Impact of Antiretroviral Therapy Duration and Intensification on Isolated Shedding of HIV-1 RNA in Semen

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Background. Effective antiretroviral therapy (ART) dramatically reduces human immunodeficiency virus (HIV) transmission. However, isolated shedding of HIV type 1 (HIV-1) in semen (IHS) can occur in the absence of detectable viremia or genital infections. We hypothesized that ART intensification with medications active in semen might prevent IHS.

Methods. Paired blood and semen samples were collected monthly for 6 months from HIV-infected men starting ART that was intensified (iART) with maraviroc and raltegravir in an open-label fashion. Semen parameters were compared to those of historical controls starting standard ART (sART).

Results. Compared with 25 controls who started sART, the semen HIV-1 load in 13 subjects who started iART was more rapidly suppressed ($P = .043$). IHS was detected at >1 visit in 2 participants (15%) receiving iART and in 12 controls (48%) receiving sART ($P = .040$). Among iART recipients, IHS was associated with lower raltegravir concentrations in blood and semen, compared with complete HIV-1 suppression ($P = .03$). Prolonged, high-level IHS (ie, shedding of >5000 RNA copies/mL) was observed in 1 iART recipient (8%), despite rapid viremia suppression and therapeutic drug levels; for 10 months, this virus remained R5 tropic, drug susceptible, and similar in sequence to virus recovered from blood. IHS was not seen after >3 years of effective ART in a parallel, prospective cohort study.

Conclusions. iART transiently reduced the occurrence of IHS early after ART initiation but did not prevent high-level IHS. IHS was not seen after more prolonged sART.

Keywords. HIV; transmission; semen; viral load; antiretroviral therapy.

The risk of sexual transmission of human immunodeficiency virus type 1 (HIV) is strongly correlated with the genital (ie, semen or cervicovaginal) HIV RNA level, and in heterosexual African couples each $\log_{10}$ increase in genital HIV RNA levels increased the probability of both male-to-female and female-to-male transmission by approximately 1.7-fold [1]. Because semen is the most common vector for sexual transmission of HIV in the global pandemic, reducing HIV levels in semen is an important public health priority.

Antiretroviral therapy (ART) reduces the HIV transmission probability by $>20$-fold within heterosexual couples [2]. However, a subset of individuals receiving effective ART continue to have detectable levels of HIV RNA in genital secretions, despite an undetectable blood HIV load and a lack of detectable sexually transmitted infections. This phenomenon is known as isolated HIV RNA shedding (IHS) and has been described in men [3–10] and women [11]. Levels
of virus during IHS are often low and of uncertain clinical importance. However, “high-level” shedding (ie, shedding of >5000 RNA copies/mL) has been described in a substantial subset of individuals [8], and this level was associated with an increased risk of HIV transmission in vitro [12]. Interestingly, the 92%-96% reduction in HIV transmission events that was seen in clinical studies of ART for prevention [2, 13] was very similar to the 92% reduction in high-level HIV semen shedding that was seen in men starting ART [14], and the rare transmission events that have occurred in recent studies all took place soon after starting therapy.

The cause of IHS has not been defined. Studies comparing antiretroviral levels in semen and blood [6,15] have documented differences in the penetration of several commonly used medications into these compartments. For example, protease inhibitors are present in semen at levels of <5% of those in blood [16], while semen levels of nucleoside/nucleotide analogues (nucleoside reverse-transcriptase inhibitors [NRTIs] and nucleotide reverse-transcriptase inhibitors) such as lamivudine may exceed those in blood by ≥600% [17–20]. While this suggests that drug regimen might play a role in IHS, no clear associations with drug regimen have been apparent in the small studies performed to date [8, 11], although the poor semen penetration of efavirenz has been implicated as a possible cause of a slow decline in the semen viral load [21]. To further study the effect of antiretroviral drug regimen on IHS, we added 2 compounds with documented high semen penetration, raltegravir and maraviroc [22, 23], to standard ART (sART) in men starting therapy and prospectively assessed the impact of this intensified drug regimen on IHS in an open-label fashion. These men were compared to a previously described group of men who had initiated 3-drug sART [8].

