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Journal

Journal of Virology, 93(13)

ISSN

0022-538X

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Publication Date

2019-07-01

DOI

10.1128/jvi.01769-18

Peer reviewed

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

PATHOGENESIS AND IMMUNITY

Comparisons of Human Immunodeficiency Virus Type 1 Envelope Variants in Blood and Genital Fluids near the Time of Male-to-Female Transmission

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ABSTRACT To better understand the transmission of human immunodeficiency virus type 1 (HIV-1), the genetic characteristics of blood and genital viruses from males were compared to those of the imputed founding virus population in their female partners. Initially serodiscordant heterosexual African couples with sequence-confirmed male-to-female HIV-1 transmission and blood and genital specimens collected near the time of transmission were studied. Single viral templates from blood plasma and genital tract RNA and DNA were sequenced across HIV-1 *env* gp160. Eight of 29 couples examined yielded viral sequences from both tissues. Analysis of these couples' sequences demonstrated, with one exception, that the women's founding viral populations arose from a single viral variant and were CCR5 tropic, even though CXCR4 variants were detected within four males. The median genetic distance of the imputed most recent common ancestor of the women's founder viruses showed that they were closer to the semen viruses than to the blood viruses of their transmitting male partner, but this finding was biased by detection of a greater number of viral clades in the blood. Using multiple assays, the blood and genital viruses were consistently found to be compartmentalized in only two of eight men. No distinct amino acid signatures in the men's viruses were found to link to the women's founders, nor did the women's *env* sequences have shorter variable loops or fewer N-linked glycosylation sites. The lack of selective factors, except for coreceptor tropism, is consistent with others' findings in male-to-female and high-risk transmissions. The infrequent compartmentalization between the transmitters' blood and semen viruses suggests that cell-free blood virus likely includes HIV-1 sequences representative of those of viruses in semen.

IMPORTANCE Mucosal transmissions account for the majority of HIV-1 infections. Identification of the viral characteristics associated with transmission would facilitate vaccine design. This study of HIV strains from transmitting males and their seroconverting female partners found that the males' genital tract viruses were rarely dis-

Citation Williams-Wietzikoski CA, Campbell MS, Payant R, Lam A, Zhao H, Huang H, Wald A, Stevens W, Gray G, Farquhar C, Rees H, Celum C, Mullins JI, Lingappa JR, Frenkel LM, for the Partners in Prevention HSV/HIV Transmission Study Team. 2019. Comparisons of human immunodeficiency virus type 1 envelope variants in blood and genital fluids near the time of male-to-female transmission. J Virol 93:e01769-18. https://doi.org/10.1128/JVI.01769-18.

Editor Viviana Simon, Icahn School of Medicine at Mount Sinai

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Received 8 October 2018
Accepted 15 March 2019
Accepted manuscript posted online 17 April 2019
Published 14 June 2019

tinct from the blood variants. The imputed founder viruses in women were genetically similar to both the blood and genital tract variants of their male partners, indicating a lack of evidence for genital tract-specific lineages. These findings suggest that targeting vaccine responses to variants found in blood are likely to also protect from genital tract variants.

KEYWORDS coreceptor usage, genetic bottleneck, HIV, heterosexual transmission, N-linked glycosylation, viral compartmentalization

Human immunodeficiency virus type 1 (HIV-1) has a remarkable tolerance of genetic variation; however, observational studies show that HIV-1 transmission results in marked genetic bottlenecks (1–6). Studies of plasma HIV-1 in recently infected individuals find that most new infections are founded by a single beta-chemokine receptor 5 (CCR5)-tropic viral variant (2, 4, 7). The transmission fitness of viral variants appears to be heavily influenced by viral fitness inferred from sequence conservation (6) but may vary by viral subtype, gender, stage of disease, HIV-1 RNA load, route and direction of transmission, male circumcision status, the presence of other sexually transmitted infectious agents, and the integrity of the immune system in the uninfected individual (2, 6, 8–20). Characterization of the viral population from the tissue or bodily fluid source of infection has been investigated in small studies (3, 21) and may help define the antigenic requirements of a vaccine to prevent the transmission of HIV-1 across mucosal membranes.

In the case of male-to-female HIV-1 transmissions, the founder virus is generally derived from exposure to the semen of the HIV-1-infected partner. HIV-1 is present both in seminal mononuclear cells and as cell-free virus in seminal plasma (3, 21), with previous studies using model systems demonstrating that both cell-associated and cell-free viruses are capable of establishing infection (22–24). While comparative *ex vivo* studies suggest that cell-free virus in human semen is more infectious than cell-associated virus, the few studies that have closely evaluated homosexual transmission *in vivo* have implicated both cell-associated and cell-free virus (25–27). Such studies are challenging in part due to the need to evaluate samples collected from both partners in the sexual transmission dyad close to the time of transmission.

In this study, we used samples from African HIV-1-serodiscordant couples (in which the male partner was HIV-1 infected and the female partner was uninfected at the time of enrollment) to compare the genetic characteristics of HIV-1 envelope glycoproteins (gp160) in non-subtype B heterosexual male-to-female transmission pairs by tissue source, including seminal plasma, seminal cells, and blood. In particular, we sought to assess whether cell-free virus in seminal plasma is favored for transmission in this cohort.

