Virologic and immunologic evidence of multifocal genital herpes simplex virus type 2 infection

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Running Title: Anatomic characterization of genital HSV-2 shedding

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Abstract

Genital HSV reactivation is thought to be anatomically and temporally localized, coincident with limited ganglionic infection. Short, subclinical shedding episodes are the most common form of HSV-2 reactivation, with host clearance mechanisms leading to rapid containment. The anatomic distribution of shedding episodes has not been characterized.

To precisely define patterns of anatomic reactivation, we divided the genital tract into a 22-region grid and obtained daily swabs for 20 days from each region in 28 immunocompetent, HSV-2 seropositive persons. HSV was detected via PCR and sites of asymptomatic HSV shedding were biopsied within 24 hours. CD4+ and CD8+ T cells were quantified by immunofluorescence, and HSV specific CD4+ T cells were identified by intracellular cytokine cytometry.

HSV was detected in 868 (7%) of 11,603 genital swabs at a median of 12 sites per person (range 0-22). Bilateral HSV detection occurred on 83 (67%) days with shedding, and the median quantity of virus detected/day was associated with the number of sites positive (p<0.001). In biopsies of asymptomatic shedding sites, we found increased numbers of CD8+ T cells compared to control tissue (27 vs. 13 cells/mm², p=0.03) and identified HSV specific CD4+ T cells.

HSV reactivations emanate from widely separated anatomic regions of the genital tract and are associated with a localized cellular infiltrate that was demonstrated to be HSV-specific in 3
These data provide evidence that asymptomatic HSV-2 shedding contributes to chronic inflammation throughout the genital tract.
Importance to field:

This detailed study of the anatomic patterns of genital HSV-2 shedding demonstrates that HSV-2 reactivation can be detected at multiple, bilateral sites in the genital tract, suggesting that HSV establishes latency throughout the sacral ganglia. In addition, genital biopsies from sites of asymptomatic HSV shedding have increased numbers of CD8+ T cells compared to control tissue, and HSV-specific CD4+ T cells are found at sites of asymptomatic shedding. These findings suggest that widespread asymptomatic genital HSV-2 shedding is associated with a targeted host immune response and contributes to chronic inflammation throughout the genital tract.
Introduction

Herpes simplex virus type 2 (HSV-2) infects over 500 million people worldwide, (1) is the leading cause of genital ulcer disease, (2) and increases the risk of HIV acquisition. (3) Herpetic genital ulcers in the immunocompetent person are localized in time and anatomic site (4), consistent with reactivation of virus from the ganglion innervating this region. However, most HSV genital shedding is subclinical with rapid onset and clearance. (5, 6) Such subclinical reactivation may be more widespread in the genital area, (7) consistent with frequent leakage of virus into the genital skin and mucosa with localized host containment. (8, 9) Mathematical models suggest that observed shedding patterns are the result of overlapping shedding episodes from different anatomic sites (10), and that amount of virus detected is both directly proportional to the number of infected cells and predictive of a long in vivo half-life of viral infected cells. (8)

Additionally, models predict that greater concentrations of host T cells are associated with viral clearance, while insufficient CD8+ T cell levels result in lesions; (11) hence T cell infiltration may be an in vivo marker of reactivation. Furthermore, tissue resident memory T cells have been shown to persist at sites of genital lesions. (9, 12) To investigate anatomic patterns of subclinical HSV reactivation in the genital tract, we prospectively studied HSV-2 seropositive persons among whom we divided the genital tract into 22 regions, swabbed each region separately on a daily basis, and assayed each sample for HSV DNA using a rapid PCR assay.

Biopsies from regions in which HSV DNA was detected were taken within 24 hours, and analyzed for HSV DNA and evidence of an inflammatory response, allowing for spatial and histologic characterization of asymptomatic HSV-2 reactivation in the genital region.
Methods

Participants and Procedures. We recruited healthy HSV-2 seropositive adults, ages 18 to 65, at the University of Washington (UW) Virology Research Clinic. Persons who were HSV-2 seropositive by the UW HSV type specific immunoblot (13) were eligible. Pregnant women, those with HIV antibodies or those who were taking immunosuppressive medications were excluded, as were persons with coagulopathy, or history of keloid formation or recurrent cellulitis. HLA typing was performed at the Puget Sound Blood Center. The protocol was approved by the UW Human Subjects Division. Written informed consent was obtained from each participant prior to initiating study procedures.

