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Characterization of Human Immunodeficiency Virus Type 1 Populations Containing CXCR4-Using Variants from Recently Infected Individuals

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Abstract

We screened 150 individuals from two recent seroconverter cohorts and found that six (4%) had CXCR4-using viruses. Clonal analysis of these six individuals, along with a seventh individual identified during clinical care as a recent seroconverter, revealed the presence of both X4- and dual-tropic variants in these recently infected adults. The ability of individual CXCR4-using variants to infect cells expressing CD4/CXCR4 or CD4/CCR5 varied dramatically. These data demonstrate that virus populations in some newly infected individuals can consist of either heterogeneous populations containing both CXCR4-using and CCR5-tropic viruses, or homogeneous populations containing only CXCR4-using viruses. The presence of CXCR4-using viruses at early stages of infection suggests that testing for viral tropism before using CCR5 antagonists may be important even in persons with known recent infection. The presence of CXCR4-using viruses in a subset of newly infected individuals could impact the efficacies of vaccine and microbicide strategies that target CCR5-tropic viruses.

Numerous studies support the widely accepted viewpoint that CCR5-using (R5-tropic) HIV-1 dominates during the early stages of infection.[1–4] The protective effect of the CCR5 Δ32 homozygous mutation against HIV-1 transmission provides compelling support for the highly selective transmission, or outgrowth, of R5-tropic viruses.[5–9] Viruses that use CXCR4 exclusively (X4-tropic) or both CXCR4 and CCR5 (dual-tropic) typically emerge during later stages of disease.[10–12] The presence of CXCR4-using viruses (X4- and dual-tropic) has been associated with rapid $CD4^+$ T cell decline and accelerated disease progression.[1,13–16] Whether this decline is a cause or consequence of disease progression is not known. Documented cases of CXCR4-using viruses in individuals recently infected with HIV-1 have raised concern because of the well-established association between CXCR4-using viruses and disease progression.[17–19] It is unclear why R5-tropic viruses dominate early HIV-1 infection. Some suggest that a higher density of CCR5-expressing cells at mucosal surfaces or in lymphoid tissues may select for R5-tropic variants during transmission or favor replication after transmission.[20]

Phenotypic characteristics of CXCR4-using viruses in newly infected individuals, and the frequency with which they occur, have not been well defined. Since the presence of CXCR4-using variants in recent infection may have implications for disease progression, antiretroviral drug treatment, development of vaccines and microbicides, and postexposure prophylaxis, we screened for CXCR4-using viruses in recent seroconverter panels and characterized the coreceptor usage and envelope (*env*) sequences of individual clones from recently infected subjects who harbored CXCR4-using subtype B viruses. Viruses were classified as R5-, X4-, or DM (dual/mixed)-tropic, based on the phenotypic results determined using the Trofile assay.[21] Briefly, full-length *env* sequences were amplified by RT-PCR and cloned into an *env* expression vector as *env* libraries. A replication-defective HIV-1 genomic vector containing a luciferase reporter gene was then used to cotransfect human embryonic kidney cell cultures with

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TABLE 1. CXCR4-USING VIRUSES WERE IDENTIFIED IN SEVEN SUBJECTS WITH RECENT HIV-1 INFECTION

Subject	Days since last HIV-neg test[a]	Viral load (copies/ml)	$CD4^+$ T cells ($cells/mm^3$)	Tropism env pools[b]	RLU of Env pools[c]		Number of clones analyzed	Number (%) of clones with R5, X4, and dual tropism (screening assay)		
					$CCR5^+$ cells	$CXCR4^+$ cells		R5	X4	Dual
1	187	N/A[d]	N/A	DM	298,606	176	57	13 (23)	0	44 (77)
2	138	N/A	N/A	DM	127,062	210	26	0	0	26 (100)
3	54	500,000	510	DM	507,257	315	80	70 (88)	1 (1)	9 (11)
4	159	N/A	N/A	DM	803,100	812,573	30	0	0	30 (100)
5	181	N/A	N/A	DM	292,83	39,126	24	0	0	24 (100)
6	14–21	362,000	399	DM	755	109,913	24	0	0	24 (100)
7	132	33,721	660	X4	98	75,006	40	0	36 (90)	4 (10)

[a]Days between last negative HIV diagnostic test result and HIV-positive sample collection. Subject 6 was diagnosed based on positive HIV RNA and negative HIV antibody test results in the setting of symptomatic HIV infection.
[b]Coreceptor tropism determined using the Trofile assay (Monogram Biosciences).
[c]RLU, relative light units of luciferase output in the Trofile assay.
[d]N/A, not available.

patient *env* expression vectors. Coreceptor tropism of pseudoviruses was evaluated by infecting CXCR4- and CCR5-expressing cells in the presence and absence of CXCR4 and CCR5 inhibitors.

