contributions by other components warrant further investigation. Cervicovaginal secretions from HIV-1–infected women manifested significantly less anti-HSV activity than was found in secretions from younger, healthier women. This provocative finding highlights the need for additional studies to determine the effects of age, HIV, other sexually transmitted infections, and hormonal changes on mediators of mucosal resistance and on the intrinsic anti-HSV defenses of the female genital tract. Moreover, assessment of the safety profile of candidate microbicides should include an evaluation of the impact of repeated application on these critical innate genital-tract defenses.

References

1. Beyrer C, Jitwatcharanan K, Natpratan C, et al. Molecular methods for the diagnosis of genital ulcer disease in a sexually transmitted disease clinic population in northern Thailand: predominance of herpes simplex virus infection. J Infect Dis **1998**; 178:243–6.
2. Chen CY, Ballard RC, Beck-Sague CM, et al. Human immunodeficiency virus infection and genital ulcer disease in South Africa: the herpetic connection. Sex Transm Dis **2000**; 27:21–9.
3. Mwansasu A, Mwakagile D, Haarr L, Langeland N. Detection of HSV-2 in genital ulcers from STD patients in Dar es Salaam, Tanzania. J Clin Virol **2002**; 24:183–92.
4. Wald A, Link K. Risk of human immunodeficiency virus infection in herpes simplex virus type 2–seropositive persons: a meta-analysis. J Infect Dis **2002**; 185:45–52.
5. King AE, Fleming DC, Critchley HO, Kelly RW. Differential expression of the natural antimicrobials, beta-defensins 3 and 4, in human endometrium. J Reprod Immunol **2003**; 59:1–16.
6. Sinha S, Cheshenko N, Lehrer RI, Herold BC. NP-1, a rabbit α-defensin, prevents the entry and intercellular spread of herpes simplex virus type 2. Antimicrob Agents Chemother **2003**; 47:494–500.
7. Yasin B, Wang W, Pang M, et al. θ Defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. J Virol **2004**; 78:5147–56.
8. McNeely TB, Dealy M, Dripps DJ, Orenstein JM, Eisenberg SP, Wahl SM. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. J Clin Invest **1995**; 96:456–64.
9. Herold BC, Siston A, Bremer J, et al. Sulfated carbohydrate compounds prevent microbial adherence by sexually transmitted disease pathogens. Antimicrob Agents Chemother **1997**; 41:2776–80.
10. Terhune SS, Coleman KT, Sekulovich R, Burke RL, Spear PG. Limited variability of glycoprotein gene sequences and neutralizing targets in

herpes simplex virus type 2 isolates and stability on passage in cell culture. J Infect Dis **1998**; 178:8–15.
11. Harwig SS, Park AS, Lehrer RI. Characterization of defensin precursors in mature human neutrophils. Blood **1992**; 79:1532–7.
12. Wu Z, Prahl A, Powell R, Ericksen B, Lubkowski J, Lu W. From pro defensins to defensins: synthesis and characterization of human neutrophil pro α-defensin-1 and its mature domain. J Pept Res **2003**; 62: 53–62.
13. Herold BC, Scordi-Bello I, Cheshenko N, et al. Mandelic acid condensation polymer: novel candidate microbicide for prevention of human immunodeficiency virus and herpes simplex virus entry. J Virol **2002**; 76:11236–44.
14. Cheshenko N, Herold BC. Glycoprotein B plays a predominant role in mediating herpes simplex virus type 2 attachment and is required for entry and cell-to-cell spread. J Gen Virol **2002**; 83:2247–55.
15. Herold BC, WuDunn D, Soltys N, Spear PG. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J Virol **1991**; 65:1090–8.
16. Melchjorsen J, Pedersen FS, Mogensen SC, Paludan SR. Herpes simplex virus selectively induces expression of the CC chemokine RANTES/CCL5 in macrophages through a mechanism dependent on PKR and ICP0. J Virol **2002**; 76:2780–8.
17. Corey L, Wald A, Celum CL, Quinn TC. The effects of herpes simplex virus-2 on HIV-1 acquisition and transmission: a review of two overlapping epidemics. J Acquir Immune Defic Syndr **2004**; 35:435–45.
18. Cheshenko N, Del Rosario B, Woda C, Marcellino D, Satlin LM, Herold BC. Herpes simplex virus triggers activation of calcium-signaling pathways. J Cell Biol **2003**; 163:283–93.
19. Daher KA, Selsted ME, Lehrer RI. Direct inactivation of viruses by human granulocyte defensins. J Virol **1986**; 60:1068–74.