Genital Swabbing. At enrollment, a digital image of the genital tract was used to create a genital map, divided into 22 sections for women and circumcised men, and 26 sections for uncircumcised men. Participants were seen daily, Monday through Friday, for four weeks. After genital examination, Dacron swabs moistened with sterile water were rubbed on the genital mucosa corresponding to each section of the grid, avoiding touching other sites. Each swab was placed in 1X PCR buffer for HSV PCR. (14) Participants were queried about genital symptoms and sexual activity during each visit. If a lesion was present, additional samples from the lesion were obtained and a biopsy was performed, if possible. To map shedding among female participants, we created a shapefile comprising the 22-region grid of our female genital map. We used ArcGIS Version 9.0 (ESRI, Redlands, CA) to create the shapefiles; and Adobe illustrator CS4 (Adobe Systems Incorporated, San Jose, CA) to later combine each participant’s daily maps into a complete participant-level shedding timeline.
Swabs at nongenital sites (thigh, abdomen, or arm) were collected as negative controls. Each examination room was thoroughly cleaned with Virex and bleach daily. In addition, examination rooms were swabbed at 12 potentially contaminated sites, including exam tables, faucets, door handles, and countertops.

**Genital Biopsies.**

Genital biopsies were performed on keratinized genital tissue. The clitoral hood, labia minora, urethra and penile shaft were excluded from biopsy. Genital tissue was cleaned with chlorhexidine and anesthetized with 1% lidocaine. A 3mm punch biopsy was obtained and placed in tissue culture media. The biopsies were sectioned for immunohistochemistry and cell culture. For sites with documented shedding in the absence of symptoms or lesions on careful examination (defined as sites of asymptomatic shedding), biopsies were performed within 24 hours of obtaining a positive PCR result. Control biopsies were performed on the contralateral genital skin or the inner arm. Biopsies were performed on a genital lesion in the first 24-48 hours after lesions were noted by participants. Participants without a genital lesion documented during the intensive sampling period were followed for up to one year after enrollment. Digital images were obtained when biopsies were performed.

**Laboratory Methods.**

**HSV PCR.** A validated, rapid quantitative real-time HSV PCR assay with primers to the glycoprotein B gene was performed on all specimens, with results available within 8 hours of specimen collection (15). DNA extraction was performed using QiaAmp 96 Blood kit. Positive and negative controls were included with samples collected from the clinic; laboratory technicians were blind to control samples. Samples with ≥2.2 log₁₀ copies/ml were considered...
positive. (16) For every 5 samples, a negative control was co-processed in the PCR lab and each DNA extraction plate had one positive control.

**Immunofluorescence.** Biopsy tissue was snap frozen and stored at -80°C in optimal cutting temperature (OCT) media. Frozen tissue was sectioned into 7 micron slices, and fixed and permeabilized in acetone. Tissue was stained with primary monoclonal antibodies specific for human CD4 (Dako) and CD8 (BD Biosciences) and counterstained with DAPI (Fluka) as previously described. (17) Tyramide signal amplification method was used for antibody detection (Invitrogen). CD4+ and CD8+ cells were autocounted (per slide) using publically available software, ImageJ and CellProfiler. (18) Slides were randomly selected for quality control by manual counting.

**HSV T cell specificity.** Bulk lymphocytes were expanded from skin biopsies using non-specific mitogenic stimulation. (19) Autologous PBMCs labeled with carboxyfluorescein diacetate succinimidyl diester (CFSE) were used as antigen presenting cells (APC) at a 1:1 ratio with expanded bulk skin lymphocyte responders as described. (20) Brefeldin A was added at 1 hour and cells labeled for CD3, CD4, and intracellular IFN-γ, TNF-α, and IL-2 were measured at 6 hours as described. (21) A FACSantoflow cytometer (BD Biosciences) with FlowJo software (Tree Star) by gating on lymphocyte forward and side scatter excluding CFSE-positive cells.

**HSV-2 ORFeome.** In biopsies from participants that had HSV-specific CD4+ T cells, we used a complete CD4+ T cell HSV-2 protein set, termed the HSV-2 ORFeome to test for proliferative responses to individual HSV proteins, as described for HSV-1. (21) Briefly, bulk expanded CD4+ lymphocytes, autologous irradiated PBMCs used as APC, and recombinant HSV-2 proteins were plated in duplicate in 96 well-U bottom plates. Primers used to generate the HSV-2 ORFeome
are appended (Supplementary Table 1). As described previously for HSV-1 (21), some HSV-2 ORFs were cloned in fragments and data are presented as positive if specific proliferation was observed for any protein fragment. [$^3$H]thymidine incorporation was measured after 3 days to detect cell proliferation. The criterion for positivity for each antigen was set as the mean plus 2.1 times the standard deviation of the [$^3$H]thymidine incorporation for 54 negative control antigens. (21) In one participant tested with both HSV-1 and HSV-2 ORFeome, CD4+ cells in both lesion and asymptomatic biopsies showed reactivity to VP16. Fragments of VP16 of HSV-1 were expressed as described (22) and tested individually; overlapping peptides from within the positive protein fragments were used to characterize the peptide epitope using the standard [$^3$H]thymidine incorporation assay. A Qdot655-labeled DRB4*0101 oligomer was made with the optimal VP16 peptide and bulk responder skin-origin cells were stained with anti-CD4 mAb and the HSV-DR4*0101 tetramer as described. (23)

