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BRIEF REPORT

Immunological Signaling During Herpes Simplex Virus-2 and Cytomegalovirus Vaginal Shedding After Initiation of Antiretroviral Treatment

Martha C. Nason,[1] Eshan U. Patel,[2] Allison R. Kirkpatrick,[2] Jessica L. Prodger,[2] Kamnoosh Shahabi,[3] Aaron A. R. Tobian,[4,5] Sara Gianella,[7] Sarah Kalibbala,[8] Paschal Ssebbowa,[8] Rupert Kaul,[3] Ronald H. Gray,[5,8] Thomas C. Quinn,[2,6] David Serwadda,[8,9] Steven J. Reynolds,[2,6,8] and Andrew D. Redd[2,6]

[1]Biostatistics Research Branch, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), and [2]Laboratory of Immunoregulation, NIAID, NIH, Baltimore, Maryland; [3]Department of Infectious Diseases, University of Toronto, Canada; [4]Department of Pathology, Johns Hopkins Hospital, [5]Department of Epidemiology, Bloomberg School of Public Health, and [6]Department of Medicine, School of Medicine, Johns Hopkins University, Baltimore, Maryland; [7]Department of Infectious Disease, University of California San Diego, La Jolla; [8]Rakai Health Sciences Program, Entebbe; and [9]School of Public Health, Makerere University, Kampala, Uganda

Vaginal proinflammatory cytokine expression during herpes virus reactivation was examined in human immunodeficiency virus-infected women before and after initiation of antiretroviral therapy (ART). Vaginal swabs were screened for levels of cytokines interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, tumor necrosis factor (TNF)-α, and interferon-γ. The relative risk (RR) of herpes simplex virus-2 or cytomegalovirus (CMV) shedding being associated with cytokine levels above the median were estimated. Herpes simplex virus-2 shedding was significantly associated with higher levels of IL-6 (RR = 1.4, $P = .003$) and TNF-α (RR = 1.3, $P = .010$), whereas CMV shedding was associated with higher IL-6 (RR = 1.3, $P = .006$) and IL-2 (RR = 1.4, $P = .01$). The association of viral shedding with higher IL-6 levels suggests that herpes virus reactivation may be playing a role in immune activation after ART initiation.

Keywords. HIV; HSV-2; CMV; Cytokine; viral shedding.

Antiretroviral therapy (ART) for the treatment of human immunodeficiency virus (HIV) has led to an unprecedented reversal of the devastating personal and public health impact of the HIV pandemic [1]. As ART becomes more commonplace around the world, a better understanding of clinical and subclinical side effects of therapy are critical to ensure optimal adherence, uptake, and positive outcomes. For some individuals, one such ramification of ART initiation is immune restoration disease (IRD), which occurs when the immune system's recovering functionality is associated with a concurrent increase in inflammation, unmasking of underlying opportunistic infections, and possible reactivation of latent viral diseases [2–4]. The most serious form of IRD is immune reconstitution inflammatory syndrome (IRIS), which manifests as a rapid clinical deterioration despite decreasing viral loads and is often associated with underlying infections of *Mycobacterium tuberculosis* and *Cryptococcus neoformans* [5].

Initiation of ART can also lead to a short-term increase of herpes virus-related illnesses [2, 4, 6–8]. In the first 3 to 4 months after ART initiation, there is an approximate 5-fold higher likelihood of reactivation of varicella-zoster virus infection, increased cytomegalovirus (CMV) retinitis, and rare occurrences of herpes simplex virus (HSV)-associated encephalitis [2, 6]. In addition, there is a 2- to 4-fold increased incidence of herpetic genital ulcers for the first 4 months after ART initiation [7, 9].

Proinflammatory cytokines are significantly associated with tuberculosis-associated IRIS events [10, 11]. In particular, interleukin (IL)-6 production by monocytes was predictive of IRIS, and it is associated with IRD in general [10, 11]. A variety of other systemic proinflammatory cytokines and immune activation markers has been found to be associated with IRIS [5, 10–12]. In addition, Stone et al [4] demonstrated that increased IL-6 levels were found in patients who experienced herpes-related IRD.

The majority of HIV-infected individuals in sub-Saharan Africa are coinfected with HSV-2 and CMV [13, 14]. A randomized clinical trial of HIV/HSV-2-coinfected individuals in Rakai District, Uganda found that treatment with the antiherpetic drug acyclovir could delay the onset of ART eligibility by approximately 6 months, and that this effect is most likely due to the direct antiretroviral effect of acyclovir on HIV viral load [9, 15]. Two subsequent studies of 96 women who participated in the trial, and who initiated ART during follow-up, found increased vaginal shedding of HSV-2 and CMV in the first 3 and 4 months after ART initiation, respectively [16, 17]. The timing of increased vaginal HSV-2 and CMV shedding is indicative of IRD, and it provided an opportunity to examine the role of vaginal proinflammatory cytokine production on herpes virus reactivation.

