MAJOR ARTICLE

Long-Term Persistence of Transmitted HIV Drug Resistance in Male Genital Tract Secretions: Implications for Secondary Transmission

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(See the editorial commentary by Stekler and Coombs, on pages 336–8.)

Background. Transmitted drug-resistant HIV slowly reverts in the blood to drug-sensitive virus. The environment of the male genital tract (MGT) may result in even slower rates of reversion to drug susceptibility.

Methods. We measured the decay of resistance in longitudinally collected blood and semen samples from 5 individuals newly infected with HIV containing resistance mutations to nonnucleoside reverse-transcriptase inhibitors (NNRTIs). We also investigated the sexual transmission of HIV to and from these participants.

Results. In 3 of the 5 individuals, NNRTI resistance persisted in blood and semen throughout follow-up (mean, 296 days after the estimated day of infection [EDI]). In the other 2 individuals, NNRTI resistance persisted in blood and semen for 871 and 1179 days after the EDI; however, even after NNRTI resistance had fully reverted in blood, it remained readily detectable in semen. Two transmission groups were identified among these participants—one as the recipient partner and the other as the source partner.

Conclusions. Transmitted drug-resistant HIV, which persists in blood for years, may revert to wild type even more slowly in the MGT. This prolonged persistence in the MGT may contribute to the high prevalence rates of transmitted drug resistance.

Under selection pressure from antiretroviral medication, drug-resistant HIV can emerge [1]. When antiretroviral pressure is removed, the circulating population of drug-resistant HIV is replaced by drug-susceptible (wild-type) strains [2–4]. This process of reversion may occur at different rates in various anatomic compartments (blood, central nervous system, and genital tract) [5–9]. The unique environment of the male genital tract (MGT), compared with blood, may result in slower viral replication kinetics and distinct selective pressures, leading to slower rates of reversion of resistance to wild type [5, 10, 11]. In such a scenario, the MGT may function as a long-lived sanctuary for high levels of drug-resistant virus, which can then be transmitted by sexual exposure. This may explain the recent reports of the high prevalence of transmitted drug resistance (TDR) in North America [12–14], given that the majority of these HIV transmissions occur from exposure to HIV in male genital secretions.

To evaluate the persistence of drug-resistant HIV in the MGT, we investigated the rates of reversion of mutations associated with nonnucleoside reverse-transcriptase inhibitor (NNRTI) resistance in blood-derived and semen-derived HIV RNA in individuals identified as having TDR during early HIV infection. To evaluate the transmissibility of drug-resistant HIV from the MGT, we investigated 3 events of drug-resistant HIV transmission after reported sexual exposure.

PARTICIPANTS, MATERIALS, AND METHODS

In these investigations, 2 sets of data were analyzed: (1) longitudinal data from 5 recently infected participants

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to determine the persistence of TDR in the MGT and (2) data from 3 transmission events of drug-resistant virus om which the donor and recipient were known. The investigations into the transmission events involved 2 individuals who also participated in the studies of the persistence of TDR. Written, informed consent was obtained from all patients, and the human experimentation guidelines of the US Department of Health and Human Services and the individual institutions were followed in conducting this research.

Participants with transmitted drug resistance. Five newly HIV-infected men enrolled in the San Diego cohort of the Acute Infection and Early Disease Research Program (AIEDRP) were identified as having transmitted resistance-associated mutations to NNRTIs (table 1). Participants were monitored while they deferred antiretroviral therapy. At study enrollment, participants were found to be negative for urethral gonorrhea and chlamydia (LCx STD system; Abbott Laboratories), syphilis (rapid plasma reagin), and active genital herpes (physical examination). No participant reported symptoms to suggest the acquisition of a sexually transmitted infection during the study. Dates of infection were based on a standardized protocol (available at: http://www.aiedrp.org) [15].