**Statistical analysis.** Participants with more than one day of collected genital swabs collected were included in the analysis. Rates of HSV shedding (defined as the number of samples with HSV detected per number collected) were calculated overall, per participant, and per day. Swabs collected from foreskin in uncircumcised men were excluded from all analyses, except during assessment of associations at concurrent sites. A shedding episode was defined as any consecutive period of shedding including no more than one consecutive negative or one consecutive missed swab; episodes were considered of known duration if they were preceded and followed by two days without shedding. (24) Most episodes were of unknown duration due to gaps in the swabbing schedule; in these cases interval censoring was used to estimate the
duration of continuous shedding, assuming an underlying Weibull hazard. Episodes were calculated in two ways: shedding during consecutive days from any site and shedding from the same site during consecutive days. Linear mixed effects regression was used to examine the association between maximum quantity of virus detected on a given day and number of sites with HSV detected. To evaluate the potential associations of previous and concurrent shedding with the risk of shedding at a given site, we created a site-level dataset including one row for each site for each day. A generalized linear mixed model with Poisson link estimated the probability of HSV detection and the association with 1) having sites positive the previous day 2) having not collected the previous day, 3) having non-adjacent sites positive concurrently 4) having adjacent sites positive concurrently and 5) gender. Adjacent sites were defined as two spatial regions sharing any portion of their edges. The analysis included a random term for individual and site. A similar model was performed to assess the influence of bilateral shedding. Sensitivity analysis was performed using >2.7 log10 copies/ml as a cutoff for the linear mixed effects model and generalized linear mixed model. The Wilcoxon signed-rank test was used to test for a difference in CD4+ and CD8+ T cell concentration between paired lesion, asymptomatic and control biopsies. Two-sided p<0.05 was considered statistically significant.
Results.

Participants and genital HSV shedding. We enrolled 29 HSV-2 seropositive persons, one provided a single day of swabbing only and was excluded from all analyses. Of the remaining 28 participants, 21 (75%) were women and 17 (61%) were white (Figure 1A). Five (71%) of 7 men were circumcised. The median age was 42 years (range 20-65). Twenty-five (89%) participants had symptomatic genital HSV-2 infection and 3 (11%) had no history of known genital HSV-2 infection. Thirteen (46%) participants were both HSV-1 and HSV-2 seropositive; fifteen (54%) were only HSV-2 seropositive. Among participants with symptomatic infection, the median time since HSV acquisition was 13 years (range <1-35 years). Twenty-five (89%) participants completed the entire 28 day intensive swabbing period (median 28 days, range 11-36).

We created anatomical grids for women (Figure 1B) and men (Figure 1D), based on the neuronal innervation patterns of the external genitalia (shown for women in Figure 1C). The grid area included innervation from multiple branches of sacral ganglia (Figure 1C). We collected 11,603 genital swabs on 528 days, with a median of 440 swabs (range 198-462 swabs) per person. Eleven (40%) participants had lesions on a total of 77 (15%) days during the follow up period. The remaining 17 (60%) participants were asymptomatic. At least one day of shedding during the follow up period was detected in 23 (82%) of participants; no HSV shedding was detected in five (18%) female participants. Overall, HSV was detected on 123 (23%) days and from 868 (7%) swabs; rates similar to those seen in previously studied cohorts of HSV-2 seropositive persons sampled once daily (24). Of 123 days with shedding, 48 (39%) were during lesions and 75 (61%) were asymptomatic. Of swabs with HSV detected, 573 (66%) were...
collected on days with lesions, and 295 (34%) were collected on days without lesions. Overall
and subclinical rates of shedding did not differ between men and women (data not shown).

**Multifocal anatomic distribution of reactivation.**

Of 23 participants with HSV genital shedding, eighteen (78%) had HSV shedding detected on
both sides of the genital tract during the study period. HSV was detected bilaterally on 83 (67%) days with shedding. Of all days with shedding, 46 (37%) days had more than one contiguous
region positive. In individual participants, we observed distinct anatomic HSV-2 shedding
patterns over time, with some days having focal sites positive or multiple sites positive in the
absence of lesions Figures 2A-2C), and widespread shedding involving the entire genital tract in
the presence of lesionsFigure 2D-2E). For example, in Figure 2A, the participant had shedding
located at focal sites on days 1-4, and again on days 18-19. The participant in Figure 2B had
focal shedding on days 1-4, 16-17, and 29-31. Widespread shedding in the presence of a lesion
is shown in Figure 2D; there is shedding throughout the genital region on day 2 and day 18; a
similar pattern is seen in Figure 2E on days 16, 17, and 31. In men, similar patterns of focal
shedding (Figure 2F, days 10 and 12, 22, and 23; Figure 2G, days 5 and 9) and widespread
shedding (Figure 2G, days 22-25) were demonstrated. Additional shedding patterns for
remaining participants are shown in Supplemental Figure 1-3. Both focal and widespread
shedding patterns were observed in 10 (36%) participants. Shedding was detected at some time
during the observation period at a median of 12 sites (range 0-22) in individual participants; 4
(14%) participants had shedding detected at all 22 sites. A median of 5 sites (range 1-22) were
positive on days when any HSV was detected. A median of 2 (range 1-22) sites were positive on
HSV detection days without lesions. In the presence of a lesion, shedding was often
disseminated throughout the genital tract, with a median of 13 sites with HSV detected (range 1-22), with gradual decline in number of sites with shedding as the lesion healed over time (Figure 2D, days 11-19, Figure 2E, days 1-3 and 16-19). Of 12 participants with lesions, 8 (67%) had lesions at more than one site, with 7 (58%) had lesions at bilateral sites.