METHODS

Study Population

The trial was described in detail previously [9]. In brief, HIV/HSV-2 coinfected individuals with a $CD4^+$ cell count between 300 and 400 cells/μL were enrolled in a double-blind, randomized placebo-controlled trial of HSV-2 suppression with

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Correspondence: A. D. Redd, Rangos Building Room 527, 851 N. Wolfe St, Baltimore, MD 21205 (aredd2@jhmi.edu).

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acyclovir to prevent HIV disease progression in Rakai, Uganda. Participants were randomly assigned to receive placebo or 400 mg of acyclovir twice daily for 24 months. The primary outcome was HIV disease progression to a CD4 cell count <250 cells/μL or World Health Organization stage IV disease, at which time ART was initiated. Acyclovir was administered throughout the trial period regardless of ART status. After ART initiation, enrollees who reached the end of the study (2 years) were observed for up to an additional 22 months. Women in the study provided monthly self-collected vaginal swabs, and those women who provided swabs within 6 months pre- and post-ART initiation were included in the previous HSV-2 and CMV shedding studies, as well as in this study (Supplementary Table 1, n = 96) [16, 17]. Menstruation status was not included in the analysis.

The trial was approved by the Uganda National Council for Science and Technology (Kampala, Uganda), Uganda Virus Research Institute Science and Ethics Committee, and the National Institute of Allergy and Infectious Diseases Intramural Institutional Review Board. The trial was registered with Clinical.Trials.Gov numbers NCT00405821.

Cytokine Measurement

Vaginal swab samples stored in specimen transport medium ([STM] Roche, Indianapolis IN) from women previously tested for HSV-2 and CMV shedding taken 1 month prior (−1), at time of ART initiation (0), and for 1–4 and 6 months after ART initiation were screened for presence of proinflammatory cytokines IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ using a sensitive multiarray platform (V-plex proinflammatory panel 1 [human] kit; Meso Scale Discovery Inc, Gaithersburg, MD). Before screening of the samples, the sensitivity of the assay to detect known spiked samples in a variety of media was examined. Four storage preparations of phosphate-buffered saline (PBS), STM, Digene, and sera (undiluted, 1:2, 1:4, and 1:8) were diluted into the appropriate assay diluent (diluent no. 2 for proinflammatory panel 1 and diluent no. 43 for custom kit) and tested for their ability to detect 4 levels of known spikes (no-spike [neat], high, medium and low) from the V-plex proinflammatory panel 1 assay and a custom kit (GM-CSF, IL-17a, IL-1β, IL-8, IFN-γ-inducible protein -10, monocyte chemoattractant protein-1, monokine induced by IFN-γ, macrophage-inflammatory protein-3α, and RANTES) (MSD, Rockville, MD). Dilution amounts refer to dilution prepared before loading into blank plate, but it does not include the 2-fold dilution that is built-in to the assay (eg, undiluted final dilution will be 1:2, 1:2 will be 1:4, etc). Sera was obtained from a red-topped vacutainer tube.

Spikes were created by adding 80 μL assay diluent (standard procedure for the V-plex assay) to 72 μL of the storage media dilutions, and 8 μL of the appropriate calibrator dilution mix for the kit as the spike (diluent alone [neat], calibrator standard 1 [high], calibrator standard 2 [medium], and calibrator standard 3 [low]). Calibrators and spikes were added to the MSD plate in duplicate wells, sealed, and incubated at room temperature for 2 hours shaking at 600 rpm. The remainder of the assay was performed according to the manufacturer's protocol.

Levels of recovery were calculated, and the measured concentration was divided by the expected concentration and multiplied by 100 to obtain percentage recovery. All assays were run independently in parallel in 2 different laboratories, and the percentage recoveries were compared for interlaboratory variation. Interlaboratory variability was calculated for the undiluted samples using Spearman correlation (SigmaPlot, San Jose, CA).

Using the results from the spiking experiments, we diluted the vaginal samples 1:4 in assay diluent no. 2 before conducting the assay to remove a previously identified inhibitory factor, and we tested in duplicate according to the manufacturer's recommendation. Samples with values below the limit of detection were considered zero. If a sample was above detection, the concentration was set to the upper standard curve limit for each plate. Samples were retested in duplicate if any of the cytokine levels were above the median value of all samples tested in the initial run and if the intra-assay coefficient of variation (CV) was >50%. If the retest CV was <50%, the result from the second run was used. If the retest CV was >50% again, data were averaged between all runs.