METHODS

Human Subjects

HIV-infected, ART-naive men who have sex with men were recruited through the Canadian Immunodeficiency Research Collaborative at the Maple Leaf Medical Clinic in Toronto, Canada, as previously described [8]. Participants were excluded if at any visit they had clinical urethritis, genital ulcer disease, laboratory evidence of infection by *Chlamydia trachomatis* or *Neisseria gonorrhoeae* by urine nucleic acid–amplification testing (Amplicor CT/NG assay, Roche Diagnostic Systems), or active *Treponema pallidum* infection detected by serologic analysis (rapid plasma reagin assay) at any study visit. A firstvoid urine dipstick for leukocyte analysis was also performed to screen for asymptomatic urethritis. All participants provided informed, written consent; ethical approval for this study was obtained through the research ethics board of the University of Toronto.

**Study Design**

This observational, open-label study of iART followed a format identical to that of a previously published study [8]. Participants consisted of men starting an iART regimen that added both maraviroc and raltegravir to a sART regimen. The use of an iART regimen was at the discretion of the participant, in discussion with their physician. Participants provided semen and blood samples at baseline (ie, before therapy initiation) and then at weeks 0, 2, 4, 8, 12, 16, 20, and 24 after therapy initiation; after this point, a subset of consenting participants were followed every 3 months, up to a maximum of 2 years. Parameters for this iART group were then compared to those for a group of historical controls who had been treated with sART during 2008–2009 [8]. All study conditions, sample processing, and virologic/immune assays were identical between studies.

In addition to this new iART group with 13 subjects and the sART group with 25 historical controls, a separate cohort was enrolled, consisting of 26 HIV-infected men who had been receiving sART for varying lengths of time (1–3 years, >3–≤5 years, and >5 years). These participants provided semen and blood samples monthly for 6 consecutive months to assess the possible association of HIV treatment duration with IHS occurrence.

Sample Acquisition, Sample Processing, and HIV Load Measurement

Paired blood and semen specimens were collected within an hour of each other at each study visit. Semen samples were collected by masturbation into 10 mL of sterile Roswell Park Memorial Institute 1640 medium (Gibco) containing 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco; transport medium). All study participants agreed to abstain from sexual intercourse or masturbation for 48 hours before sample donation. All samples were processed within 2 hours of collection. Seminal plasma specimens were cryopreserved at −80°C after sample centrifugation at 850 × g for 10 minutes. Blood plasma was collected and cryopreserved after Ficoll density gradient centrifugation at 500 × g for 25 minutes. Blood and seminal plasma HIV RNA concentrations were measured in the Mount Sinai Hospital Department of Microbiology (accredited by the Ontario Public Health Laboratory for clinical HIV load measurement), using the Versant HIV RNA 3.0 assay (bDNA; Bayer Diagnostics; lower limit of detection, 50 RNA copies/mL). Correction for semen dilution was calculated on the basis of a mean semen volume of 2 mL, as in previous studies [24].

**Antiretroviral Levels**

Blood and semen plasma concentrations of maraviroc, raltegravir, etravirine, lopinavir, efavirenz, and ritonavir were determined using validated high-performance liquid chromatography with tandem mass spectrometry, after protein precipitation.
This method was adapted from previous studies [6] by adding maraviroc, raltegravir, and etravirine in the original calibration standards. All detected drug concentrations in blood and semen were within the calibration curve ranges, and levels of recovery, accuracy, and precision were well within the predefined limits. All semen drug levels were corrected for dilution, as described above.

**HIV RNA Amplification, Sequencing, and Analysis**

Total nucleic acids were extracted from baseline plasma and longitudinal semen specimens from a patient exhibiting high-level IHS, using a standard commercial kit (Invitrogen). HIV RNA sequences spanning (1) the protease gene and the first 400 codons of the reverse transcriptase gene, (2) codon 401 of the reverse transcriptase gene through the end of the integrase gene, and (3) the envelope V3 loop were amplified in independent nested reverse transcription polymerase chain reaction assays, using sequence-specific primers optimized for HIV subtype B. Reactions were performed in triplicate (at minimum). Successful amplicons were “bulk” (directly) sequenced bidirectionally, using an ABI 3130xl genetic analyzer (Applied Biosystems). Chromatograms were analyzed using Sequencher 4.10.1 Software (GeneCodes). Bases were called as mixtures if the height of the secondary peak was ≥25% of the height of the primary peak. Sequences were aligned using MUSCLE [25]. HIV coreceptor use was inferred by means of the geno2pheno [coreceptor] 2.5 method (available at: http://coreceptor.bioinf.mpi-inf.mpg.de/), using optimized false-positive rate cutoffs. Drug resistance genotype interpretation was performed using the Stanford HIV Drug Resistance Database (available at: http://hivdb.stanford.edu/). Twenty-seven sequences have been deposited in GenBank (accession numbers JX519532–JX519558).

