Valacyclovir Therapy Does Not Reverse Herpes-Associated Alterations in Cervical Immunology: A Randomized, Placebo-Controlled Crossover Trial

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Herpes simplex virus type 2 (HSV-2) infection is associated with a 3-fold increase in the risk of human immunodeficiency virus (HIV) acquisition, perhaps through alterations in mucosal HIV-susceptible target cells. We performed a clinical trial to assess the impact of herpes therapy on cervical immunology in HSV-2–infected, HIV-uninfected women from Africa or the Caribbean who were living in Toronto, Canada. Thirty participants received 1 g of valacyclovir orally each day for 2 months in a randomized double-blind, placebo-controlled, crossover trial. Valacyclovir did not reduce the number of cervical CD4+ T cells, the number of dendritic cells, or the expression of proinflammatory cytokines and tended to increase the expression of the HIV coreceptor CCR5 and the activation marker CD69. Short-term valacyclovir therapy did not reverse HSV-2–associated alterations in genital immunology.

Clinical Trials Registration. NCT00946556.

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Herpes simplex virus type 2 (HSV-2) infection is associated with a 3-fold increase in HIV acquisition risk after sexual exposure [1]. While there is a prolonged infiltration of HIV-susceptible CD4+ T cells and dendritic cells (DCs) in skin at the site of HSV-2 ulcers [2], HSV-2 infection is generally asymptomatic, and perigenital skin is not the site of most HIV acquisition. The endocervix may be particularly susceptible to HIV infection [3], and while this is not a classical site for herpetic ulcers, asymptomatic HSV-2 infection is associated with a substantial increase in these same HIV-susceptible cell types within the endocervix [4].

Despite strong epidemiological and immunological evidence that HSV-2 infection enhances HIV susceptibility, HSV-2 suppression with acyclovir had no impact on the incidence of HIV acquisition, with a trend toward an increased risk of HIV acquisition in the acyclovir arms [5, 6]. We hypothesized that persistence of HSV-2–associated increases in the number of mucosal cells targeted by HIV underlies the failure of herpes suppression to reduce the incidence of HIV acquisition. To directly address this question, we performed a randomized, double-blinded, placebo-controlled, crossover trial of daily oral treatment with 1 g of valacyclovir in asymptomatic, HSV-2–infected women. Overall, we demonstrate that short-term valacyclovir therapy had no impact on the number of putative HIV-target cells in the endocervix.

METHODS

Study Participants
Participants were recruited into this randomized, double-blinded, placebo-controlled, crossover trial (clinical trials registration NCT00946556) from a larger study of genital infections involving women from Africa or the Caribbean who were living in Toronto, Canada [7]. HIV-uninfected women who were seropositive for HSV-2, based on an adjusted threshold value of 3.5 determined by the HerpeSelect gG-1 and gG-2 enzyme-linked immunosorbent assay (ELISA; Focus Technologies, Cypress, CA), were approached. Exclusion criteria included any symptomatic genital infection during the past 3 months; asymptomatic Trichomonas vaginalis, Neisseria gonorrhoeae, or Chlamydia trachomatis infection; and use of herpes medication. Informed written consent was obtained from participants, and the study protocol was approved by the HIV Research Ethics Board at the University of Toronto.

Study Protocol and Sampling
Enrolled participants were randomly assigned to receive 1 g of valacyclovir orally once daily or identical placebo during 2-month period A. After a 1-month washout, participants...
crossed over to the alternate regimen during period B. Drug and placebo were purchased from Apotex (Toronto). Only the study biostatistician had access to randomization codes; research personnel and participants were blinded to group allocation.

Participants were followed monthly for 6 visits, with blood and cervical cytobrush specimens collected 10–18 days after the last day of menstrual period. Blood, cervicovaginal secretions (Instead Softcup; EvoFem, San Diego, CA), and a first-void urine sample were collected at each visit. The study physician collected 2 cotton-tipped swab samples and 1 polyethylene terephthalate–tipped swab (Dacron) sample from the posterior vaginal fornix, and 1 polyethylene terephthalate–tipped swab sample and 2 cytobrush specimens from the endocervix. Cytobrushes were inserted into the cervix and rotated 360°; the presence of any visible blood was formally recorded. A questionnaire regarding sexual behavior, medication, and genital symptoms was completed at each visit. Adherence was estimated by pill count and urine drug levels.

