High-Sequence Diversity and Rapid Virus Turnover Contribute to Higher Rates of Coreceptor Switching in Treatment-Experienced Subjects with HIV-1 Viremia

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Abstract

Coreceptor switching from CCR5 to CXCR4 is common during chronic HIV-1 infection, but is even more common in individuals who have failed antiretroviral therapy (ART). Prior studies have suggested rapid mutation and/or recombination of HIV-1 envelope (*env*) genes during coreceptor switching. We compared the functional and genotypic changes in *env* of viruses from viremic subjects who had failed ART just before and after coreceptor switching and compared those to viruses from matched subjects without coreceptor switching. Analysis of multiple unique functional *env* clones from each subject revealed extensive diversity at both sample time points and rapid diversification of sequences during the 4-month interval in viruses from both 9 subjects with coreceptor switching and 15 control subjects. Only two subjects had *env*s with evidence of recombination. Three findings distinguished *env* clones from subjects with coreceptor switching from controls: (1) lower entry efficiency via CCR5; (2) longer V1/V2 regions; and (3), lower nadir CD4 T cell counts during prior years of infection. Most of these subjects harbored virus with lower replicative capacity associated with protease (PR) and/or reverse transcriptase inhibitor resistance mutations, and the extensive diversification tended to lead either to improved entry efficiency via CCR5 or the gain of entry function via CXCR4. These results suggest that R5X4 or X4 variants emerge from a diverse, low-fitness landscape shaped by chronic infection, multiple ART resistance mutations, the availability of target cells, and reduced entry efficiency via CCR5.

Keywords: HIV-1 coreceptor switching, sequence diversity, sequence evolution

Introduction

HIV ENTRY INTO TARGET CELLS is mediated by interactions between the envelope protein, the CD4 receptor, and either the CCR5 or CXCR4 coreceptor.¹ Coreceptor switching refers to the evolution of HIV-1 envelope sequences^{2,3} that change the target cell coreceptors utilized for viral entry from CCR5 (R5) to CCR5 and CXCR4 (R5X4) or, more rarely, CXCR4 (X4). Sequence changes in the third variable loop (V3) of envelope (*env*) are the most important for coreceptor tropism shifts from R5 to R5X4 or X4,^{4–7} but other regions of envelope also contribute.^{8–11} Primary HIV-1 infection is usually dominated by R5 virus isolates,^{12,13} and R5X4 or X4 viruses are detected late in chronic infection in 50%–70% of subtype B

infections.^{14,15} The introduction of CCR5 inhibitors into the clinical setting^{16–19} has made tropism testing by either phenotypic^{20,21} or genotypic^{22–24} methods routine, and deep sequencing of V3 regions had allowed the identification of rare predicted R5X4 or X4 sequences.^{25–27} Since the frequency of coreceptor switching is influenced by both HIV-1 subtype^{28,29} and host CCR5 genotype,^{30–32} both viral and host factors must contribute. Coreceptor switching is less frequent in subtype C HIV-1 infection^{29,33} but the frequency increases^{34–36} following antiretroviral therapy (ART), as it does in subtype B infection.^{17,37} Subjects heterozygous for the CCR5 $\Delta 32$ mutation tend to have lower viral loads^{38,39} but earlier emergence of R5X4 or X4 variants,³² suggesting that entry fitness and selective pressure to use CXCR4 are influenced by CCR5 density.⁴⁰

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Prior work from our laboratory has suggested that loss of entry fitness via CCR5 and increased CD4 binding may precede coreceptor switching.^{3,41} Low nadir CD4 T cell count is a strong predictor of coreceptor switching,^{37,42} as is a history of ineffective ART.^{37,43} The increase in the frequency of coreceptor switching following ART could have trivial explanations such as a longer duration of infection, or it might be explained by changes in reverse transcriptase (RT) fidelity associated with drug resistance mutations^{44,45} that increase *env* mutations, increased diversity of latently infected CD4 T cells from which X4 virus can be rescued,⁴⁶ or greater depletion of CCR5⁺ target cells.^{32,47–49} A long history of ART with only intermittent responses might lead to repeated seeding of the latent reservoir,^{50–52} providing more stochastic options for R5X4 virus archiving or generation.