Statistical Analysis

Differences in cytokine detection pre-ART and post-ART were examined with a Pearson's χ^2. Associations between cytokine levels and HSV-2 or CMV shedding over time were modeled using generalized estimating equations (GEE) with an independent correlation structure. Relative risk (RR) and *P* values were based on dichotomizing cytokine expression levels as above or below the median and fitting longitudinal models to estimate the relative risk of HSV-2 or CMV shedding using cytokine levels as time varying covariates adjusting for sample month (ie, −1, 0, 1, 2, 3, 4, and 6) and ART status. A Poisson model with a log link and robust standard errors was used for the GEE algorithm, although similar results were obtained with binomial models.

Associations between cytokine levels and the women's shedding status were visualized with heat maps using geometric means for cytokine levels, and they were analyzed using Wilcoxon signed-rank test. Exploratory Classification Trees were also built to explore the relationships between shedding status and cytokine levels post-ART initiation, using the rpart package in R (reference CRAN).

RESULTS

Women who had 6 months of vaginal swabs available before and after initiation of ART that were previously tested for levels of HSV-2 and CMV shedding were included (n = 96; Table 1).

Table 1. Participant Demographics and Characteristics

Characteristic	Number of Individuals (%), n = 96
Trial arm	
Placebo	53 (55%)
Acyclovir	43 (45%)
Age at enrollment	
20–29	23 (24%)
30–39	41 (43%)
40+	32 (33%)
Viral load pre-ART (log copies/mL)	
<4.0	15 (16%)
4.0–5.0	34 (35%)
5.0–6.0	41 (43%)
>6.0	6 (6%)
CD4 count at ART initiation (cells/μL)	
<200	40 (42%)
>200	56 (58%)

Abbreviation: ART, antiretroviral therapy.

Herpes simplex virus-2 and CMV shedding were previously measured by real-time polymerase chain reaction (PCR). Levels of shedding of HSV-2 increased 158% for the first 3 months after ART, and CMV shedding increased by 44% for months 2–4 after ART initiation [16, 17]. No clinical evidence of IRIS was seen in the women in this study [9]. Vaginal samples pre-ART (−1 and 0 months) and post-ART (+1, +2, +3, +4, and +6 months) were tested for cytokine levels (n = 657 sample visits). Specimen transport medium demonstrated a consistent and strong inhibition of recovery in both kits for virtually all of the spiked biomarkers concentrations. This was reduced significantly with a 1:4 dilution from all the biomarkers in the V-plex kit (Supplementary Figures 1 and 2). The probability of detection varied between cytokines, and there were no statistically significant differences in cytokine detection pre- and post-ART ($P > .05$; Table 2). Due to low levels of detection, IFN-γ and IL-12p70 were removed from further analyses (Table 2). The remaining cytokines were dichotomized (> or ≤ median; IL-1, IL-2, IL-4, IL-10, and TNF-α median = 0 pg/mL; IL-6 median = 2.95 pg/mL; IL-13 median = 9.9 pg/mL; IL-8 median =1137 pg/mL) and were independently analyzed for an association with HSV-2 and CMV shedding status while adjusting for month of sample and ART status (Table 2). Herpes simplex virus-2 shedding was significantly associated with IL-6 (RR = 1.35, $P = .003$) and TNF-α (RR = 1.31, $P = .010$), whereas CMV shedding was associated with IL-2 (RR = 1.35, $P = .007$) and IL-6 (RR = 1.30, $P = .006$; Table 2). These 3 cytokines were significantly correlated with each other to varying degrees (Supplementary Figure 3).

The associations between HSV-2 and CMV shedding and cytokine detection were also examined independently for women in both trial arms. This reduced the power to detect associations between cytokine levels and shedding, but the associations with HSV-2 shedding and IL-6 (RR = 1.64, $P = .0002$) and TNF-α (RR = 1.50, $P = .003$) remained statistically significant in women participating in the placebo arm of the original trial, but not in the women in the treatment arm (IL-6: RR = 1.07, $P = .7$ and TNF-α: RR = 1.14, $P = .5$). In contrast, CMV shedding was associated with IL-2 (RR = 1.47, $P = .04$) and IL-6 (RR = 1.48, $P = .002$) in the treatment arm but not in the placebo arm (IL-2: RR = 1.27, $P = .1$ and IL-6: RR = 1.15, $P = .3$).