**Quantity and duration of shedding episodes**

As shown in Figure 2, participants had several episodes of focal shedding in different regions during the observation period. Overall, we observed 44 episodes of shedding. However, if each of the 22 sites was considered separately, there were 454 shedding episodes. Overall, the median number of consecutive days with HSV detected at the same site was 3.3 days and with HSV detected at any site was 4.1 days. In the absence of lesions, HSV was detected for a median of 2.3 consecutive days at the same site and 3.1 days at any site. In the presence of lesions, shedding at the same site was often prolonged, with a median of 4.0 consecutive days of shedding at the same site and 6.1 days of shedding at any site.

Over all swabs with HSV detected, the median quantity of HSV detected was 3.3 log<sub>10</sub> copies/ml (range 2.2-8.3 log<sub>10</sub> copies/ml) (Figure 3A). The median per-day maximum quantity of HSV detected was 2.8 log<sub>10</sub> copies/ml (range 2.2-7.3 log10 copies/ml) without lesions and 5.9 log<sub>10</sub> copies/ml (range 2.2-8.3 log<sub>10</sub> copies/ml) with lesions. We observed a significant positive association between the median quantity of virus detected and the number of sites positive on any given day (Figure 3B). For each additional site positive on a given day, the median day-level copy number was 0.09 log<sub>10</sub> copies/ml greater in the absence of lesions (p<0.001) and 0.06 log<sub>10</sub> copies/ml greater in the presence of lesions (p<0.001).
Predictors of shedding.

The finding that shedding was found simultaneously at multiple anatomic genital sites could be explained by: 1) contiguous viral spread through epithelial cells; 2) contamination through infected fluids; or 3) simultaneous, multifocal reactivation within sacral ganglia innervating the genital tract. Shedding at an adjacent site could be consistent with all three possibilities, while shedding at non-adjacent sites is most consistent with simultaneous shedding from more than one ganglion. Of 892 positive swabs, 34 (3.8%) occurred on a day with only one site positive, 58 (6.5%) occurred when shedding was present at non-adjacent sites only, and 800 (90%) occurred with concurrent adjacent shedding. The shedding rate increased 60% if the prior day was positive (RR= 1.6, 95% CI=1.3-1.9, p<0.0001). The risk ratio for having any non-adjacent site positive concurrently was 11.0 (95% CI=7.1-17, p<0.0001), and for having an adjacent site positive was 91 (95% CI=63-131, p<0.0001). In another model, we looked at the risk ratio for concurrent shedding on the same or opposite side, and found that the shedding risk increased if shedding was present the day prior (RR=1.9, 95% CI=1.5-2.3), if shedding was present on the opposite side concurrently (RR=13.0, 95% CI=7.7-22.0, p<0.0001) and if shedding was present on the same side concurrently (RR=57, 95% CI=40-83, p<0.001). Therefore, shedding at an adjacent site or same side was associated with very high risk of concurrent shedding at a given site, but shedding at non-adjacent sites or contralateral site was also associated with increased risk of shedding, consistent with simultaneous ganglionic reactivation.

To evaluate whether self-contamination contributed to the results, control swabs were obtained from the leg or arm. Of 283 swabs collected from these sites, 12 (4%) were positive, with median of 2.6 log_{10} copies/ml (range 2.2-3.0). To assess environmental contamination, we...
swabbed examination rooms: of the 60 swabs obtained, 2 (3%) were positive, with viral loads of
2.27 and 2.36 log_{10} copies/ml, respectively. These data suggest that environmental or self-
contamination was rare and low level, but that contamination could account for some of our
findings. We performed a sensitivity analysis in which we used a cutoff of 2.7 log_{10} copies/ml.
This lowered the overall frequency of shedding from 123 days (23%) to 92 days (17%). We
found that the results of the spatial analyses and the association between median HSV quantity
detected and number of sites with HSV detected were, however, unchanged. In addition, since
high quantities of virus are associated with lesions, which may be more likely to contaminate
other areas, we performed an analysis restricted only to non-lesion days, and the results were
similar (RR non-adjacent sites positive=6.9, 95% CI=3.3-14.1; RR adjacent sites positive=94, 95%
CI=58-151).

Biopsies of sites with asymptomatic shedding.