These hypotheses prompted the current studies to examine both the evolution of *env* sequences and the entry competence via CCR5 or CXCR4 for full-length *env* clones isolated from nine treatment-experienced, viremic subjects selected from the SCOPE (Study of the Consequences of the Protease Inhibitor Era) cohort³⁷ just before and just after coreceptor switching. The results were compared to similar studies of 15 control subjects who maintained the same coreceptor use at the two sample time points. These studies are unique with respect to the short, 4-month interval between sample collection, the large number of unique full-length *env* clones with phenotypic entry data to correlate with sequences, and the extensive data on the status of the subjects enrolled in the SCOPE cohort.

Materials and Methods

Subject characteristics

Full-length *env* clones were amplified from plasma samples previously collected from 24 subjects enrolled in the SCOPE cohort with tropism results reported in studies by Hunt *et al.*³⁷ and Reeves *et al.*⁵³ Informed consent was obtained from all participants, and ethical approval was obtained from the ethics boards of each institution participating in the SCOPE at the University of California, San Francisco. Subjects were identified by code to allow linkage to clinical

data, and the current study was approved by The Scripps Research Institute IRB (IRB 13-6137). Most subjects contributed two samples collected at roughly 4-month intervals; two subjects had four serial samples, and one subject had three serial samples. All subjects were classified into three groups according to their previous results from testing with an enhanced sensitivity tropism assay (21, 53) at the two (or more) sample time points as R5/R5, R5/DM (dual/mixed), or DM/DM. Table 1 presents data for each subject group. The R5/R5 (non-switch [NS] control) and R5/DM (switch [S]) groups were well matched for duration of HIV diagnosis, CD4 T cell counts, and viral loads. The two subjects in the DM/DM group had a longer duration of diagnosis, lower CD4 T cell counts, and higher viral loads than the other groups. All subjects were ART experienced and all had multiple drug-resistant mutations with poor or intermittent responses to therapy. None of the subjects had been treated with the CCR5 inhibitor Maraviroc.¹⁸ All subjects were viremic at the time of sample collection.

Envelope cloning and coreceptor typing

Samples that had been polymerase chain reaction (PCR) amplified with primers containing a 5' MluI site and a 3' NotI site were received from Monogram. Samples were cut with MluI and NotI and ligated into pC1neo from Addgene. Colonies were picked from transformations of ligations and grown up and purified. *Env* clones were coexpressed with the NL4.3 *env*-negative, luciferase-positive reporter plasmid⁵⁴ in 293T cells (American Tissue Culture Collection CRL-3216). Coreceptor use of viruses or envelope clones was evaluated by infection of NP2.CD4.CCR5 and NP2.CD4.CXCR4 cell lines (generously provided by Prof. H. Hoshino) that were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1 μ g/mL of puromycin, and 500 μ g/mL of G418. The luciferase activities were determined as previously described⁴¹ and are reported as relative light units (RLU) expressed as log₁₀ units. Relative entry efficiency of individual *env* clones via CCR5 was calculated by using the RLU value of the laboratory R5 isolate BaL *env* as the standard defining 100% efficiency.⁴¹

TABLE 1. SUBJECTS, SAMPLE COLLECTION, AND FUNCTIONAL HIV ENVELOPE CLONES ANALYZED

	R5/R5	R5/DM ^a	DM/DM	Total (T)/ Average (A)
Subjects	13	9	2	24 (T)
Years seropositive	13.0	12.45	23	15.67 (A)
Mean days between samples 1 and 2	141	107	112	132 (A)
Mean functional <i>Env</i> clones/subject	10.9	27.5	27.5	445 (T)
Sample 1 CD4 count	302 $\pm$ 41 ^b	311 $\pm$ 60	135 $\pm$ 65	
Sample 2 CD4 count	268 $\pm$ 42	327 $\pm$ 51	118 $\pm$ 58	
Sample 1 vRNA	16,246 ^c (6,483–40,709)	15,211 (6,782–34,112)	52,097 (12,889–210,572)	
Sample 2 vRNA	14,597 (5,438–39,182)	10,710 (4,011–28,602)	60,072 (989–3,650,000)	

^aDM entry phenotype in the enhanced Trofile assay as reported in Reeves *et al.*^{21,53}

^bMean $\pm$ standard error CD4 T cells/mL.