RESULTS

Study group. Twenty-nine phylogenetically confirmed male-to-female HIV-1 transmission pairs were evaluated. All individuals were of sub-Saharan African origin (Kenya, Uganda, or Zambia) and had non-subtype B HIV-1 infections. Seventeen pairs had specimens available for sequence analysis, and eight pairs yielded sufficient viral sequences for analyses from both blood and genital tissues (Table 1). The larger group of 17 males' blood and semen had a median of 5.2 $\log_{10}$ copies/ml (range, 2.6 to 5.8 $\log_{10}$ copies/ml) and 3.6 $\log_{10}$ copies/ml (range, 2.7 to 5.8 $\log_{10}$ copies/ml), respectively, which were 0.3 to 0.6 $\log_{10}$ copies/ml lower, respectively, than those in the blood and semen of the final group of eight (Table 1). Thus, the possibility exists that our study was biased to men with higher seminal viral loads. However, the nine men not used in the final analysis had other specimen limitations. Their seminal plasma volumes available to be studied were often low (down to <1 μl), and the seminal plasma was often previously thawed. The eight men that we studied had median HIV RNA blood and seminal plasma loads of 5.5 and 3.4 $\log_{10}$ copies/ml, respectively, which suggests that the specimens from these individuals were representative of the specimens in the

TABLE 1 Male-to-female transmission study participant demographics and laboratory characteristics

Partner pair	Location	Age (yr)		Log_{10} HIV-1 RNA load (no. of copies/ml)			HIV-1 subtype	Time (mo) separating collection of partners' specimens
		Transmitter	Seroconverter	Transmitter plasma[a]	Transmitter semen[a]	Seroconverter plasma		
1	Ndola, Zambia	29	27	5.5	3.5	4.9	C	1.3
2	Kampala, Uganda	36	21	4.8	4.9	5.4	A1	2.6
3	Kampala, Uganda	30	25	5.8	4.9	4.5	A1	14.7
4	Kampala, Uganda	32	29	5.5	2.9[b]	5.1	A1C	7.3
5	Kisumu, Kenya	23	19	4.8	3.0	5.1	A1D	10
6	Kampala, Uganda	26	19	5.1	3.4[b]	4.1	D	7
7	Kampala, Uganda	32	27	5.7	TND[b,c]	5.7	A1D	6
8	Kampala, Uganda	26	21	5.6	2.6[b]	3.6	D	0.5
Median		29.5	23.0	5.5	3.4	5.0		7

[a]RNA was measured in most seminal plasma samples and from a blood plasma sample collected at the same visit.
[b]HIV-1 RNA data were not assessed by clinical assay; values were calculated from limiting dilution PCR amplification.
[c]TND, target not detected.

greater cohort. The median HIV-1 RNA level in the blood plasma of the females was 5.0 log_{10} copies/ml. The median age of the transmitting males in the group of eight was 29.5 years, and the median age of their female partners was 23.0 years.

Yield and features of HIV-1 *env* sequences. A median of 21.5 full-length HIV-1 *env* sequences summed across three tissues (blood plasma, seminal plasma, semen cells) were obtained from each transmitting male, and a total of 22.5 were obtained from their female partners' blood and cervical os (Table 2). CXCR4 (X4)-tropic viral genotypes were detected in 4 of the 8 transmitting men; these were found as minority variants ($<$5%) in three men and as the majority variant in the blood and semen of one man (Table 2). All viruses from the seroconverting female subjects were predicted to be CCR5 tropic.

As expected for newly infected individuals, the diversity of sequences from the 8 seroconverting females was low, with 7 of 8 having a median genetic diversity of $<$1% across all fluids sampled. As the participants' fluids were not sampled at the moment of transmission, a most recent common ancestor (MRCA) derived from seroconverting female sequences was used as the imputed founder of infection. One woman (partner pair 3) had two founder variants (see Fig. 2C) that had a median of 2.70% divergence but only 0.4% to 1.0% divergence within each variant population. All viral populations

TABLE 2 HIV-1 gp160 single-genome amplification, predicted CCR5, CXCR4 coreceptor usage, and nearest transmitted founder variant

Partner pair	Transmitter				Seroconverter		
	No. of sequences			No. of CXCR4 sequences/total no. of sequences (% of CXCR4 sequences)	No. of sequences		Genetic distance (%) of seroconverter's MRCA to nearest transmitter variant[a]
	Blood plasma RNA	Seminal plasma RNA	Seminal cellular DNA		Blood plasma RNA	Cervical nucleic acids[f]	
1	18	9	1	0/38 (0)	10	2	0.44
2	13	4	20[b]	1/37 (2.7)	0	15	1.13
3	13[b]	21	3	1/37 (2.7)	14	18	1.50
4	13	17	19	0/49 (0)	14	16	1.10
5	11	2	0	0/13 (0)	6	0	1.99
6	13	16[b]	0	1/29 (3.4)	0	15	1.57
7	12	0	4	0/16 (0)	8	17	2.20
8	15[c]	5[d]	15[e]	28/35 (80)	0	17	1.71
Median	13.0	6.5	2.0		7.0	15.5	1.54

[a]Pairwise genetic distances measured between the transmitter's sequences and the seroconverter's imputed MRCA.
[b]A single CXCR4 variant was detected.
[c]Twelve CXCR4 variants were detected.
[d]Three CXCR4 variants were detected.
[e]Thirteen CXCR4 variants were detected.
[f]Sequences derived from total nucleic acids, including DNA and RNA.