We hypothesized that sites of asymptomatic reactivation would be associated with a host
immune response. We obtained genital biopsies from 15 persons at sites of asymptomatic
shedding within 24 hours of HSV shedding; control skin biopsies were also obtained. We did not
observe lesions prior to biopsies at asymptomatic sites of shedding in any case. The median
quantity of HSV DNA detected prompting the biopsy was 3.4 log_{10} copies/ml (range 2.1-6.7 log_{10}
copies/ml). Nine (60%) biopsies were negative by surface HSV PCR on the day of biopsy,
indicating rapid clearance of the shedding episode (Table 1). One (6.7%) biopsy had HSV DNA
detected within biopsy tissue. Ten participants also had a lesion biopsy performed.
High densities of CD4+ and CD8+ cells were detected in the tissue of several biopsies of
asymptomatic areas compared to control (Figure 4A). The focal distribution of CD4+ T cells was
observed in the dermis, with some areas showing intense infiltration of CD4+ T cells (Figure 4A) while other areas of the same biopsy lacked detectable CD4+ T cells. Asymptomatic biopsies had higher CD8+ T cell density than did controls (median 27 cells/mm² vs. 13 cells/mm², p=0.03). However, we found no significant difference in overall CD4+ T cell density between asymptomatic and control biopsies (20 cells/mm² vs. 10 cells/mm², p=0.52) (Figure 4B), despite the focal enrichment of CD4+ T cells seen in tissue of some asymptomatic biopsies (Figure 4A). As expected, lesion biopsies had highest concentrations of CD8+ cells (median 396 cells/mm² vs. 27 cells/mm², p=0.016) and CD4+ T cells (median 698 cells/mm² vs. 20 cells/mm², p=0.016) compared to asymptomatic biopsies (Figure 4C) and control biopsies (data not shown).

To determine if CD4+ T cells present in asymptomatic and lesion biopsies were HSV-2 specific, we performed intracellular cytokine cytometry (ICC) to assess IFN-γ, TNF-α, and IL-2 production in response to whole HSV antigen (UV-treated HSV-2). Representative findings from a single participant are shown in Figure 5. Among 8 asymptomatic biopsies with sufficient cellular expansion, 3 (38%) had HSV-2 specific CD4+ T cells present. By comparison, in 4 (66%) of 6 lesion biopsies HSV specific CD4+ T cells were present and HSV specific CD4+ T cells were not detected in control biopsies.

Antigen and epitope mapping
To map the antigen specificity of the CD4+ T cells in genital biopsies, we used the HSV-2 ORFeome, a tool which can be used to study proliferative responses to all HSV-2 open reading frames and which allows dissection of antigen-specific T cell responses.(21) We observed reactivity to several HSV-2 antigens (range 3-18) in lesion biopsies in three of three participants.
who had HSV specific cells detected from genital biopsies (Supplemental Table 2). In one of three participants, the asymptomatic biopsy also had HSV-specific T cells. In this participant, T cells from both the lesion biopsy (Figure 6A) and the asymptomatic biopsy (Figure 6B) reacted to VP16 (gene UL48), a late tegument protein that transactivates HSV gene transcription and may be required for initiating replication from latency.(26) The asymptomatic biopsy produced IFN-γ to whole HSV antigen (Figure 6C). We fine mapped the epitope in the asymptomatic biopsy to UL48/VP16 AA 187-199. After creating Qdot-655 conjugated HSV-2 specific DRB4 multimers, as previously reported for HLA class I reagents,(9) we showed that this participant had CD4+ HSV specific T cells reactive to this epitope at the site of asymptomatic genital shedding (Figure 6D) and also in blood (data not shown). These results demonstrate that HSV-specific CD4+ T cells can be recovered from sites of asymptomatic genital HSV shedding, suggesting that asymptomatic shedding episodes are associated with a targeted host immune response.
Discussion

Our intensive study to characterize spatio-temporal patterns of genital HSV-2 shedding and the immune response to asymptomatic HSV shedding episodes showed: 1) widespread viral shedding throughout the genital tract, suggesting that reactivation of HSV-2 often occurred simultaneously from distinct regions of the genital tract and 2) asymptomatic reactivations were associated with increased focal concentrations of CD8+ T cells and the detection of HSV-2 specific T cells at the site of subclinical shedding in nearly 40% of biopsies, providing evidence of robust host-pathogen interactions. In one case, we found that an asymptomatic shedding site biopsy contained HSV-2 specific CD4+ T cells, targeting a specific viral epitope, confirming HSV-2 immunologic specificity in areas of asymptomatic shedding.

Our findings are consistent with the body of literature showing HSV reactivates frequently. (6, 24) We have previously observed a high correlation between shedding duration and peak quantity of virus detected during a shedding episode. (27) In this study, we add a spatial component that shows a positive correlation between increasing virus quantity and larger anatomic area with shedding. Mathematical models have predicted this relationship, as low-copy shedding episodes are associated with few infected cells. (8) However, we were surprised to find evidence of widespread shedding, regardless of the presence of lesions. This was most dramatic with the high frequency of contralateral shedding, suggesting HSV-2 infection may be present in multiple anatomic areas: areas often innervated by different sacral ganglia. These findings are consistent with previous findings in the guinea pig model of vaginal HSV-2 infection as well as studies of human sacral ganglia which indicate HSV-2 latency is widespread.