Diagnostic Tests
Vaginal swab specimens were evaluated for bacterial vaginosis on the basis of Nugent score criteria and for T. vaginalis by the OSOM Trichomonas Rapid test (Sekisui, San Diego, CA). Treatment was not provided for asymptomatic bacterial vaginosis based on Nugent score alone. A urine sample was evaluated for N. gonorrhoeae and C. trachomatis by a nucleic acid amplification test (Mount Sinai Hospital, Toronto). Cervical secretions were screened for HSV-2 shedding. Miniprep Qiagen DNA kits were used to extract DNA (Qiagen, Valencia, CA). HSV-2 shedding was detected using a real-time polymerase chain reaction (PCR) assay described elsewhere [8] with minor adjustments: SuperMix (Invitrogen, Burlington, Canada) and Rotorgene 6000 (Corbett Life Science, Australia) were used.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic</td>
<td></td>
</tr>
<tr>
<td>Age, y, median (range)</td>
<td>44 (24–66)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>10 (33.3)</td>
</tr>
<tr>
<td>Behavioral</td>
<td></td>
</tr>
<tr>
<td>Sex within the past week</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Oral hormonal contraceptive use</td>
<td>2 (6.7)</td>
</tr>
<tr>
<td>Condom use during last sex</td>
<td>5 (16.7)</td>
</tr>
<tr>
<td>Intravaginal washing (douching)</td>
<td>5 (16.7)</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
</tr>
<tr>
<td>Previous HSV-2 symptoms</td>
<td>2 (6.7)</td>
</tr>
<tr>
<td>Bacterial vaginosis*</td>
<td>4 (13.3)</td>
</tr>
<tr>
<td>Altered vaginal flora*</td>
<td>12 (33.3)</td>
</tr>
</tbody>
</table>

Data are no. (%) of participants, unless otherwise indicated.

* Bacterial vaginosis was defined as a Nugent score of 7–10, and altered vaginal flora was defined as a Nugent score of 4–6.

Cervical Immune Cell Populations
The T-cell panel consisted of α4–fluorescein isothiocyanate (FITC; Miltenyi Biotec, Bergisch Gladbach, Germany), CD4–ECD (Beckman Coulter, Marseille, France), CCR5–phycocerythrin (PE), β7–allophycocyanin (APC), CD38–AlexaFluor700, HLA–DR–APC-Cy7, CD69–eFluor450 (BD Biosciences, Franklin Lakes, NJ), Live/Dead Aqua (Invitrogen), CD25–peridinin chlorophyll protein (PerCP)-Cy5.5, CD39–PE-Cy7, and CD3–eFluor650 (eBiosciences, San Diego, CA). The DC panel consisted of BDCA2–FITC (Miltenyi), CD207–PE (Beckman Coulter), DC–SIGN–PerCP-Cy5.5, CD206–APC (BD Biosciences), CD83–Streptavidin, CD123–PE-Cy7, CD11c–AlexaFluor700, CD14–AlexaFluor780, CD1a–v450, CD3–eFluor650 (eBiosciences), and Live/Dead Aqua (Invitrogen). Cells were enumerated using a BD LSR-2 (BD Systems, San Jose, CA) and analyzed with FlowJo 9.0 (Treestar, Ashland, OR).

Cytokine Assays
Cervicovaginal secretions were diluted 10-fold in phosphate-buffered saline and stored at −80°C. Levels of interleukin 1α, interleukin 8, monocyte chemotactic protein 1, macrophage–derived chemokine, monokine induced by interferon γ, macrophage inflammatory protein 3α, regulated on activation, normal T-cell expressed and secreted, interleukin 1β, and interferon γ–induced protein 10 were measured using an electrochemiluminescence ELISA platform (Meso Scale Discovery, Rockville, MD), corrected for dilution.