Transmission participants. Two individuals with NNRTI TDR, who participated in the persistence studies described above, also participated in the San Diego HIV Transmission study. One individual (subject 1, also recipient 1) recruited a sex partner (source 1) for participation whom he identified as potentially transmitting HIV infection to him (transmission pair 1). Another individual (subject 2, also source 2) identified 2 sex partners (recipients 2A and 2B) who developed acute HIV infection 9 days after sexual exposure (transmission pair 2). Each partner agreed to participate and was enrolled pursuant to local institutional review board standards. However, the 2 recipients (2A and 2B) in transmission pair 2 chose not to be monitored longitudinally and were not included in the reversion analyses, even though they also harbored TDR.

Sample collection and processing. Participants were asked to abstain from sex for at least 48 h before specimen collection. They submitted paired blood and semen samples at variable intervals during this time. Blood from peripheral venipuncture in acid-citrate-dextrose tubes was collected and processed within 2 h. Blood plasma (BP) was aliquoted, frozen, and stored at $-80°C$ until processing. Semen samples were collected in the morning by masturbation without lubrication. Two milliliters of viral transport medium (80% RPMI 1640, 9% fetal bovine serum, 9% penicillin/streptomycin, and 2% nystatin) was added to the samples at the time of collection [7]. Seminal plasma (SP) was separated from the seminal cells by centrifugation at 700 *g* for 12 min within 2 h of specimen collection. SP was stored at $-80°C$ until further processing.

HIV RNA extraction and quantitation. HIV RNA was extracted and quantified from 500 μL of BP in accordance with the manufacturer's protocol (50 copies/mL minimal level of detection; Amplicor HIV-1 Monitor Test; Roche Molecular Diagnostics). HIV RNA was extracted from 1 mL of SP from each sample by use of the Boom method (NucliSens Extractor; bioMeriéux) and then quantified (25 copies/mL minimal level of detection; Amplicor HIV-1 Monitor Test; Roche Molecular Diagnostics). HIV RNA quantities were normalized to ejaculate volume by subtracting the volume of viral transport medium [7].

Sequencing. The ViroSeq HIV genotyping system (Applied Biosystems) was used for population-based *pol* sequencing. This system includes components for RNA extraction from 500–1000 μL of sample fluid (BP and elution from SP extraction, as described above), followed by cDNA synthesis using Moloney murine leukemia virus reverse transcription. This was followed by polymerase chain reaction (PCR) amplification to generate a 1500-bp amplicon including the entire protease and first two thirds of the reverse transcriptase. Sequencing was performed on an ABI 3100 Genetic Analyzer. To avoid potential contamination, BP and SP samples were processed, amplified, and sequenced in separate areas and on separate days. Sequences were manually reviewed and edited using ViroSeq genotyping software (version 2.4.2; Celera Diagnostics). Genotypic drug resistance was interpreted using the algorithm

Table 1. HIV resistance-associated mutations in semen and blood of individuals identified with transmitted resistance who were followed up longitudinally.

	Resistance mutations					
Subject	NRTI	NNRTI	PI	Resistance testing performed in SP and BP, days after EDI	DSV first detected in SP, days after EDI	DSV first detected in BP, days after EDI
1	V118I, T215F	Y188L	D30N	95, 102	>102	>102
2		K103N		45, 74, 280, 444	>444	>444
3		K103N		475, 875, 1193	1193	875
4		K103N, Y181C		235, 1179	>1179	1179
5	T215Y	K103N	I84V, L90M	44, 342	>342	>342

NOTE. Subject 1 is also recipient 1, and subject 2 is also source 2 in the transmission investigations. BP, blood plasma; DSV, drug-sensitive virus; EDI, estimated date of infection; NNRTI, nonnucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; PI, protease inhibitor; SP, seminal plasma.

available with the ViroSeq program. A mixture at an amino acid residue within the viral population that was sequenced was determined by both the Basecaller program in the Viroseq package and through manual interrogation of the sequence electropherograms.