**Statistical Analysis**

All analyses were performed using IBM SPSS Statistics software for Mac (version 18; SPSS). Data were statistically analyzed using Wilcoxon matched-pairs signed rank, Mann-Whitney, and χ² tests. Statistical significance was defined as P < .05. We hypothesized that starting iART with 2 antiretroviral agents active in semen would prevent IHS. Were this to be the case, the study would be powered to see a significant difference between groups with a sample size of just 5 iART recipients, given the IHS frequency of 48% in our previous study [8]. However, in case occasional IHS occurred, we expanded enrollment to include 13 participants.

**RESULTS**

**Study Participants**

This prospective, observational study enrolled 13 men starting an iART regimen (Table 1) and compared these participants with 25 previously described men starting sART [8]. Compared with men starting iART, men starting sART had a lower median nadir (baseline) CD4⁺ T cell count (213 vs 340 cells/mm³; P = .001) and a higher median baseline blood HIV RNA load (50 000 vs 7122 copies/mL; P = .05). Herpes simplex virus type 2 and cytomegalovirus seroprevalences were 36% (9 of 25 men) and 100% (25 of 25), respectively, in the sART group and 69%

### Table 1. Baseline Demographic Characteristics and Antiretroviral Regimens of Participants Receiving Intensified Antiretroviral Therapy (ART)

<table>
<thead>
<tr>
<th>Participant</th>
<th>T-Cell Count, cells/mm³</th>
<th>HIV Load, RNA copies/mL</th>
<th>Initial ART Regimen</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CD4⁺</td>
<td>CD8⁺</td>
<td>In Blood</td>
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<tr>
<td>001</td>
<td>340</td>
<td>570</td>
<td>7122</td>
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<tr>
<td>013</td>
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<td>860</td>
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Abbreviations: ABC, abacavir; ATZ, atazanavir; AZT, zidovudine; EFV, efavirenz; FTC, emtricitabine; HIV, human immunodeficiency virus type 1; LPV, lopinavir; MVC, maraviroc; NVP, nevirapine; RAL, raltegravir; RTV, ritonavir; SOV, saquinavir; TDF, tenofovir; 3TC, lamivudine.

* Individuals with subsequent isolated semen HIV RNA shedding.
(9 of 13) and 84.6% (11 of 13), respectively, in the iART group. The 2-drug NRTI “backbone” was similar for both sART and iART groups and was composed of combinations of tenofovir, abacavir, zidovudine, and lamivudine; however, all participants in the iART group were receiving a boosted protease inhibitor, while participants in the sART group were more evenly split between those receiving a nonnucleoside reverse-transcriptase inhibitor (efavirenz or nevirapine; 12 of 25 men) and those receiving a boosted protease inhibitor (lopinavir, atazanavir, or saquinavir; 13 of 25) [8].

**Overall Impact of ART on Blood and Semen HIV RNA Level**

The median baseline levels of HIV RNA in semen were similar between groups, at 2979 copies/mL (range, 300–70 710 copies/mL) in the iART group and 5136 copies/mL (range, 300–86,856 copies/mL) in the sART group (P = .36; Figure 1). The semen viral load dropped more rapidly in the iART group and was more likely to be undetectable by 2 weeks after treatment initiation, compared with that in the sART group at the same time point (P = .043). However, the majority of participants had achieved undetectable blood and semen HIV RNA loads by week 16 in both the sART group (20 of 25 men) and the iART group (12 of 13).

**Overall and High-Level IHS**

As previously reported [8], IHS was detected at ≥1 visit for 12 of 25 participants (48%) in the sART group, with high-level shedding in 4 (16%); IHS was present during 19 of 116 study visits (16.4%) with an undetectable blood HIV RNA load and at a high level in 5 of 116. In the iART group, IHS was seen in fewer participants (2 of 13 [15%]; P = .048), but there was no reduction in the occurrence of high-level shedding (1 of 13 [8%]; P = .472; Figure 2). IHS was present during 5 of 73 study visits (6.8%) in the iART group with an undetectable blood HIV RNA load (odds ratio, 2.6 vs the sART group; P = .072) and at a high level in 4 of 73 (OR, 0.94 vs the sART group; P = 1.0).