We tested 150 individuals from two separate cohorts of recent seroconverters. Four subjects (1, 2, 4, and 5) were identified in a cohort of 126 seroconverters,[22] and two others (3 and 7) were identified in a second cohort of 24 seroconverters. Overall, 4% (95% CI 3.1–7.1%) of the 150 recent seroconverters had CXCR4-using viruses. We also studied an additional subject (6) who was a newly infected individual identified in routine clinical care.[23] All seven subjects were men who are believed to have acquired HIV-1 through sexual contact with other men. Viruses were isolated from plasma collected at the time of diagnosis (years 2000 and 2003). One subject (7) was infected with a virus population that was predominantly comprised of X4-tropic variants, while the remaining six subjects were infected with DM-tropic virus populations that exhibited notably different levels of infectivity (relative light units, RLU) in $CCR5^+$ and $CXCR4^+$ cells (Table 1). The DM-tropic virus populations from subjects 1, 2, and 3 displayed lower levels of infectivity in $CXCR4^+$ cells compared to $CCR5^+$ cells. In contrast, the DM-tropic virus population from subject 6 exhibited higher levels of infectivity in $CXCR4^+$ cells compared to $CCR5^+$ cells. The DM-tropic virus populations from subjects 4 and 5 infected both $CCR5^+$ and $CXCR4^+$ cells with similar efficiencies.

To understand the components of the CXCR4-using virus populations in these recently infected individuals, we performed *env* clonal analyses on viruses obtained from each of the subjects. We began by screening numerous clones from each virus population for their abilities to infect CCR5- and CXCR4-target cells to estimate the relative proportion of R5-, dual-, and X4-tropic clones (Table 1). The virus populations from subjects 2, 4, 5, and 6 were comprised exclusively of dual-tropic variants, whereas the virus populations from the remaining three subjects were comprised of mixtures of R5- and dual-tropic variants (1), X4- and dual-tropic variants (7), or R5-, X4-, and dual-tropic variants (3). To confirm the coreceptor tropism of these variants, we analyzed a subset of representative clones (103 clones total, 13–16 clones per sample) derived from each of the seven subjects using the Trofile assay. Infectivity levels (RLU) of the clones in $CXCR4^+$ and $CCR5^+$ cells are shown in Fig. 1. Both X4- and dual-tropic clones were identified in these seven newly infected subjects, and dual-tropic clones exhibited different abilities to infect $CXCR4^+$ and $CCR5^+$ cells, with infectivity ranging from 10^2 to 10^6 RLU.

We previously reported that dual-tropic *env* clones of subtype D viruses varied in their ability to use CXCR4 and CCR5, and created two new designations, dual-X and dual-R, to describe dual-tropism based on coreceptor use and V3 amino acid sequence.[24] Dual-X refers to dual-tropic clones that infect $CXCR4^+$ cells efficiently and have V3 sequences that are distinct from R5-tropic clones in the same virus population; dual-R refers to dual-tropic clones that infect $CXCR4^+$ cells poorly and have V3 sequences that are similar, or identical, to R5-tropic clones from the same virus population. To determine whether dual-tropic, subtype B variants from recent infections have similar characteristics, we sequenced the gp160 *env* of the 103 clones described above using conventional dideoxy-chain termination chemistry, and in-