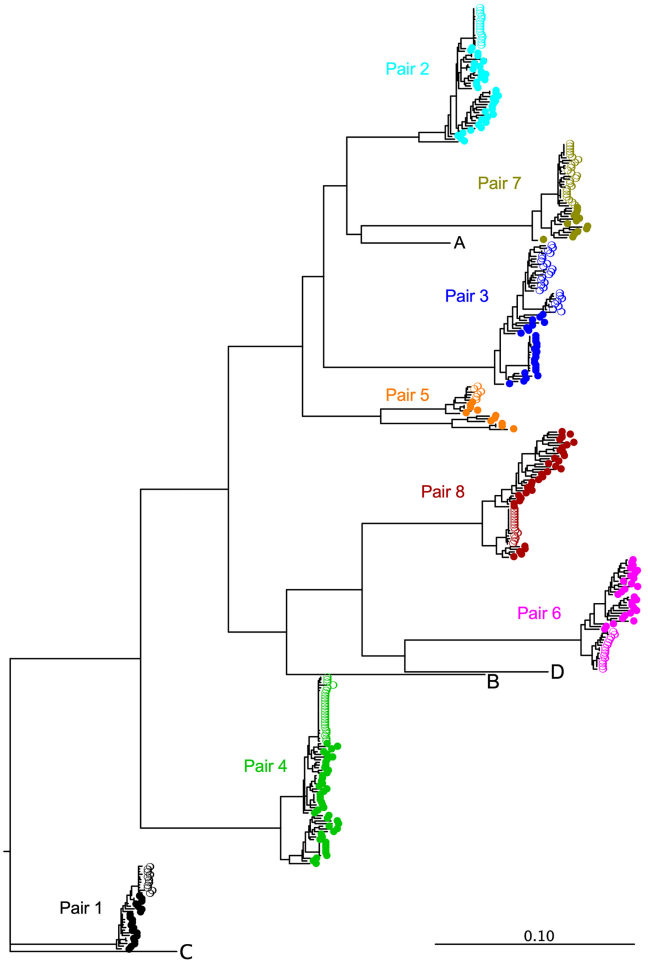

FIG 1 Maximum likelihood phylogenetic analyses of HIV-1 gp160 sequences from all male-to-female transmission pairs. Unaligned, hypervariable regions were removed from the alignment. Generalized time-reversible phylogenetic maximum likelihood trees of the transmitting male's blood plasma RNA, semen RNA, and semen DNA (■) and the seroconverting female's blood plasma RNA and cervical sequences (○), derived by single-genome sequencing, are shown. The scale bar indicates the horizontal branch length, corresponding to 1 substitution per 100 sites. Teal, partner pair 1; blue, partner pair 2; red, partner pair 3; orange, partner pair 4; magenta, partner pair 5; brown, partner pair 6; green, partner pair 7; black, partner pair 8.

in the men were more diverse than those in their female partners ($P = 0.041$) (data not shown).

V1V2 variable region lengths were not different among the transmitters (median = 69 amino acids) and seroconverters (median = 70 amino acids) or within transmission partners (the transmitters' V1V2 regions were, on average, 1 amino acid shorter; range, −7.5 to 7 amino acids shorter). Similarly, no difference was found between the number of N-linked glycosylation sites across the five *env* variable regions between transmitter (median = 11) and seroconverter (median = 10) viruses ($P = 0.08$) or between genital and blood viruses ($P = 0.35$). No distinct sequence signatures distinguished the viruses from seroconverting women from viruses from their transmitting partners.

Compartmentalization analyses of HIV-1 *env* within men and women. Phylogenetic analysis revealed that sequences from each pair represented a distinct clade, confirming transmission linkage and a lack of sample mix-up across pairs (Fig. 1).

Maximum likelihood phylogenetic trees (Fig. 2) and distance matrices were also generated for each of the 8 individual transmission pairs. Compartmentalization of the men's blood and semen HIV sequences was then evaluated by six different methods. First, permutation tests of HIV-1 gp160 sequence distances in semen RNA versus semen