Bernstein and Stanberry performed a detailed analysis of primary vaginal HSV-2 infection in the
guinea pig, and found that HSV-2 was detected from ipsilateral dorsal root ganglia on days 2-3 post infection, and from contralateral dorsal root ganglia on days 4-5, (28) and that the majority (9/12) of animals shed virus on the contralateral genital skin from the initially infected ganglion, suggesting that HSV-2 infection can spread throughout neuronal tissues. Although data on the distribution of HSV-2 in human sacral ganglia are limited, Obara, et al described PCR and in situ hybridization evidence of widespread sacral ganglia infection in three persons co-infected with HSV-1 and HSV-2, with HSV-2 detected in 21 of 23 sacral ganglia and HSV-1 in 22 of 23 ganglia (29) and concluded that both HSV-1 and HSV-2 infection are latent bilaterally throughout the sacral ganglia. Croen et al also detected evidence of HSV-2 infection in the sacral ganglia of 9 persons at autopsy using in situ hybridization; HSV-2 was detected in more than one sacral ganglion in 3 of 5 persons (30).

Shedding at an adjacent site increased the risk of site specific shedding nearly 100-fold, consistent with either viral spread through epithelial viral replication and spread through contaminated fluids from adjacent sites. While this form of spread or contamination would be reasonably expected over a small anatomic area, it is difficult to envision spread or contamination to include all 22 sites, as was seen on many days in the presence of lesions. This suggests a pattern of diffuse reactivation. The finding that the risk of shedding is increased 10-fold when only non-adjacent sites were positive supports this hypothesis, and is corroborated by evidence of an inflammatory response in tissue biopsies from areas of reactivation.

Whether these shedding patterns reflect either a simultaneous loss of control of latency at multiple neurons or a defect in local immunologic control resulting in increased peripheral viral replication will require further study. From a transmission standpoint, the linkages between...
shedding episode duration, virus quantity, and localization of shedding are likely to be critical. Still, it is difficult to determine if the primary driver of shedding duration is quantity (which could reflect amount of virus released from neurons), space (which may reflect number of neurons releasing virus), or time (which may reflect mucosal replication). Our participants shed on a median of 12 sites throughout this short study, suggesting that it may be important to inform patients that shedding, and therefore the possibility of transmission, is not confined to the site of genital herpes recurrences. This widespread genital shedding even in the absence of symptoms may explain the partial efficacy of condoms in preventing HSV acquisition, as viral shedding frequently occurs in areas not protected by condoms. That CD8+ T cells were significantly more numerous in asymptomatic biopsies than control biopsies suggests that asymptomatic HSV shedding is associated with a targeted immunologic response in tissue, and offers additional evidence that observed shedding patterns are not an artifact of contamination. We recently described a CD8-αα+ T cell population at the dermal-epidermal junction that function in immune surveillance and early containment at sites of previous genital HSV-2 lesions. We are conducting ongoing studies to determine associations between the quantity of CD8+ T cells and the rate of HSV shedding at anatomically defined sites. At asymptomatic biopsy sites, we observed HSV-2 specific CD4+ T cells that had an antiviral and proliferative phenotype, producing IFN-γ, TNF-α and IL-2 in response to HSV-2 antigen. We previously reported that HSV-2-specific CD4+ T cells can persist at sites of healed HSV-2 lesions for several weeks. It is possible, then, that the HSV-2-specific responses described in this report represent memory T cells that were holdovers from previous cycles of HSV-2 replication.
rather than T cells responsive to the current shedding episode. Alternatively, HSV specific CD4+
T cells may have migrated from the circulation to the skin in response to viral shedding
detected the day prior to the biopsy, which would be consistent with mouse models of herpes
showing that CD4+ T cells rapidly traffic to mucosal sites after antigen stimulation. (33) It is
critical to determine whether it is tissue resident memory or circulating CD4+ T cells that
contain HSV, particularly for therapeutic vaccine development.
Both incident and prevalent HSV-2 infection are associated with increased risk of HIV
acquisition, (3) and CD4+ T cells found at the sites of HSV lesions bear the HIV coreceptors CCR5
and CXCR4. (17, 34) We found HSV specific CD4+ T cells that produce pro-inflammatory
cytokines in response to HSV stimulation at asymptomatic shedding sites in nearly 40% of
asymptomatic biopsies; this supports the hypothesis that subclinical shedding episodes may
augment the inflammatory milieu that increases HIV susceptibility. Since rapidly cleared
episodes of subclinical shedding are the most common form of HSV reactivation, (35) this
immunologic response may contribute substantially to increased HIV risk. HSV-specific CD4+ T
cells require further study to learn if they also express HIV coreceptors. While we were unable
to assess the anatomic distribution of short episodes of shedding, we hypothesize that more
frequent sampling would demonstrate additional short episodes of anatomically defined
shedding, as has been described with mixed anogenital sampling (35).
This is the first study to collect samples in defined geographic areas. Each patient in our study
had over 400 separate collections over a 30-day period. Our study was directed at obtaining
samples carefully from each region, hence requiring office visits for each collection. Weekend
and holidays interrupted sampling, leaving the true length of some shedding episodes
unknown. Strengths of our study include excellent adherence, especially in light of the complex
protocol, time demands on participants, and careful acquisition of clinical, virologic, and
histopathologic data. The HSV-2 shedding frequency on a per-day basis is similar to that
described in several previous cohorts with HSV-2 infection (24, 36), suggesting that the
population studied in this report is similar to the large number of persons sampled in a less
detailed fashion. In addition, the duration of shedding episodes is similar to what has been
reported in other once daily sampling studies (27). Use of the rapid HSV PCR assay allowed us
to capture asymptomatic shedding episodes and obtain biopsies at sites with shedding within
24 hours. Our sampling grid may have been too coarse to allow biopsies to capture all areas of
focal HSV shedding; a finer grid could have improved our ability to target small areas of HSV
shedding. While the study team worked to minimize contamination during sample collection, it
is possible that participants self-contaminated different regions through daily activities such as
dressing and toileting. However, our sensitivity analysis suggests that low-level contamination
would not alter our findings that reactivation of HSV-2 occurred simultaneously at multiple
regions of the genital tract in immunocompetent men and women.