^cGeometric mean (95% confidence interval) viral RNA copies/mL.

DM, dual/mixed.

Sequence analysis

Sequences were compiled, visualized, and aligned using Lasergene 8.1 software (DNASTAR, Madison, WI). To first rule out contamination or dual infection within each subject, we combined the *env* sequences obtained in this study with the HIV-1 2014 Web Alignment for *env* (3,551 sequences) from the Los Alamos National Laboratory HIV sequence database (www.hiv.lanl.gov), followed by alignment with MUSCLE,⁵⁵ manual adjustment and maximum-likelihood phylogenetic reconstruction using FastTree 2.1.5,^{56,57} within Geneious v8 (Auckland, NZ).⁵⁸ We note that analysis of diversity is identical for *env* sequences generated by bulk PCR amplification or single-genome amplification,^{59,60} so the method of generation of these sequences should not influence the outcome. In only one instance was a duplicate sequence observed, and this was excluded from analysis.

We also created amino acid alignments for each individual subject using MUSCLE within Geneious v8, followed by manual adjustment. Subject-specific maximum-likelihood phylogenetic trees were reconstructed from amino acid alignments using PhyML,⁶¹ assuming the LG⁶² amino acid replacement matrix, also within Geneious v8. Pairwise amino acid distance estimates were calculated for each person, including each set of sampling dates, using the LG matrix with the DIVEIN web server for sequence analysis.⁶³ For longitudinally sampled sequence sets, for each subject, amino acid divergence was estimated by comparing pairwise distance to the calendar time elapsed between sampling times. To assess the possibility of superinfection, sequences from subject 3,102 were aligned in Geneious v8 with 290 randomly chosen HIV-1 subtype B *env* sequences from the Los Alamos HIV database 2014 compendium. A neighbor-joining phylogenetic tree was generated. Sequence data will be deposited in GenBank on article acceptance.

Results

Subject characteristics

The interval between sample collection and the number of unique *env* clones with confirmed entry via CCR5 and/or CXCR4 are presented in Table 1. The interval between samples was slightly longer in the R5/R5 control group than the R5/DM or DM/DM groups (although this difference was not significant; Fig. 1), and the number of functional *envs* evaluated was lower in the R5/R5 group. This latter difference was a result of most *env* clones from the second sample in the R5/DM group mediating entry only via CCR5 (Fig. 1), so that evaluation of entry phenotypes continued until an R5X4 or X4 clone was identified. In only one subject where the second sample was originally typed as DM did we fail to identify an R5X4 or X4 clone after testing 151 functional and nonfunctional *env* clones. Since we had coreceptor entry data for unique *env* clones from each subject, the ambiguous DM classification was replaced by R5X4 or X4. Only 8/445 *env* clones from three subjects typed as X4, so the vast majority of unique clones from subjects with DM tropism results from populations of *envs* that were R5X4. We noted, as has been reported,²⁰ that a proportion of *env* clones from plasma were nonfunctional. All subsequent sequence analyses were performed only on the 445 *env* clones with confirmed entry function.

FIG. 1. (A) Distribution of entry phenotypes by coreceptor use for all functional HIV envelope (*env*) clones with unique sequences. Subjects with R5X4 or X4 *env* clones still had a predominance of R5 *env* clones (e.g., see Fig. 2 and Supplementary Figs. S1–S24). (B) Interval between sample collections for subjects who maintained R5 use, who switched from R5 to R5X4 (or X4), or who maintained R5X4 use.