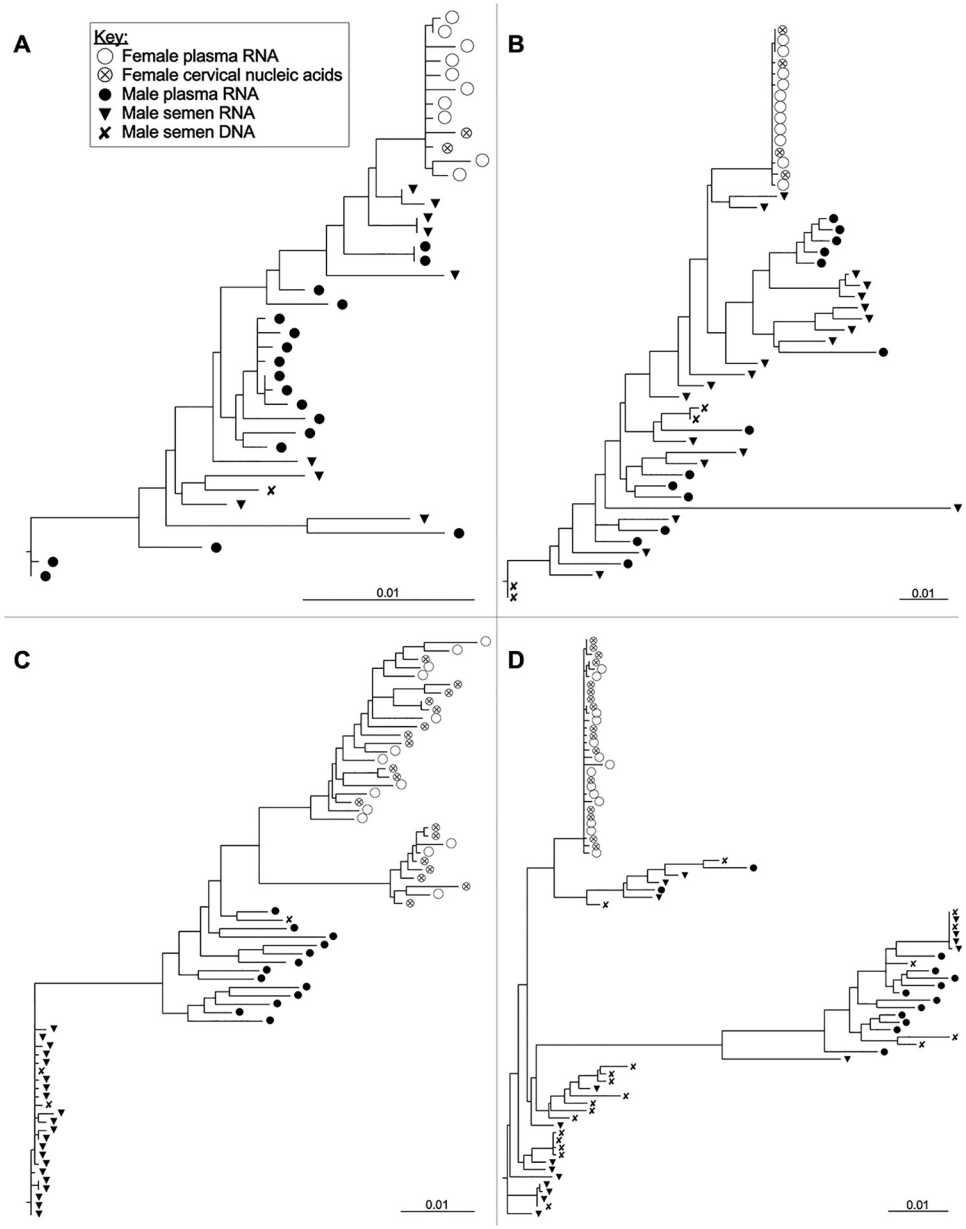

FIG 2 Maximum likelihood phylogenetic analyses of HIV-1 *env* gp160 sequences from male-to-female transmission pairs. Generalized time-reversible phylogenetic maximum likelihood trees of HIV *env* sequences derived from each of the eight partner pairs are shown, with pairs labeled A to H, respectively. Hypervariable regions were included in the alignments. See the key for symbol designations. Phylograms were rooted using subtypes A (GenBank accession number AF484509.1), B (GenBank accession number K03455.1), C (GenBank accession number AY772699.1), and D (GenBank accession number U88824.1) sequences from GenBank (not shown). Bootstrap proportions of ≥75% are labeled with an asterisk. The scale bar indicates the horizontal branch length, corresponding to 1 substitution per 100 sites.

DNA sequences and in semen (pooled RNA and DNA) versus blood sequences were performed. The genetic distances between seminal plasma RNA and seminal mononuclear cell DNA sequences were not significantly greater than the distances within those samples ($P = 0.27$), and thus, seminal RNA and DNA were considered a single compartment for comparison with blood viral sequences in the men. The mean genetic distances between blood and semen sequences were significantly greater than the distances within either tissue ($P < 0.001$), suggesting the occurrence of local virus production or some compartmentalization between blood plasma and semen.