**Maraviroc and Raltegravir Levels**

Median maraviroc and raltegravir levels after 6 months of therapy were 109 ng/mL (range, 44.4–377 ng/mL) and 246 ng/mL (range, 40.3–1380 ng/mL) in blood and 804 ng/mL (range, 153–4920 ng/mL) and 723 ng/mL (range, 282.6–1890 ng/mL) in semen, respectively (Figure 3). Levels of maraviroc and raltegravir in both blood and semen were above the minimal therapeutic concentration required to suppress HIV in all samples tested (90% inhibitory concentration for maraviroc, 2.03 nM; 95% inhibitory concentration for raltegravir, 31 nM) [26, 27]. Median semen-to-plasma ratios for maraviroc and raltegravir were 9.7 (range, 0.49–46.9) and 4.9 (range, 0.49–8.9), respectively (P < .05).

We compared samples from 2 individuals with IHS to timematched samples from 11 participants who had never had IHS. In keeping with results of our prior study [14], no differences were observed in blood or semen plasma levels of maraviroc (blood, P = .637; semen, P = .346), etravirine (blood, P = .670; semen, P = .530), lopinavir (blood, P = .478; semen, P = .166), or ritonavir (blood, P = .157; semen, P = .530). However, lower levels of raltegravir were observed in both blood and semen plasma (P = .03; Figure 4). Within these 2 individuals, raltegravir levels during the IHS episode were then compared to levels at flanking time points with no detectable semen virus, and no significant differences were observed. This suggests that raltegravir levels were consistently lower in participants with IHS, although the IHS episode itself was not associated with further reductions.

**Sequence Analysis of Virus Detected in Semen From a Patient With Prolonged High-Level IHS**

One individual had prolonged, high-level IHS despite undetectable viremia in blood plasma (Figure 2C). Follow up for this individual was extended to 2 years, with persistent IHS detected until month 14. Viruses from pre-ART semen and blood specimens, as well as virus from semen obtained during IHS episodes at month 2 and month 10, were assessed for drug resistance mutations. Bulk sequencing of blood plasma HIV V3 sequences revealed at least 2 baseline circulating variants, whereas all HIV V3 sequences from semen samples were identical and matched one of these blood plasma variants (Figure 5). Blood and semen viruses were identified as CCR5 tropic by V3 genotyping. Sequencing of reverse transcriptase–integrase revealed greater HIV diversity, as measured by the presence of nucleotide mixtures [28], in plasma compared...
with semen, but no evidence of any major or minor drug resistance mutations were found in HIV from in any sample at any time point (not shown). Overall, there was no evidence for the development of drug resistance or virus evolution in the semen of this individual despite prolonged, high-level IHS for >10 months.

Figure 2. A, Median levels of human immunodeficiency virus type 1 (HIV) RNA in the blood and semen of all 13 participants starting an intensified antiretroviral therapy (iART) regimen, demonstrating rapid viral load suppression in semen and blood. One participant demonstrated a single episode of low level IHS (B), while another demonstrated sustained high-level semen shedding even beyond the 6-month duration of the study (C).
Duration of sART and Incidence of IHS

Despite prolonged IHS in this one individual, semen virus levels fell below the level of detection by 16 months, suggesting that IHS may resolve after sustained ART. Therefore, to explore whether a longer duration of sART would be associated with reduced IHS, we recruited a new cohort of ART-experienced men for this purpose. Monthly semen and blood samples were collected for 6 months from 26 HIV-infected, sART-experienced men classified into 3 groups on the basis of duration of past effective sART: 1–≤3 years (group 2; 10 men), >3–≤5 years (group 3; 9 men), and >5 years (group 4; 7 men). All men enrolled in each of groups 2–4 had had an undetectable blood viral load and no sexually transmitted infection for 6 months before enrollment. These groups were compared to the previously described [8] participants initiating sART (group 1; 25 men). Overall, the occurrence of IHS during the 6 months of follow up was associated with a shorter past duration of effective sART. At least 1 episode of IHS was observed in 12 group 1 participants (48%), in 2 group 2 participants (20%), and in no group 3 or 4 participants (P < .001, by the Mann-Whitney test; Figure 6). Among clinical visits with an undetectable blood viral load, the proportion in which IHS was detected also declined with the duration of past treatment (19 of 116 visits for group 1, 2 of 60 for group 2, 0 of 54 for group 3, and 0 of 42 for group 4; P < .001). IHS was much more likely to occur during the first year on therapy (OR, 15.1; P < .001).