This study demonstrates our ability to precisely define the spatial localization of HSV
reactivation from tissue at sites of asymptomatic HSV shedding in the human host. Our findings
reveal HSV genital infection is widespread throughout the genital tract and that genital HSV
shedding is associated in an HSV-specific inflammatory response in the genital skin, even in the
absence of lesions.
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Conflict of Interest: CJ has received research support from Aicuris GmbH. DMK is listed as a co-inventor on patents describing T-cell responses to HSV-2 and is a consultant to Agenus Inc. AW has received research funding from Gilead, Agenus, and Genocea. She has been a consultant for Aicuris. LC is on the scientific advisory board for, and holds stock (<1% of company) in, Immune Design Corp. and is a co-inventor on several patents involving potential HSV vaccine development.


Acknowledgements: The authors are indebted to the dedicated participants and clinicians (Michael Remington, Steve Kuntz, Negusse Obamichael, and Michael Stern) who made this study possible.
References


Figure Legends.

Figure 1A. Study design and participant flow.

Figure 1B. Example female grid.

Figure 1C. Example male grid. Grid outlines in red reflect sites that were not considered to be safe for genital biopsy.

Figure 2. Shedding patterns from individual participants collected over the swabbing period. Timeline indicates days on study. Empty boxes indicate days without shedding, boxes with an “A” indicate days with asymptomatic shedding, boxes with a “L” indicate days with lesions, shaded boxes indicate days without swab collection. Quantity of virus shed in each region is shown according to the heat map (see legend).

Figure 2A. Focal and widespread asymptomatic shedding in women

Figure 2B. Widespread shedding in the presence of a lesion.

Figure 2C. Shedding patterns in men (L=left, R=right, Dor=dorsal, Pen=penis, Ven=ventral).

Figure 3. 3A. Histogram of quantity of HSV detected in swabs. N=868.

Figure 3B. Association between maximum HSV detected and number of sites with HSV detected on single days. Light gray dots= days without lesions, black dots=days with lesions. The lines indicate the regression slope calculated using linear mixed effects. P<0.001.

Figure 4. 4A. CD4 and CD8 staining in asymptomatic, lesion, and control biopsies. CD4 (green) and CD8 (red) immunofluorescence staining from 2 participants who had biopsies collected from an asymptomatic site, lesion site, and control skin. 20X view. Scale bar: 100 micron.

Figure 4B & 4C. CD4 and CD8 T cell densities in asymptomatic, lesion, and control biopsies. Results are from computerized counting of individual slides. Each dot represents counts from a
single participant. Median quantities are indicated by the solid bar, interquartile range indicated by dotted lines. P value for pairwise comparison indicated above.

Figure 5. Representative intracellular cytokine cytometry using bulk expanded lymphocytes stimulated with UV-HSV-2 demonstrates that asymptomatic biopsies contain HSV-2 specific T cells. Cells produce interferon-γ, (IFN-γ), IL-2, and TNF-α. Percentages of positive cells, gated on live CD3+, CD8-, CD4+ cells are provided.

Figure 6. 6A. T cells expanded from a lesion biopsy showed strong proliferative responses to full-length VP16 (gene UL48), UL37, and UL26.

Figure 6B. T cells expanded from an asymptomatic biopsy from the same participant showed strong proliferative response to full-length VP16 (gene UL48). 6A, 6B. The average of 2 reads is shown. Red bars represent ORFs that met the criterion for positivity in 2 replicates; pink bars represent ORFs that were positive in only one replicate.