To examine changes within women for both HSV-2 and CMV shedding, women were categorized according to shedding status pre-ART (months −1 and 0) and post-ART (months 1–4). Women who never shed during these windows were classified as nonshedders (HSV-2, n = 51; CMV, n = 30), and they were compared with women who did not shed before ART but did shed during 1 or more of the 4 months post-ART (HSV-2, n = 34; CMV, n = 25). Women who shed pre-ART (HSV-2, n = 11; CMV, n = 41) were excluded from these analyses. The patterns of cytokine levels were compared between the nonshedders

Table 2. Cytokine Detection Pre- and Post-ART Initiation and Associations of Cytokine Levels Greater Than the Median With HSV-2 and CMV Shedding

Cytokine	% Detection (Samples)	% Detection Pre-ART	% Detection Post-ART	RR of HSV-2 Shedding	*P* Value[a]	RR of CMV Shedding	*P* Value[a]
IL-1β	30.9% (203/657)	28.0%	32.1%	1.28	.12	1.28	.08
IL-2	40.3% (265/657)	43.9%	38.9%	1.05	.73	**1.35**	**.01**
IL-4	13.1% (86/657)	12.2%	13.5%	0.67	.32	1.02	.92
IL-6	77.8% (511/657)	79.9%	76.9%	**1.35**	**.003**	**1.30**	**.006**
IL-8	99.4% (653/657)	98.9%	99.6%	1.20	.13	0.97	.50
IL-10	47.2% (310/657)	47.6%	47.0%	1.20	.12	1.14	.28
IL-12p70[b]	2.1% (14/657)	2.6%	1.9%	—	—	—	—
IL-13	62.3% (409/657)	61.9%	62.4%	1.17	.20	1.12	.23
IFN-γ[b]	0.6% (4/657)	0.5%	0.6%	—	—	—	—
TNF-α	46.6% (306/657)	48.7%	45.7%	**1.31**	**.01**	0.99	.30

Abbreviations: ART, antiretroviral therapy; CMV, cytomegalovirus; GEE, generalized estimating equations; HSV, herpes simplex virus; IFN, interferon; IL, interleukin; RR, relative risk; TNF, tumor necrosis factor.

[a] Poisson models with GEE were used to estimate the RR of HSV-2 and CMV shedding. The primary exposure was cytokine levels above the median, and models were adjusted for month and ART status. $P < .05$ is indicated in bold.

[b] Due to limited detection, IL-12p70 and IFN-γ were removed from further analyses.

and the post-ART shedders for both HSV-2 and CMV (Figure 1A and 1B). Women who shed HSV-2 post-ART appeared to have a pattern of higher proinflammatory cytokine levels (IL-2, −6, −13, −10, and TNF-α) after ART initiation, which dissipates in intensity by 6 months post-ART initiation (Figure 1A). A more subtle effect was observed in women who shed CMV post-ART only (Figure 1B). These differences in cytokine levels between shedders and nonshedders post-ART were not statistically significant, although IL-2 ($P = .08$) and IL-6 ($P = .09$) trended to higher levels in CMV and HSV-2 shedders, respectively.

CONCLUSIONS

Antiretroviral therapy is a life-saving intervention, but IRD immediately after initiation of therapy remains a serious clinical problem in some individuals [2]. The association of HSV-2 and CMV shedding with higher IL-6 levels, which is strongly associated with herpetic and bacterial forms of IRIS, suggests that subclinical herpes virus reactivation in the genital tract of African women may be an additional factor associated with this problem [4, 10, 11]. There was not an association between cytokine detection and ART status, even though all of these women were seropositive for both viruses, suggesting that HSV-2 or CMV shedding is an essential component of the immunological response observed in this study. The association between patterns of viral shedding and increased levels of multiple proinflammatory cytokines indicate that this is a dynamic process that likely involves multiple aspects of the immune response. The strengthening of the association between HSV-2 shedding and increased levels of IL-6 and TNF-α in the placebo arm, but not in the acyclovir treatment arm, of the original study supports the hypothesis that these cytokines are interacting with the virus in a direct manner. Unfortunately, this study was not designed to determine the causal nature of this relationship. It will be important for future studies to explore whether IL-6, TNF-α, or IL-2 directly stimulates HSV-2 and CMV reactivation in vivo, or conversely if CMV and HSV-2 reactivation stimulates cytokine production. It is also possible that both are markers for a more complicated immunological mechanism. Understanding the role of IL-6 specifically may be critical because it has been shown that increased levels of IL-6 is associated with increased mortality in treated HIV-infected patients [18].