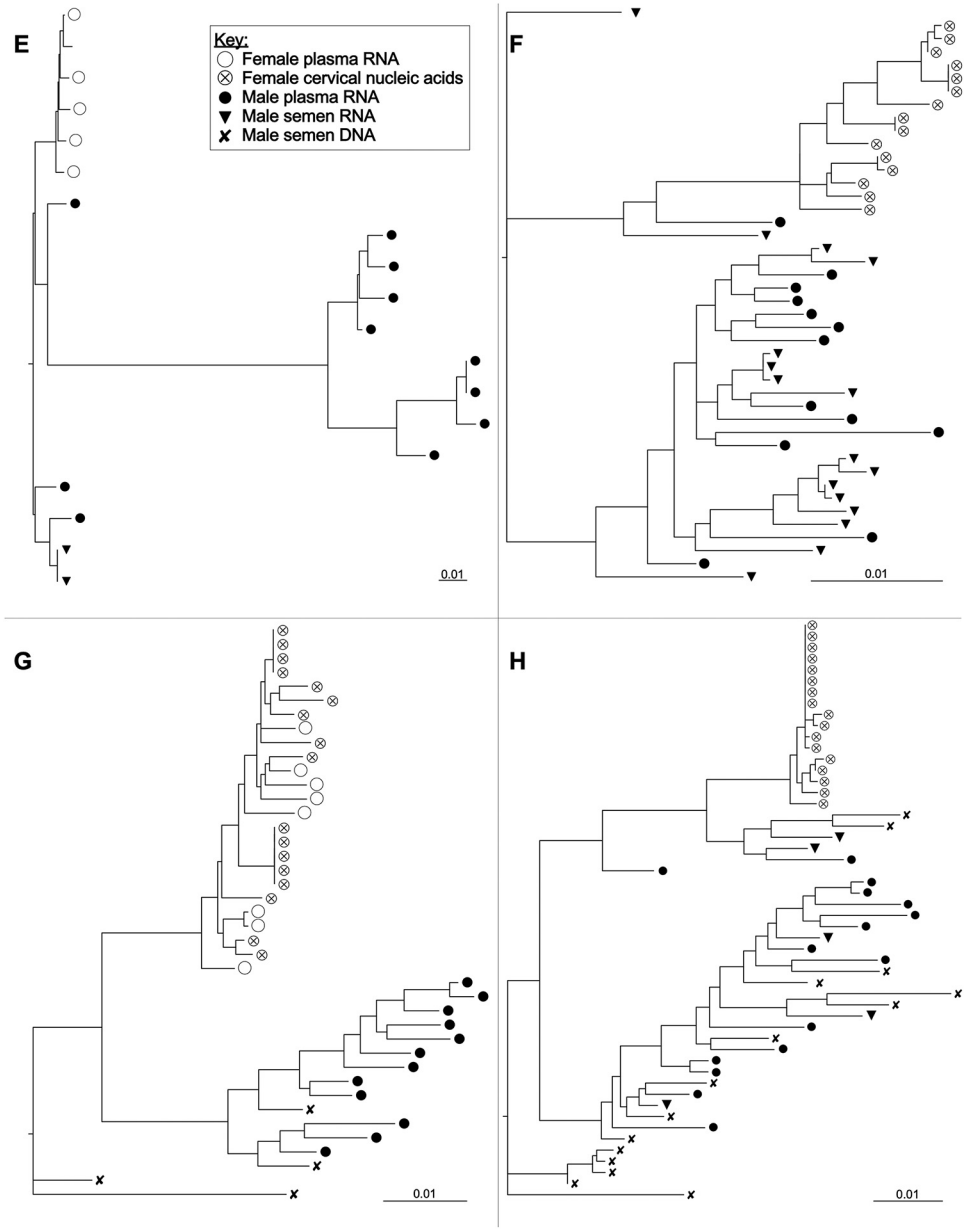

FIG 2 (Continued)

Permutation testing of blood versus genital tract viral sequences found compartmentalization of the viral sequences in one of the eight men (partner pair 3). The other seven men showed intermixing of HIV-1 blood- and semen-derived sequences. Similarly, no compartmentalization was observed between the genital tract and blood viruses from the four women from which both virus populations were sampled.

Additional analyses of each male's blood versus semen viruses performed by the use of multiple models (28) indicated compartmentalization by a majority of tests in three of eight men (from partner pairs 1, 3, and 4) and by only one of five models for men in pairs 2 and 8 (Table 3, left columns). Because bursts of viral replication would release multiple identical (or similar) viruses, the identical sequences were collapsed into one and the data were reanalyzed. These analyses consistently found compartmentalization in pairs 3 and 4, and compartmentalization was otherwise detected by one of five models in pair 5 (Table 3, right columns).

TABLE 3 Evaluation of HIV-1 *env* compartmentalization between blood plasma and pooled semen sequences[a]

Partner pair	*P* value[b]									
	All blood plus semen sequences					Identical sequences collapsed				
	Tree based			Distance based		Tree based			Distance based	
	r_b	r	SM	S_{nn}	F_{ST}	r_b	r	SM	S_{nn}	F_{ST}
1	0.995	**0.006**	0.009	**<0.001**	**0.001**	0.131	0.080	0.115	0.028	0.054
2	0.953	0.048	0.015	**0.002**	0.379	0.393	0.021	0.010	0.018	0.392
3	**0.001**	**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
4	0.992	0.010	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**0.001**	**<0.001**
5	1	0.020	0.037	0.014	0.098	0.172	0.367	1.000	0.620	**<0.001**
6	0.936	0.068	0.055	0.066	0.039	0.026	0.019	0.140	0.732	0.017
7	0.784	0.228	0.021	0.061	0.022	0.014	0.203	0.028	0.085	0.024
8	0.998	**0.004**	0.093	0.051	0.221	0.107	0.011	0.097	0.021	0.200

[a]Viral compartmentalization was assessed by 5 methods, including tree-based methods that determined the Critchlow correlation coefficients (r_b and r) and the Slatkin and Maddison measure (SM) and distance-based methods that determined the nearest neighbor (S_{nn}) and the fixation index (F_{ST}).
[b]*P* values of <0.01 (in bold) were considered significant after a Bonferroni correction for multiple comparisons.

Linkage of male sequences to the imputed founder variants in their partners. Permutation testing of the nine pooled transmission clusters (two were from the woman in partner pair 3) showed that the mean genetic distance from the imputed female founder to the male semen sequences was significantly less than that to the blood plasma sequences ($P < 0.001$). These results appear to be largely driven by partner pairs 4 and 5 (Fig. 3), which had two plasma clades, and one of the males' clades was more closely related to the female founder than the other clade. A distance analysis within individual pairs shows that the closest sequence to the imputed female founder virus was approximately equally likely to be present in each male's semen or blood viral population.