Figure 6C. CD4+ T cells from the asymptomatic biopsy produce IFN-γ in response to whole HSV-2 antigen. Figure 6D. The VP16 (gene UL48) epitope was mapped to AA 187-199 and a Qdot655 oligomer containing DRB4*0101 and peptide was created. Cells expanded from the asymptomatic biopsy show strong reactivity to the DRB4*0101-VP16 187-199 multimers.
Table 1. Quantity of HSV (log10 copies/ml) detected before and on day of asymptomatic biopsies

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<th>Participant</th>
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Johnston et al. Anatomic characterization of genital HSV-2 shedding
Figure 1A. Study design and participant flow.

HSV-2 seropositive
HIV seronegative
N=50 screened, 29 enrolled

Clinic visits 5 days per week (M-F) for exam and site specific swabbing for HSV by rapid PCR for 28 day period

If positive swab

3 mm biopsy of area with HSV detected within 24 hours

Monitor for genital HSV recurrence for 1 year; biopsy

Of 29 participants enrolled:
1 (3%) collected swabs only 1 day - excluded

Of 28 remaining participants:
3 (11%) lost to follow up or withdrew prior to asymptomatic genital biopsy

Of 25 remaining participants, 15 (60%) had a biopsy performed at a site of asymptomatic shedding
5 (20%) did not have HSV shedding
5 (20%) did not have shedding at an area that could be biopsied

Of 15 remaining participants, 10 (67%) had a genital biopsy at the site of a clinical HSV recurrence
5 (33%) did not have HSV recurrence

Figure 1B. Example female grid.

Figure 1C. Innervation of female genitalia

Figure 1D. Example male grid.

Note: Grid outlines in red reflect sites that were not considered to be safe for genital biopsy.
Figure 2. Shedding patterns from individual participants collected over the swabbing period. Timeline indicates study day. Empty boxes indicate days without shedding, boxes with “A” indicate days with asymptomatic shedding, boxes with “L” indicate days with lesions, shaded boxes indicate days without swab collection. If lesions are present, the site is outlined with bold to clearly indicate the location. Quantity of virus shed in each region is shown according to the key.

Focal and widespread asymptomatic shedding in women

2A. Shedding patterns in men

2G. A lesion was present days 22-25, as indicated by outlined sites

2D. A lesion was present days 1-7 and 16-20 and on day 31 as indicated by outlined sites

2B. Shedding patterns in women

Widespread genital shedding in presence of lesion in women

2C. A lesion was present days 1-7 and days 11-21 as indicated by outlined sites

2F. Shedding patterns in men

2E. A lesion was present days 1-7 and 11-21 as indicated by outlined sites
Figure 3A. Histogram of quantity of HSV detected in swabs. N=868.

Figure 3B. Association between median log10 copies HSV DNA detected/ml and number of sites with HSV detected on single days.

Hollow dots= days without lesions, black dots= days with lesions. The lines indicate the regression slope calculated using linear mixed effects. P<0.001.
Asymptomatic Lesion Control

Figure 4A. CD4 and CD8 staining in asymptomatic, lesion, and control biopsies.

CD4 (green) and CD8 (red) immunofluorescence staining from 2 participants who had biopsies collected from an asymptomatic site, lesion site, and control skin. 20X view. Scale bar: 100 micron.

Figure 4B and 4C. CD4 and CD8 T cell densities in asymptomatic, lesion, and control biopsies. CD4 (4B) and CD8 (4C) T cell densities in biopsy tissues. Results are from computerized counting of individual slides. Each dot represents counts from a single participant. Median quantities are indicated by the solid bar, interquartile range indicated by dotted lines. P value for pairwise comparison indicated above.
Figure 5. Representative intracellular cytokine cytometry using bulk expanded lymphocytes stimulated with UV-HSV-2 demonstrates that asymptomatic biopsies contain HSV-2 specific T cells. Cells produce interferon-γ (IFN-γ), IL-2, and TNF-α. Percentages of positive cells, gated on live CD3+, CD8+, CD4+ cells are provided.
HSV specific T cells are found in lesion and asymptomatic biopsies.

A. T cells expanded from a lesion biopsy showed strong proliferative responses to full-length VP16 (gene UL48), UL37, and UL26.

B. T cells expanded from an asymptomatic biopsy from the same participant showed strong proliferative response to full-length VP16 (gene UL48), 6A, 6B. The average of 2 reads is shown. Red bars represent ORFs that met the criterion for positivity in 2 replicates; pink bar represents ORF that was positive in only one replicate.

C. CD4+ T cells from the asymptomatic biopsy produce IFN-γ in response to whole HSV-2 antigen.

D. The VP16 (gene UL48) epitope was mapped to AA 187-199 and a Qdot655 oligomer containing DRB4*0101 and peptide was created. Cells expanded from the asymptomatic biopsy show strong reactivity to the DRB4*0101-VP16 187-199 multimers.