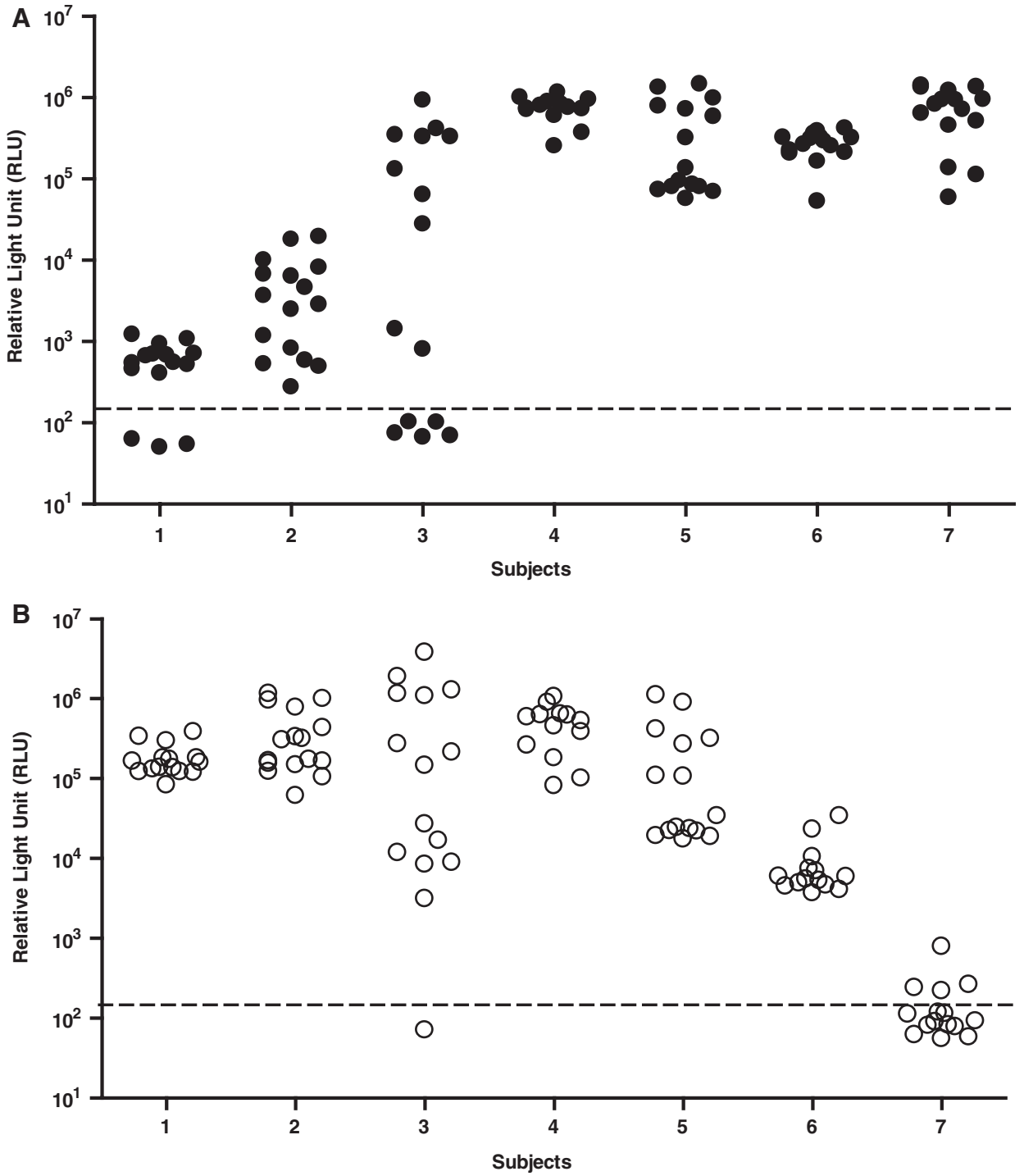

FIG. 1. Infectivity of *env* clones isolated from seven subjects recently infected with CXCR4-using HIV-1. (**A**) Infectivity in CXCR4-expressing cells is indicated by closed circles. (**B**) Infectivity in CCR5-expressing cells is indicated by open circles. Infectivity was measured as relative light units (RLU). The horizontal, dashed line indicates the lower limit of detectable infection.

vestigated the relationship among tropism, infectivity, and V3 amino acid sequence for each of the clones (Table 2). All clones from subject 2 were dual-R-tropic and contained closely related V3 sequences. All clones from subjects 4, 5, and 6 were dual-X-tropic with nearly identical V3 sequences within each population. The virus population of subject 1 included a mixture of dual-R- and R5-tropic clones sharing nearly identical V3 sequences. The virus population of subject 7 included a major subpopulation of X4-tropic and a minor subpopulation of dual-X-tropic clones with low levels of infectivity in CCR5-expressing cells; all but one of the clones shared identical V3 sequences. Subject 3 had the most complex virus