Statistical Analysis
The primary end point was the difference in the change in numbers of CD4+ T cells per cytobrush from baseline to the 2-month follow-up visit between valacyclovir and placebo phases. Secondary end points were changes in (1) the absolute number of immature DCs and (2) proinflammatory cytokine/chemokine levels in cervicovaginal secretions between placebo and valacyclovir phases. The change from baseline to month 2 in the percentage and absolute number of T cells and DCs between the valacyclovir and placebo phases was compared using paired t tests. Repeated measurements were analyzed using generalized linear regression models with random effects, to account for carryover and period effects. The chemokine and cytokine analysis used a Wilcoxon signed rank test. Statistical analyses were performed using SAS, version 9.3 (SAS Institute, Cary, NC), or SPSS, version 20 (IBM, Armonk, NY).

RESULTS
Participant Demographic Characteristics
Participants were recruited from 419 African/Caribbean women who had participated in a clinic-based study of HIV/STI epidemiology [7]. Of these, 119 met inclusion criteria and were invited to participate in the study; 35 consented and were enrolled. Five participants (9%) did not complete the protocol: 1 became
pregnant, 1 withdrew because of headaches, and 3 were lost to follow-up. Data from the 30 participants (91%) who completed both phases were included in the final analysis. The median age was 44 years (range, 24–66 years; Table 1), and all participants were infected by both HSV-1 and HSV-2. Adherence was 89% based on pill count and 80% (47/59) based on the detection of urine acyclovir. All participants had asymptomatic HSV-2 infection, and none reported HSV-2 lesions during the study.

Impact of Valacyclovir on the Absolute Number of Cervical CD4+ T Cells and DCs

There were means of 2.57 log10 and 2.47 log10 CD4+ T cells/cytobrush at the start of the placebo and valacyclovir phases, respectively (Supplementary Table 1; representative flow cytometry gating is shown in Supplementary Figure 1). There was significant intraindividual variability in cervical cell numbers between visits (Mean was 2.51 and unit was log10 CD4+ T cells per cytobrush, SD = 0.14), although this variability was lower than the interindividual variability (SD = 0.36). Visible blood was present on 5 of 180 cervical cytobrush specimens (2.8%).

The change in the absolute number of CD4+ T cells from baseline to 8 weeks was −0.06 log10 cells/cytobrush during the placebo phase and +0.08 log10 cells/cytobrush during the valacyclovir phase, with no difference between the 2 phases (P = .45; Figure 1A).

Insufficient plasmacytoid DCs were present for meaningful analysis (data not shown). The change in the absolute number of Langerhans cells, CD11c+ myeloid–derived DCs (mDCs), and CD14+ monocytes did not differ between the placebo and valacyclovir phases (all P > .1; Figure 1B–D).

Impact of Valacyclovir on T-Cell and DC Subsets Relevant to HIV Susceptibility

T-cell subset analysis demonstrated a trend to an increase in the proportion of cervical CD4+ T cells expressing the HIV

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**Figure 1.** Changes in the number of various immune cell subsets in the endocervix between valacyclovir versus placebo phase. The difference in the change of CD4+ T cells (A), Langerhans cells (B), myeloid–derived dendritic cells (mDCs; C), and monocytes (D) between placebo and treatment phases. Values were normalized through log10 transformation. E and F, Change in the percentage expression of CCR5 and CD69 by CD4+ T cells.
coreceptor CCR5 during valacyclovir therapy ($P = .10$; Figure 1E) and a significant increase in expression of the early activation marker CD69 ($P = .01$; Figure 1F). Representative gating for these subsets are depicted in Supplementary Figure 1. However, this change did not remain significant in a multivariate analysis controlling for study period and crossover effects (Supplementary Table 1). No differences between placebo and valacyclovir phases were seen in the proportion of cervical CD4+ T cells expressing regulatory markers CD25/CD39, chronic immune activation markers CD38/HLA-DR, or the mucosal homing integrin $\alpha 4\beta 7$ (all $P > .1$; Supplementary Table 1).