DISCUSSION

HIV-1 *env* genotypes were studied in eight couples who were enrolled when they were found to be serodiscordant for HIV-1 infection and in whom the male sexual partner was found to have transmitted the virus to the female sexual partner. Our goal was to identify the probable source of virus (cellular or cell-free virions) and the sequence characteristics that were most closely related to those of the founder virus in the females and that may have allowed certain viral variants to establish infection. Our primary findings were that (i) the male donors' blood and semen viruses were infrequently compartmentalized, and thus, it was not possible to distinguish these two potential sources; (ii) female founder viruses were exclusively CCR5 tropic, despite the presence of CXCR4-tropic variants in the male partners; (iii) most women had a single

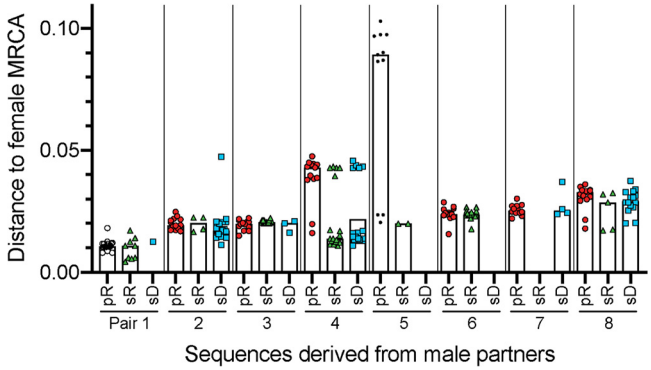

FIG 3 Genetic distances of male partner sequences to the imputed founder of infection in the female seroconverters. Pairwise genetic distances from each male-derived HIV *env* gp160-coding sequence relative to the imputed founder sequence in their female partners are shown. The founder was taken to be the calculated most recent common ancestor (MRCA) of infection. Male-derived sequences corresponded to plasma RNA (pR), semen RNA (sR), and semen DNA (sD).

founder; and (iv) while we found that the genetic distance of semen viruses to the partner's founder viruses was less than that of blood viruses, this was due to some men harboring in their blood multiple HIV-1 clades that were not detected in their semen. Additionally, (v) no unique sequence signatures were identified across the HIV-1 *env* sequences of the women's founder viruses, including (vi) their V1V2 amino acid lengths and (vii) the number of potential N-linked glycosylation sites, which were not statistically significantly different from those for the viruses in their partners.

Male-to-female HIV-1 transmissions are presumed to occur primarily through vaginal sexual intercourse, with infectious HIV-1 existing in both a proviral state and a cell-free state in semen (21–24, 29). Consistent with this transmission route, we found the virus in semen to have less genetic distance than virus in blood to the imputed female founder virus. These closer distances to the semen viruses, while statistically significant, appeared to be driven in large part by a subset of males with two plasma virus clades, with one being more closely related to the female founder.

HIV-1 compartmentalization to the genital tract has been a concern to public health officials and vaccinologists. Discordant shedding from the genital tract of individuals with suppression of virus replication by antiretroviral treatment, as measured by the plasma HIV-1 RNA load, could promulgate transmission. Furthermore, if the viruses transmitted across mucosal surfaces differ from those in the blood, then identifying these differences would be relevant to facilitate vaccine design. To point out the complexity of examining compartmentalization, we tested our sequences by multiple methods. We used a permutation analysis across all the male participants and separately across the female participants to address the unequal sampling of sequences across participants' bodily fluids. In addition, we compared multiple other methods based on distance matrices and phylogenetic trees to assess compartmentalization. Our permutation method detected HIV-1 compartmentalization between blood and genital tract viruses in one male participant and no women. In contrast, the other methods (30–34) identified compartmentalization by all five tests in the man in whom compartmentalization was identified by the permutation analysis plus two additional men in whom compartmentalization was identified by three or four tests and two additional men in whom it was identified by one test. As phylogenetic analyses of each of these individuals showed small clades of genetically similar and/or identical viruses, we interpreted these sequences to be suggestive of a burst of virus replication or cell proliferation. To account for the bias that this can bring to compartmentalization analysis, we reanalyzed the data after collapsing identical sequences into one representative sequence. These results found consistent compartmentalization signals in two men but not in the three others (plus, one additional man's sequences were positive in one of five tests). Furthermore, our and others' previous studies demonstrated that compartmentalization could largely be attributed to monotypic (identical) HIV-1 variants in cross-sectional comparisons of genital tract sequences (3, 35), as this apparent compartmentalization did not persist over time in longitudinal studies, indicating the relatively free flow of viruses between the blood and genital tract (36, 37).

Our detection of exclusively CCR5 coreceptor-utilizing (R5) founder viruses, despite the presence of CXCR4-tropic variants in the transmitter, is consistent with others' studies (2, 7). Previous work has identified multiple possible gatekeeping mechanisms that permit infection of T lymphocytes, macrophages, Langerhans cells (LCs), and, possibly, dendritic cells (DCs) by R5 viruses during penetrative vaginal sexual transmission and that are hypothesized to explain the low rate of transmission of X4-tropic strains, despite their presence in their transmitting partner (38–44): expression of CCR5 but not CXCR4 on the surface of spermatozoa, hypothetically enabling the shuttling of virus in semen to target cells in the upper female genital tract (45); epithelial cell secretion of the CXCR4-binding chemokine SDF-1, blocking access of X4 variants beneath the epithelial cell layer (40); enrichment of CCR5-expressing T cells in cervical tissues (39); extension of LCs and DCs, which are responsible for antigen presentation to T cells exclusively expressing CD4 and CCR5, into the vaginal lumen (39); the presence of CCR5-expressing cells at higher concentrations than CXCR4-expressing

cells in the epithelial layer (45); and the more efficient opsonization and neutralization of X4 variants within the transmitter, rendering such viruses noninfectious (38).