Table 2. Coreceptor Usage and V3 Sequences of *env* Clones from Seven Subjects Infected with CXCR4-Using Viruses

Subject	Number of clones[a]	Tropism (Trofile)	Infectivity measured by luciferase activity[b]		Predictions[c]		V3 sequence analysis[d]		
			R5 RLU (median)	X4 RLU (median)	11KR/25KR	PSSM	Net charge	PNGS	V3 amino acid sequences
1	11	Dual-R	174,105	769	R5	R5	4	1	CTRPNNNTRK**S**IHMGPGKAFYATG**E**IIGDIRKAHC
	2	R5	120,891	59	R5	R5	4	1	.....
	1	R5	83,223	50	R5	R5	4	1	.....V.....
	1	Dual-R	164,877	461	R5	R5	4	1	.....R.....
2	10	Dual-R	374,986	4,593	R5	R5	4	1	CTRPNNNTRK**G**IHMGPGRVFYTTG**E**IIGDIRKAHC
	1	Dual-R	305,047	18,029	R5	R5	5	1	.....R.....
	1	Dual-R	104,486	4,637	X4	R5	6	1	.....K.....
	1	Dual-R	123,164	2,484	R5	R5	4	1	.I.....
	1	Dual-R	164,741	3,654	R5	R5	4	1	.....V.....
	2	Dual-R	240,359	677	R5	R5	4	1	.....A...V.....
3	5	R5	1,082,926	74	R5	R5	4	1	CTRPGNNTRR**S**ITMGPGRAFYTTG**E**IIGDIRKAHC
	3	Dual-R	1,278,284	1,426	R5	R5	4	1	.....
	5	Dual-X	8,862	349,224	R5	R5	7	1	.....H..-H.R....KN.....
	1	Dual-X	11,893	27,700	R5	R5	6	1	..G.....H..-H.R....KN.....
	1	X4	71	328,864	R5	X4	7	1	......S...G.LV.-T.R....RN.....
4	13	Dual-X	529,308	759,363	R5	R5	5	1	CTRPNNNTRK**G**IVIGPGRSFYAAR**S**IIGDIRQAHC
5	14	Dual-X	29,245	208,732	X4	X4	4	1	CTRPNNNTIK**G**IRIGPGRAIYATE**K**IVGDIRQAHC
	1	Dual-X	106,948	135,564	X4	X4	4	1	.....V.....
6	13	Dual-X	5,930	313,126	X4	X4	6	0	CTRPNNNIRR**R**IHIGPGRAFYAT**D**ITGSIRRAYC
	1	Dual-X	5.294	207,153	X4	X4	5	0	.....G.....
7	10	X4	86	770,172	X4	X4	5	0	CTRPNEYRTR**R**IHIGPGRAFVTTK**S**ITGDIRQAYC
	4	Dual-X	252	1,149,581	X4	X4	5	0	.....
	1	X4	82	454,024	X4	X4	5	0	.....D.....

[a]The number of clones with identical V3 sequences.
[b]RLU, relative light units of luciferase output in the Trofile assay.
[c]11KR-25KR, positively charged lysine and/or arginine residue at position 11 and/or 25 in the V3 loop. PSSM, position-specific scoring matrices.[26]
[d]PNGS, potential N-linked glycosylation site. PNGS site located at amino acids 6–8 in V3 are underlined; amino acid positions 11 and 25 are highlighted in bold.