DISCUSSION

Semen is the most common vector for HIV transmission, and the semen viral load is an independent predictor of HIV transmission risk [1]. While sART generally reduces the semen viral load to undetectable levels, IHS may be seen despite an undetectable blood viral load, occasionally at levels high enough to represent a significant transmission risk [8]. The present study evaluated the ability of an iART regimen, defined as the addition of 2 antiretroviral drugs with enhanced semen penetration to sART, to reduce the incidence of IHS during the first 6 months of therapy. We confirmed that both maraviroc and raltegravir were present in the semen at high levels, as has been shown in previous studies [22, 23]. In addition, we found that the incidence of IHS was significantly reduced in participants receiving iART, generally due to the prevention of low level semen HIV-1 RNA shedding (ie, <1000 HIV RNA copies/mL). (ie, <1000 HIV RNA copies/mL). However, one participant demonstrated high-level IHS (often >10 000 HIV RNA copies/mL) that persisted for 14 months.
after starting therapy. This indicates that sexual transmission of HIV might still be possible on occasion despite effective ART with an undetectable blood HIV load, although semen infectivity was not directly assessed [6].

The cause of IHS is not known. In this one individual, we found no drug resistance mutations or evidence of viral evolution despite more than a year of sustained, high-level semen viral RNA in the presence of therapeutic semen and blood drug levels. We hypothesize that the virus may have originated from a latent mucosal reservoir without active replication cycles, perhaps due to the activation of immune cells in the genitourinary mucosal compartment that contains integrated HIV DNA. This would be in keeping with previous studies showing that IHS in men receiving sART was associated with transient, compartmentalized T-cell activation [14] and may be plausible in the semen compartment, since drugs that act after integration (ie, protease inhibitors) were present at very low or undetectable levels [8], while levels of several drugs that act before integration (ie, lamivudine, maraviroc, and raltegravir) exceeded those seen in blood [8].

Which cell subset constitutes the mucosal HIV reservoir, if such a reservoir exists, is not known. IHS became progressively less common with an increasing duration of effective ART, with no IHS seen beyond 3 years of ART. We hypothesize that the size of the genital reservoir may decrease over this period in the absence of productive virus replication, as has been seen in both the blood and gut reservoirs of individuals receiving ART [29]. However, this decay in other reservoirs is too slow to permit eradication; whether this is also the case for the putative genital reservoir requires future study. The high virus levels seen during some IHS episodes suggest that the mechanism is distinct to that of low-level viral RNA “blips” in blood, which are thought to reflect random biological and statistical variation around a mean HIV level that falls just below the sensitivity of current clinical assays [30].

Individuals initiating an iART regimen were more likely to achieve virologic suppression in semen (HIV RNA load, <300 copies/mL) by 2 weeks, in keeping with prior studies showing delayed semen virus suppression in men receiving an efavirenz-based ART regimen [21]. However, the majority of participants in both the sART and iART arms had an undetectable semen HIV load within 2 months of starting treatment, so it is not clear that the marginal benefit of more rapid semen virus suppression in iART-treated participants would have any significant public health benefit as compared to sART in reducing secondary transmission of HIV. The fact that
participants with IHS had significantly lower semen (and blood) levels of raltegravir suggests that low semen levels of this medication may play a role, although in the 2 iART recipients with IHS there was no difference in raltegravir levels between visits with and visits without IHS, and clinical trials have shown no significant association between blood viral load suppression and raltegravir trough levels [31, 32].

The sample size of groups treated with iART and sART was relatively small, and data regarding IHS frequency in sART-treated individuals come from historical controls in a previously published study [8]. This introduces potential bias and reduces, but cannot wholly prevent, high-level IHS. Whether effective ART has been documented by many groups [7, 8, 33–36], with at least 1 case of transmission documented in this context [37]. We previously described the clinical and mucosal content of the manuscript have been disclosed.

In summary, the persistence of HIV RNA in semen despite effective ART has been documented by many groups [7, 8, 33–36], with at least 1 case of transmission documented in this context [37]. We previously described the clinical and mucosal associations of IHS in men receiving effective ART [8, 14] and now provide observational evidence that an iART regimen reduces, but cannot wholly prevent, high-level IHS. Whether the phenomenon of IHS relates to actual transmission is not known. Although the HIV RNA levels seen in one individual during effective iART were well above a previously defined in vitro threshold for HIV infection [12], it is important to note that the frequency of high levels of HIV in semen was reduced by 89% in participants receiving iART and by 92% in participants receiving sART, compared with previously described ART-naïve men [8]. This implies that ART, whether provided as a regimen of sART or iART, will have a substantial effect in reducing HIV sexual transmission early after ART initiation, as has been shown in both observational and randomized clinical studies [2, 13]. It is also encouraging that IHS was not observed after prolonged ART, regardless of initial regimen intensification. Nonetheless, the biological basis of IHS, including the nature of a possible HIV reservoir within the semen compartment, merits further investigation.

Notes

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