We did not detect selection for shorter V1V2 loop lengths in the seroconverters, nor did we detect fewer potential N-linked glycosylation sites (PNLGS) across the 5 variable regions of gp120. This is in agreement with the findings of prior studies of clade B infections (46–48) but not with those of non-clade B infections (1, 49). A meta-analysis of transmission pairs found that amino acids associated with the stabilization of the Env protein and immune escape were more prevalent in the founder viruses of men but less so in founders of women and couples at high risk of transmission (6). The men that we studied appeared to have acquired HIV-1 infection relatively recently, given their low viral diversities (medians, 1.04%, 1.84%, 1.89%, 3.43%, 8.79%, 1.40%, 1.55%, and 2.21%). As suggested in the meta-analysis, the recent HIV-1 acquisition would provide relatively less time for the selection of variable loop extension and PNGLS. Furthermore, the larger and more vulnerable mucosa of women, in whom sexually transmitted infections and trauma have been associated with the acquisition of HIV-1 infection (20), likely results in features of HIV-1 Env having less relevance in transmission.

While sequence signatures in HIV-1 *env* and *pol* have been reported to segregate between the cerebrospinal fluid or semen and blood plasma (37, 50, 51), no comparable signatures were identified among transmitted compared to nontransmitted variants in our participants. Generally, the imputed founder appeared to be linked to the most frequently observed male-derived variant, suggesting that, except for selection against X4-tropic variants, the founding populations resulted from stochastic events.

A critical limitation of our and other studies of HIV transmission is the difficulty in sampling viral populations at the time of HIV-1 transmission. Our couples had a range of 2 weeks to 1.2 years between the times of collection of paired samples from the partners. This time lapse likely prevented us from identifying the precise founder of infection and allowed time for the transmitted variant(s) to evolve toward a more ancestral and fit population (52), which was potentially the case in females from partner pairs 7 and 8, or to undergo immune selection. Another significant limitation of our study was the focus solely on HIV-1 *env* sequences, as analyses of infectious clones have shown greater transmission fitness encoded by residues encoded within multiple viral genes (6). Our study did not evaluate inflammation of the female genital tract, which, along with trauma, has been shown to lessen the genetic sequence bottleneck (20) and which is associated with an increased risk of HIV acquisition. Our study was also limited by our inability to amplify virus from the blood and semen of all of the 17 selected partner pairs with specimens available.

In summary, this study of male-to-female HIV-1 transmissions found a strong selection bottleneck for HIV-1 variants that utilize the CCR5 coreceptor, similar to the findings of previous studies (2, 7), but did not reveal characteristic differences in HIV-1 *env* sequences previously reported (1, 53). The absence of differences in the donor and recipient HIV-1 *env* sequences is most likely due to the recency of the male transmitter acquisition of HIV infection. Our observation of an overall tendency for less genetic distance between the female founder viruses and their partners' genital tract variant than between the female founder viruses and their partners' blood variants, while suggestive of compartmentalization, appeared to be driven by detection of multiple viral clades in the blood but not the genital tract of two of the eight men in our cohort. Furthermore, compartmentalization of virus between the blood and genital tract of men was infrequently observed by phylogenetic-based methods. This, combined with longitudinal studies showing that compartmentalization is often an artifact of cross-sectional sampling (36, 37), suggests that while founder viruses likely derive from genital variants, the relatively free exchange of viruses between the genital tract and blood indicates that the blood likely includes viruses representative of transmitted variants.

MATERIALS AND METHODS

Selection of participants. Male-to-female HIV-1 transmission events identified in African serodiscordant heterosexual couples from the Partners in Prevention HSV/HIV Transmission Study (54) and the Couples Observational Study (55) were selected for study. Written, informed consent was obtained from all participants and the research was conducted according to the principles in the Declaration of Helsinki. The following ethics committees reviewed the Partners in Prevention HSV/HIV Transmission Study (ClinicalTrials.gov no. NCT00194519) and the Couples Observational Study (55): from local and national African study sites, the Kenya Medical Research Institute Ethics Committee, Kenyatta National Hospital Ethics Committee, Kilimanjaro Christian Medical College, Moi University Ethics Committee, Republic of Botswana Ministry of Health, South Africa Medicines Control Council, Uganda National Council for Science & Technology, University of Witwatersrand Ethics Committee, and University of Cape Town Institutional Review Board; from site-affiliated international institutions, the Harvard School of Public Health, Indiana University Institutional Review Board, University of California San Francisco Institutional Review Board, and University of Washington Institutional Review Board. The University of Washington Institutional Review Board was also the institutional review board for the UW coordinating center application (University of Washington Human Subjects Division STUDY00000172 [formerly HSD no. 32528]). Transmission linkage between sexual partners was previously confirmed by analysis of HIV-1 *env* C2-V3-C3 and *gag* p17-p24 sequences from blood plasma (56). Partner pair selection criteria included the availability of semen specimens, a cervical os swab specimen within 3 months of diagnosis of HIV-1 infection, and peripheral blood plasma specimens from both partners within 6 months of infection.

Specimen processing, HIV-1 RNA quantification, and nucleic acid extraction for sequence analysis. Semen samples were centrifuged within 4 h of collection at 600 to 800 $\times$ *g* for 10 min to pellet the cells. Blood and seminal plasma HIV-1 RNA was quantified using a Cobas TaqMan HIV-1 (v1.0) system (Roche Diagnostics). Cervical HIV-1 RNA was not quantified.