Evolution of *env* sequence and coreceptor use

Figure 2 presents data for one subject with R5 virus at the first sample time point (S5) and R5X4 virus 151 days later (S6). Figure 2A presents the entry data via either CCR5 or CXCR4 for each unique full-length *env* clone, with entry data presented as $\log_{10}$ RLU. Three *env* clones with robust R5X4 entry were identified in the second sample, and three more weak R5X4 clones and one weak X4 clone were also detected. Figure 2B shows the average pairwise distance in gp120 amino acid sequence from the first sample R5 sequences to R5X4 *env* clone S6.58, which conferred the most efficient entry via CCR5 and CXCR4. S6.58 was a genetic outlier (with no evidence of hypermutation). Hence, there was an overall, high (7.41%) divergence in all of gp120, with even more dramatic changes in V1/V2, V3, and C3. Figure 2C presents the phylogenetic tree of entire gp160 amino acid sequences from most functional *env* clones shown in Figure 2A. Note that S6.58 is most closely related to an R5 *env* clone from the prior sample time point, S5.2, whereas the other two robust R5X4 *env* clones (also outliers) were most closely related to each other and then to the R5 *env* clone S5.18.

Similar analyses were performed on functional *env* clones from all 24 subjects and are presented in the same format in Supplementary Figures S1–S24 (Supplementary Data are available online at www.liebertpub.com/aid) except that panel B is only included where there was a switch from R5 to R5X4 or X4 between samples. Data from all subjects were pooled for subsequent analysis of entry function and sequence evolution between the two (or more) sample time points.

Figures 3–5 present data for mean amino acid divergence (amino acid distance/time between samples) and mean

FIG. 2. (A) Entry data for HIV *env* clones from one subject before coreceptor switching (S5) and 151 days later (S6) when enhanced Trofile assay results revealed DM viral variants. Data are entry of pseudotyped *env* clones into target cells expressing either CCR5 (x-axis) or CXCR4 (y-axis) with a reporter construct expressing luciferase. Data are plotted as log₁₀ RLU with significant entry function being greater than 10³ RLU (indicated by gray line on each axis). (B) Amino acid pairwise distance of regions of gp120 from all S5 sequences to the unique sequence of R5X4 *env* clone S6.58 (see A). (C) Phylogenetic tree of full-length/gp160 *env* sequences from time point S5 and S6. Scale is 0.01% or 1% per 100 amino acids. Note that R5X4 *env* clone S6.58 is the most divergent sequence identified. Mean pairwise distance per 100 amino acids is given at the bottom of (C). A similar format is used for Supplementary Figures 1–24. DM, dual/mixed; RLU, relative light units.

pairwise amino acid distance between sample time points for full-length, functional *env* sequences for the three subject groups, now categorized as R5 to R5 (controls), R5 to R5X4 (switch), and R5X4 to R5X4 based on entry data for each *env* clone. One set of related sequences from one subject in the R5 to R5X4 group is omitted from the data because it represented superinfection or dual infection from a second source partner (Fig. 9, below). Figure 3 shows no significant difference in diversity or distance between the three groups, although the median value is slightly higher in the R5 to R5X4 group than the control group. Figure 4 shows the same data converted to diversity and distance per year to correct for differences in the interval between collecting sample 1 and 2. Again there is no significant difference between the three groups, although the two subjects (with three samples) in the R5X4 to R5X4 group show much higher values and the median values remain higher in the R5 to R5X4 group than the R5 to R5 controls. It should be

noted that all *env* sequences from the two sample time points were included in the data presented in Figures 3 and 4, and the majority of *env* clones in the R5 to R5X4 group retained the R5 phenotype at the second time point (e.g., see Fig. 2).