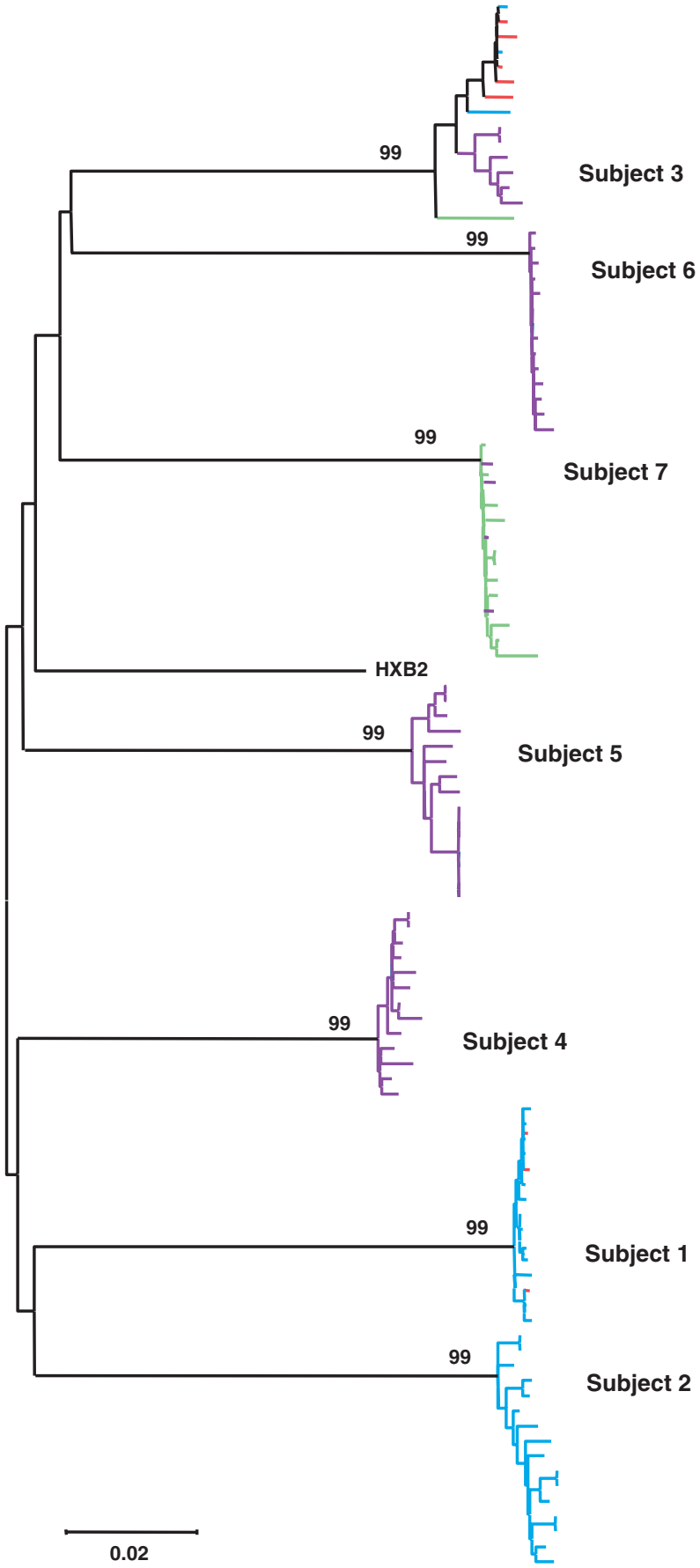

FIG. 2. Phylogenetic analysis of full-length *env* sequences (gp160 nucleotide sequences) from seven subjects recently infected with CXCR4-using HIV-1. Replicate bootstrap resampling (1000×) of the data revealed >99% support for all seven subject nodes in neighbor-joining trees. Individual *env* clones are color coded for coreceptor tropism (red, R5; green, X4; blue, dual-R; purple, dual-X).

population, containing R5-, dual-R-, dual-X-, and X4-tropic clones. The R5- and dual-R-tropic clones had identical V3 sequences, and the dual-X- and X4-tropic clones had similar V3 sequences (Table 2). These data are highly consistent with our previous observations in subtype D virus populations[24] and extend the dual-R and dual-X subclassifications to subtype B viruses, as well as to virus populations in recently infected adults.