It should be pointed out that this study is a secondary analysis of a randomized clinical trial, and therefore it contains some limitations [9]. In particular, the collection method for vaginal swabs was originally designed to maximize the detection of viral shedding by PCR [16, 17]. The storage media used was found to inhibit the detection of cytokines, and therefore it was diluted 1:4 for this analysis. This limited the detectability of the multiplexed cytokine assay, and future studies should use a more direct collection method for vaginal samples to guarantee more accurate determination of cytokine levels. In addition, serum and cellular populations were not collected in tandem with the vaginal swabs, making it impossible to explore the full role of systemic immune activation on increased herpetic vaginal shedding. Finally, as a secondary analysis, the study

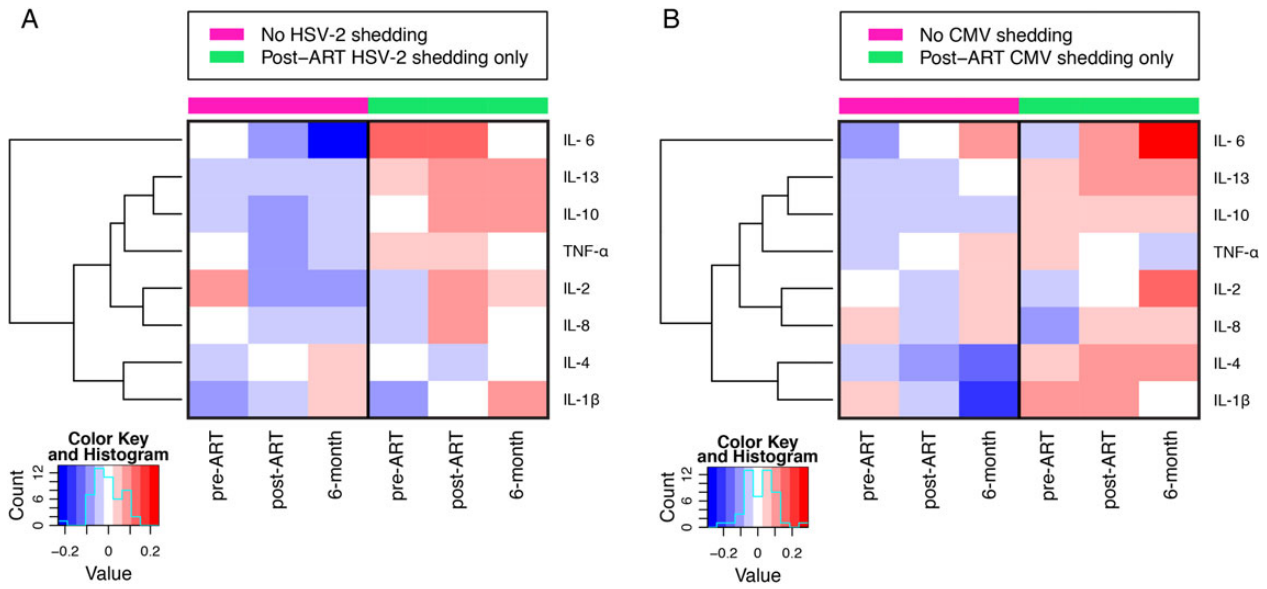

Figure 1. Heat maps of geometric means of cytokine levels for women who did not shed (A) herpes simplex virus (HSV-2) and (B) cytomegalovirus (CMV) pre-antiretroviral therapy (ART) (month −1 and 0), post-ART initiation (month 1–4), or at month 6 (indicated with pink line) compared with women who did not shed pre-ART, but did shed at some point post-ART initiation (indicated with green line). Blue indicates lower relative cytokine levels and red indicates higher cytokine levels for the specific time frame indicated. The intensity of the colors reflects the likelihood of a woman in that group having a cytokine level above the median for that specific time point. Abbreviations: IL, interleukin; TNF, tumor necrosis factor.

size and sample timing was not optimized to examine these changes in cytokines. Therefore, the results presented here should be viewed as being hypothesis generating.

These data provide intriguing clues into a possible role for herpes virus reactivation as an associated factor in the inflammatory response seen after ART initiation. Given that HIV-infected individuals in Africa are most likely exposed to a high number of viral, bacterial, and parasitic coinfections, it will be important to better understand (1) their exact role in immune reconstitution and (2) whether they contribute to continued immune activation seen in individuals who are successfully treated.

Supplementary Data

Supplementary material is available online at Open Forum Infectious Diseases online (http://OpenForumInfectiousDiseases.oxfordjournals.org/).

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