Expression of DC-SIGN or CD206 (mannose receptor) was then assessed on CD11c+ mDCs and CD14+ monocytes. The change in the absolute number of CD11c+ mDCs or CD14+ monocytes expressing DC-SIGN or CD206 did not differ between placebo and valacyclovir phases (all $P > .1$; Supplementary Table 1).

**Cytokine and Chemokine Levels in the Female Genital Tract**

Genital cytokine and chemokine levels were measured at enrollment and the end of each phase (placebo and valacyclovir; Supplementary Figure 2) by personnel blinded to the treatment assignment. No significant changes were seen in any cytokine between enrollment and the end of the placebo or valacyclovir phases (all $P > .1$).

**Genital HSV-2 Shedding**

An in-house PCR was used to screen for the presence of HSV-2 DNA in cervicovaginal secretions collected at 174 of 180 asymptomatic participant visits (97%). HSV-2 shedding was detected at 4 study visits (2.3%), with 1 visit (of 58 [1.7%]) occurring during valacyclovir therapy and 3 (of 116 [2.5%]) occurring during no treatment or placebo ($P = .721$).

**DISCUSSION**

This randomized, placebo-controlled, double-blinded, crossover study demonstrated no impact of short-term valacyclovir on our primary end point, the change in the absolute number of CD4+ T cells per endocervical cytobrush. Our secondary end points focused on cervical immune cell parameters that may be important in HIV susceptibility, including CD4+ T-cell immune activation and expression of CCR5 [9] and $\alpha 4\beta 7$ [10] and the expression by DCs of the HIV-binding lectin receptors CD206 and CD209 [11]. Again, valacyclovir therapy did not reduce these immune parameters, and it increased expression of the activation marker CD69 and the HIV coreceptor CCR5 in univariate analysis. Although the significance of the latter findings was lost in multivariate analysis, the findings are interesting in light of the trend toward increased HIV acquisition that was observed among participants receiving acyclovir in clinical trials of HSV-2 suppression (hazard ratios of 1.16 and 1.08) [5, 6]. Such an effect, if real, might have been mediated by enhanced host anti-HSV immune responses in the context of effective therapy, a hypothesis that may merit further study. However, cervicovaginal levels of proinflammatory cytokines were not elevated, including those prospectively linked with HIV acquisition (eg, interleukin 6 and interleukin 1) [12], arguing against the induction of a significant inflammatory response by valacyclovir.

Participants in our study had asymptomatic HSV-2 infection, and although asymptomatic infection was strongly associated with altered endocervical immunology in this same community [13], one might hypothesize that a greater immune effect would be seen in women with symptomatic infection or after a longer course of valacyclovir. However, frequent episodes of HSV-2 shedding occur in people with asymptomatic infection (16.5 episodes/year despite high-dose valacyclovir therapy [14]), and we believe that HSV-associated mucosal immune alterations are induced by this asymptomatic HSV-2 reactivation. Since HSV-2–associated immune changes lasted for >2 months at the site of a previous ulcer [2], this suggests that longer courses of therapy are unlikely to have an effect and that valacyclovir (either in standard or higher doses) does not provide sufficiently potent virus suppression to reverse HSV-induced immune changes.

Although a prior clinical trial demonstrated a substantial disconnect between self-reported adherence and the presence of study drug in urine [5], the 80% compliance that we found based on urine analysis may even have been an underestimate, because of the short half-life of acyclovir [15], and so noncompliance is not likely to have undermined our study outcome. The relatively low frequency of HSV-2 reactivation we observed may relate to our focus on cervical and cervicovaginal secretions, where our immune measurements were performed, with the exclusion of possible reactivation sites, such as the labia and perianal areas.

In summary, this randomized, placebo-controlled, double-blinded, crossover trial demonstrates that standard herpes suppressive therapy with valacyclovir in asymptomatic HSV-2–seropositive women does not alter cervical immune alterations associated with enhanced HIV susceptibility. This may explain the inability of this clinical strategy to reduce HIV acquisition. Coupled with the recent finding that very-high-dose valacyclovir therapy also provided suboptimal suppression of herpes reactivation [14], these results suggest that currently available herpes antivirals are unlikely to impact HIV acquisition.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.
Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References