Transmission confirmation. Transmission was confirmed through maximum-likelihood phylogenetic analyses of the *pol* sequences. For transmission to be confirmed through phylogenetic analysis, viral sequences from partner pairs should cluster together (share a most common recent ancestor) among the background of other possible sources of the infection sufficiently strongly that the association could not be due to chance and should be so similar relative to the other sequences as to reject the possibility that transmission occurred through a third party. The sequence length necessary to detect and establish linkage of infections is dependent on the information content, specifically the average divergence between strains from unrelated infections, which is ~5% in conserved genes such as *pol* [16–18]. To ensure an unbiased assessment of linkage, background sequences from non–epidemiologically linked individuals who participated in the San Diego AIEDRP cohort were included in the phylogenetic analyses. Phylogenetic trees for *pol* sequences were obtained from a matrix of synonymous nucleotide distances, as is appropriate to avoid clustering by drug-resistance mutations that have arisen in epidemiologically unlinked infections. In confirmatory phylogenetic analyses, amino acid residue sites associated with drug resistance (for reverse transcriptase, K103, Y181, T215, V118, and Y188; for protease, D30, I84, and L90) were stripped from all sequences to evaluate linkage independent of resistance-associated mutations. Sequences were initially compiled, aligned, and edited in BioEdit using the Clustal W alignment tool [19, 20]. The alignment was then manually edited to preserve frame insertions and deletions. Phylogenetic analyses were performed using PHYLIP [21, 22], HyPhy [23], and FastDNAml [19] software. Phylogenetic trees were viewed with TreeView software [24].

RESULTS

Participants. All individuals lived in San Diego and reported sex with other men as their only HIV risk factor. They all enrolled into the AIEDRP cohort after having received a diagnosis of primary HIV infection. The average age of the participants at enrollment was 37 years (range, 22–59 years). All participants had NNRTI resistance by genotype when they enrolled in the AIEDRP cohort. Two individuals (subjects 1 and 5) had resistance to 3 classes of antiretrovirals by genotype (table 1).

Persistence of resistance. In 3 of the 5 participants, NNRTI resistance persisted in both blood and semen throughout follow-up (mean, 296 days after the estimated day of infection [EDI; range, 102–444 days after EDI). These individuals have been monitored for the shortest amount of time. The other 2 individuals started to display reversion to drug-sensitive strains, at least in blood, >800 days after the EDI. One individual (subject 3) had full reversion of his NNRTI resistance mutation (K103N) in blood and semen between days 875 and 1193 after the EDI; however, drug-sensitive virus was detectable in blood but not in semen 875 days after the EDI. Similarly, another individual (subject 4) had a mixture of resistant and wild-type sequences (K103N/K and Y181C/Y) 1179 days after the EDI in blood, but only a drug-resistant sequence was detected in semen (table 1).

Transmission. TDR commonly occurs by the transmission of a drug-resistant variant from an individual who developed drug resistance secondary to drug exposure. To investigate this, we identified the source partner (source 1) of an individual with TDR harboring high-level resistance to 3 classes of antiretrovirals (subject 1, also recipient 1) (table 1). The recipient with TDR (subject 1, also recipient 1) was enrolled in the study during acute HIV infection, probably within 12 weeks of HIV infection. His source partner (source 1) was enrolled in the study soon after, probably within 14 weeks of HIV transmission, which reportedly occurred after male-to-male anal sexual exposure. At the time of exposure, the source partner (source 1) had discontinued antiretroviral therapy (ART) ~6 months previously. He reported multiple different ART regimens.

Transmission linkage was confirmed with phylogenetic analysis of the *pol* sequences (data not shown). Analysis of HIV RNA extracted from the partner's BP and SP revealed that the circulating viral population in blood was a mixture of drug-resistant and drug-sensitive virus; however, only drug-resistant virus could be identified in semen (table 2). Phylogenetic analysis of the source and recipient *pol* sequences revealed all partner sequences to be very closely related (<1% genetic distance), consistent with the reported sexual transmission (data not shown).