Prior to sequencing, HIV-1 DNA was isolated from seminal cell pellets using an ArchivePure DNA purification kit (catalog number FP2300720; Fisher). DNA was eluted into 100 μl DNA hydration solution. HIV-1 RNA was extracted from blood plasma using silica (57). An elution volume of 70 μl was used for every 200 μl of extracted plasma. Seminal plasma was diluted 1:5 in RNase-free RPMI and then centrifuged for 1 h at 23,000 $\times$ *g* at 4°C to pellet the virions. Pelleted seminal virions were then extracted using the same protocols used for blood plasma. Seminal plasma RNA was treated with Turbo DNA-free DNase (catalog number AM1907; Ambion) following the kit's instructions to avoid amplification of cell-associated HIV-1 DNA that may have contaminated the specimen. Nucleic acids were extracted from endocervical swabs using a NucliSENS miniMag nucleic acid purification system (bioMérieux) following a pretreatment lysis with guanidinium thiocyanate. Due to difficulties recovering endocervical RNA following DNase treatment, RNA and DNA were jointly extracted for amplification and sequencing.

Two primers were used for reverse transcription of gp160: oligo(dT) and an HIV-1 envelope-specific primer (data not shown). cDNA was synthesized using 10 μl of RNA and a TaKaRa BluePrint First Strand synthesis kit (catalog number 6115; Clontech) according to the manufacturer's protocol.

Single-genome amplification (SGA) by PCR and DNA sequencing. Amplifiable copies of the gp160 region were quantified by limiting dilution of the nucleic acid followed by nested PCR (58). DNA and cDNA were diluted across 96-well plates so that they contained an average of 0.3 copy/well. HIV gp160 was PCR amplified using an Advantage 2 PCR kit (catalog number 639201; Clontech) to create a 3.1-kb first-round product, followed by a second round of PCR, using primers whose sequences are available from the authors, resulting in a 2.7-kb product.

PCR products were identified on agarose gels and purified, using the ExoSAP-IT reagent (catalog number 78201; Affymetrix), to remove primers and deoxynucleoside triphosphates prior to sequencing. Dideoxynucleotide sequencing was performed using primers corresponding to the HIV-1 subtype infecting each individual (data not shown), with forward and reverse coverage across the full length of gp160. Sanger sequencing was performed on an ABI 3730xl DNA analyzer by the University of Washington High-Throughput Genomics Center.

Data analysis. Sequence data files were assembled using Geneious (v6.1.3) software to produce 2.6- to 2.7-kb gp160 contigs (59) and evaluated for mix-ups using an all-inclusive maximum likelihood phylogenetic analysis (Fig. 1). Sequence assemblies containing more than a single position with mixed bases were interpreted to be from $\geq$2 viral templates and were omitted from further analysis. Maximum likelihood phylogenetic trees were generated using the PhyML tool within Geneious software and the generalized time-reversible model of evolution (60). Genetic distances between sequences and between an imputed most recent common ancestor (MRCA) were calculated for each subject using the DIVEIN web server (61).

Permutation testing of HIV-1 gp160 sequence distances by source compartment was performed to evaluate tissue compartmentalization in semen RNA sequences versus semen DNA sequences and in semen (pooled RNA and DNA) sequences versus blood sequences. For each transmitting man, the mean distance between sequences from the same compartment was subtracted from the mean genetic distance between two sequences sampled from different compartments. The test statistic was the mean of this quantity across individuals, weighted by the number of distances contributed by each individual. The null distribution of the test statistic was obtained under within-person permutation of viral sequence compartment labels. *P* values were calculated as the proportion of test statistic values under permutation that were less than or equal to the observed test statistic value. Similarly, permutation testing was used to compare the distances between the respective imputed MRCA of the seroconverting woman and the blood versus seminal sequences of the transmitting man. The test statistic for this analysis compared the mean genetic distance between the MRCA and male blood sequences to the mean genetic distance

between the MRCA and male semen sequences. Compartment labels were permuted, and *P* values were computed as described above.

Compartmentalization between each man's blood and semen was also analyzed using three tree-based methods that determined the Critchlow correlation coefficients (r_b and r) (30) and the Slatkin and Maddison measure (SM) (31) and two distance-based methods that determined the nearest neighbor (S_{nn}) (32) and the fixation index (F_{ST}) (33). These methods were performed using HyPhy software (34). Following Bonferroni correction, *P* values of <0.008 were considered evidence of compartmentalization. These analyses were also performed after collapse of identical sequences into one taxon.

Viral coreceptor usage was predicted using the Geno2pheno system and the standard 10% false positivity threshold (62). The length of the V1V2 variable region and the number of potential N-linked glycosylation sites in blood plasma and genital tract sequences were determined using the HIV N-linked glycosylation site analyzer (63). Env gp160 amino acid sequences were aligned, and the VESPA program (64) was used to identify signature sequences between the male- and female-derived sequences within pairs. The sequences were then visually inspected and tallied for unique signatures across the seroconverting women that were present as minority variants in their corresponding transmission partner or found across multiple seroconverting women.

Accession number(s). The HIV-1 sequences determined in this study were deposited in GenBank under accession numbers KU865691 to KU866086.

ACKNOWLEDGMENTS

We thank the men and women who participated in this study and the clinical personnel, without whom this study would not have been possible.

This study was supported by National Institutes of Health (NIH), National Institute of Allergy and Infectious Diseases, grants P01 AI057005 (principal investigator [PI], J. I. Mullins); Seattle Centers for AIDS Research Retrovirology and Molecular Data Sciences Core grant P30 AI027757 (PI, J. Baeten, University of Washington); the Bill & Melinda Gates Foundation (grants 26469 and 41185; PI, C. Celum); and NIH grant K08 AI074424 (PI, M. S. Campbell).

We have no conflicts of interest to disclose.

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