Next, we evaluated the accuracy of tropism predictions generated using V3 sequence-based algorithms by analyzing the V3 sequences characterized in this study. Both the 11RK/25RK rule[25] and position-specific scoring matrices (PSSM)[26] correctly assigned coreceptor tropism in some, but not all cases (Table 2). For example, all X4- and dual-X-tropic clones from subjects 5, 6, and 7 were correctly predicted as CXCR4-using by the 11KR/25KR rule and PSSM. Conversely, all of the dual-X-tropic clones from subjects 3 and 4 were incorrectly predicted as R5-tropic. Furthermore, all of the dual-R clones analyzed were predicted to be R5-tropic by the 11RK/25RK rule and PSSM, except for one dual-R clone from subject 2 that had a positively charged lysine (K) residue at position 25. In prior studies, a higher net charge and fewer potential N-linked glycosylation sites (PNGS) in V3 have been associated with CXCR4 use.[25,27] In this study, X4- and dual-X-tropic clones generally had higher net charges (+4 to +7, median: +5) than R5- and dual-R-tropic clones (+4 to +6, median: +4). Five of the 11 V3 sequences found in X4- and dual-X-tropic clones lacked PNGS, while the remaining six had a single PNGS at amino acids 6–8. All V3 sequences in the R5- and dual-R-tropic clones also had only one PNGS at this position. Overall, the associations between V3 genotype and coreceptor tropism were observed for some, but not all of the clones analyzed (Table 2).

Phylogenetic analyses of gp160 nucleotide sequences of all 103 *env* clones from the seven subjects were performed using neighbor-joining methods (MEGA V3.0). These analyses revealed that each of the subjects was infected with phylogenetically distinct viruses (Fig. 2). All clones analyzed were subtype B (data not shown). The median within-patient *env* nucleotide diversity ranged from 0.19% to 1.33%, consistent with recent reports of early HIV infection.[28,29] Similar to the V3 data, full-length *env* sequences from subject 3 exhibited the most heterogeneity among all subjects; dual-R- and R5-tropic clones clustered and were distinct from the dual-X- and X4-tropic clones in phylogenetic trees (Fig. 2). Our data indicate that individuals infected with CXCR4-using viruses can contain phylogenetically homogeneous or heterogeneous virus populations.

The presence of X4-tropic variants in individuals with recent seroconversion implies that either CXCR4-expressing cells exist at sites of transmission or X4-tropic viruses are carried to remote lymphoid tissues, where such target cells are available. However, our observations do not rule out the possibility that some transmitted R5-tropic variants are rapidly adapted to efficient CXCR4 use after infection. Clinical follow-up was available for subjects 6 and 7, with dual-X- and predominantly X4-tropic virus populations, respectively. Both individuals experienced rapid $CD4^+$ T cell declines within 1 year of HIV-1 diagnosis (from 399 to 73 cells/mm^3 for subject 6; 660 to 192 cells/mm^3 for subject 7). Thus, these two cases support the linkage between CXCR4 use during primary or early-stage infection and accelerated disease progression. The efficiency of CXCR4 use of individual variants and the proportion of CXCR4-using variants in virus populations may impact the pathogenesis and clinical course of HIV-1 infection in recently infected individuals. Unfortunately, no follow-up information was available for the other five subjects in this study. Based on observations in sexual transmission reported here and vertical transmission reported elsewhere,[30] we speculate that in addition to evolution from R5-tropic viruses, the emergence of CXCR4-using viruses during later stages of HIV-1 infection could, in some cases, result from the outgrowth of transmitted CXCR4-using variants.

The presence of CXCR4-using variants may have important implications for treatment regimens that include CCR5 inhibitors. In clinical evaluations of the CCR5 antagonists, maraviroc (Pfizer) and vicriviroc (Schering-Plough), highly treatment-experienced patients harboring only R5-tropic virus showed significant reductions in viral load,[31,32] whereas patients with CXCR4-using virus did not.[33–36] The utility of CCR5 antagonists in treatment-naive or early treatment settings is currently under investigation.[37] Recent surveys have reported CXCR4-using virus was detected in approximately 20% of antiretroviral drug-naive HIV-1-infected patients.[15,38] Here our results suggest that although R5-tropic viruses predominate in early HIV-1 infection, CXCR4-using viruses are not rare. Thus, testing for viral tropism to reduce the risk of treatment failure could be important prior to initiating CCR5 inhibitor therapy, even for patients in early stages of infection.

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Disclosure Statement

Wei Huang, Jonathan Toma, Eric Stawiski, Signe Fransen, Terri Wrin, Neil Parkin, Jeanette Whitcomb, Ean Coakley, and Christos Petropoulos are employees and shareholders of Monogram Biosciences. Susan Eshleman is a Clinical Advisor for Monogram Biosciences.

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