As TDR becomes more prevalent, the transmission of drug resistance from individuals who had drug resistance transmitted to them (serial transmission of resistance) will also become

Table 2. Population-based *pol* sequencing of HIV extracted from blood and seminal plasma from transmission pair 1.

29 July 2002 samples	Reverse-transcriptase resistance mutations	Protease resistance mutations
Source 1		
Blood plasma	V118I, Y188L, T215F/L	D30D/N
Seminal plasma	V118I, Y188L, T215L	D30N
Recipient 1		
Blood plasma	V118I, Y188L, T215L	D30N
Seminal plasma	V118I, Y188L, T215L	D30N

NOTE. Blood and semen from the source and both recipients of transmission pair 2 harbored identical resistance patterns without mixtures; all had K103N.

more prevalent, especially the longer that resistant variants persist at high levels in the MGT. To investigate this scenario of TDR being serially transmitted, we identified 2 recipient sex partners (recipients 2A and 2B) with acute HIV infection and TDR (<4 weeks since the EDI) from a source partner (subject 2, also source 2). This source partner was previously identified as having TDR, and he remained antiretroviral naive (transmission pair 2). Transmission was confirmed through phylogenetic analysis of the *pol* sequences from blood and semen (data not shown). Similar to the first transmission pair, phylogenetic analysis of the source and recipient *pol* sequences revealed all partner sequences to be very closely related and to harbor the same resistance-associated mutation (K103N), consistent with reported sexual transmission (data not shown). Together, these transmission events demonstrate how the persistence of drug resistance in the MGT allows the transmission of drug resistance from source partners with either acquired resistance (transmission pair 1) or transmitted resistance (transmission pair 2).

DISCUSSION

These investigations confirmed previous reports that the MGT represents a different environment for HIV replication than the rest of the body. During HIV infection, the MGT can act as (1) a viral compartment with restricted gene flow and a slower molecular clock [5, 10], (2) a viral reservoir with viral persistence [25, 26], or (3) a drug sanctuary with variable antiretroviral penetration [7, 27, 28]. These characteristics allow for the possible development of drug resistance, its long-lived persistence, and its subsequent transmission [29]. Once transmitted, the drug-resistant variant reverts very slowly, over the course of years, to a drug-susceptible phenotype in blood [12, 30–32]. Here, we demonstrate that the drug-resistant variant persists at least as long in the MGT, which allows for a prolonged opportunity to transmit drug-resistant virus. The decay rate of resistance detectable by population-based sequencing indicated that NNRTIs can persist for >3 years in both blood and the MGT in some individuals. Drug resistance persisted longer in the MGT than in blood; however, given the small number of participants, the actual delay cannot be extrapolated to all individuals with TDR.

Population-based sequencing of *pol* using the Viroseq method allows for the detection of minority viral populations only if they comprise at least 30% of the total circulating population [33]. Most likely, if we were to use highly sensitive detection techniques, such as ligase chain reaction [34], allele-specific PCR [35], or terminal dilution PCR [36, 37], we would detect the persistence of NNRTI resistance in blood and semen for even longer [38]. Additionally, given that SP viral loads are generally lower than BP viral loads, as reviewed by Coombs et al. [40], population-based sequencing may be more susceptible to sampling bias [41]. Although this may be true, a more clinically significant persistence for transmission would be when the NNRTI resistance persisted at higher levels [39], which would be detected by our population-based sequencing methods.

These data demonstrate that drug-resistant HIV persists at higher proportions in the MGT longer that it does in blood. This long persistence could contribute to the high prevalence of TDR [12–14], as evidenced in transmission pair 1. Once transmitted, the resistant variants can remain in the circulating viral populations in blood and semen for >3 years at relatively high levels and, as seen in transmission pair 2, the persistence of TDR in the MGT also allows for the further transmission of drug resistance.

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