Figure 5 shows the percent divergence in gp120 amino acids from the consensus R5 sequence in sample 1 to the R5X4 (or X4 in two subjects) *env* clone that mediated the most efficient entry via CXCR4 in sample 2. Figure 5A shows the data with or without the highly divergent S26.15 sequence. If the outlier 26.15 (and two closely related sequences; see Fig. 9 below) is excluded, the median divergence from consensus R5 to the R5X4 variant that mediated the most efficient entry via CXCR4 4 months later is 6.1%, which is not significantly different than the median value (7.6%) for all R5 to R5X4 *env* clones shown in Figure 3A. Examination of all phylogenetic trees (Supplementary Figs. S1–S24) explains this somewhat surprising result. Although several subjects

FIG. 3. (A) Mean amino acid divergence/100 amino acids (AA) for all subjects who maintained CCR5 use at both sample time points (R5 to R5), all subjects who had some R5X4 or X4 *env* clones at the second sample time point (R5 to R5X4), two subjects who had R5X4 *env* clones at multiple time points (R5X4 to R5X4). All data points are displayed in a box and whisker plot with the box representing the upper and lower quartile, the line in the box representing the median value, and the whiskers the data range. (B) Mean pairwise distance/100 amino acids for the same data. One subject with extreme divergence was excluded from these data because of suspected superinfection or dual infection (Fig. 9). AA, amino acids.

had substantial divergence between the first sample R5 consensus and the second sample R5X4 or X4 variant, other subjects had R5X4 sequences that were more closely related to the earlier R5 consensus than most contemporaneous R5 *env* sequences. Figure 5B compares divergence in the V3 sequence in the R5 to R5 control group to the divergence in the R5 to R5X4 switch group. This difference is highly significant as might be expected, given the importance of the V3 sequence in determining coreceptor use. However, genotypic predictors based on the V3 sequences performed poorly for these samples (data not shown), perhaps because of the recent (<4 months) gain of entry via CXCR4.

Functional changes in envelope properties in coreceptor switch subjects versus NS controls

We evaluated the entry efficiency of all *env* clones capable of CCR5 use from subjects in the R5 to R5 NS group compared to the R5 to R5X4 coreceptor switch (S) group. The

FIG. 4. (A) The same data as presented in Figure 3 but plotted as divergence/100 AA/year to correct for differences in the duration between the two sample time points. (B) Data from Figure 3 plotted as mean pairwise distance/100 AA/year to correct for differences in the duration between the two sample time points (Table 1).

results are shown in Figure 6. Virus entry via CCR5 was significantly higher for the NS subjects compared to the S subjects (Fig. 6A) when results were pooled for both sample time points. The decreased entry via CCR5 was evident both before and after coreceptor switching (Fig. 6B), but the lower entry function in the preswitch samples no longer achieved significance. There were no consistent trends toward improved or declining entry efficiency during the interval between samples that distinguished NS controls from R5 to R5X4 S subjects (data not shown).

We also determined if there were any consistent changes in *env* sequence properties associated with coreceptor switching, and found no significant differences in potential N-linked glycosylation sites that distinguished *env* sequences from NS controls and sequences from S subjects (data not shown). However, V1/V2 regions were significantly longer in *env* sequences from R5 to R5X4 switching subjects (Fig. 7A) than control subjects when sequences from both samples were compared. When the change in V1/V2 length between the two sample times was compared (Fig. 7B), the NS controls tended to maintain the shorter V1/V2 length, while the S subjects showed a trend toward shorter V1/V2 length at the second sample point.

Many subjects in this study had prior analysis of viral replicative capacity⁶⁴ due to changes in protease (PR) and RT

FIG. 5. (A) Percent divergence in amino acid sequence of gp120 (535–560 amino acids) from the consensus of all R5 *env* clones at the first sample time point to the R5X4 *env* clone that mediated the most efficient entry via CXCR4 at the second time point (e.g., clone 6.58 in Fig. 2). (B) Changes in V3 sequences between the first and second sample point for R5 to R5 controls and R5 to R5X4 coreceptor switch variants.

genes from their multidrug-resistant virus. Drug resistance mutations for most subjects are presented in Table S1. We used these data to determine if there was any correlation between reduced replication capacity (RC) and entry efficiency via CCR5, as suggested by a recent publication by Mohri *et al.*⁶⁵ We observed a trend in our sampling toward improved CCR5 entry as PR/RT RC declines, in agreement with Mohri *et al.*,⁶⁵ but this trend was not statistically significant ($p = .0603$).

Clinical data from subjects that correlated with coreceptor switching

We evaluated two clinical parameters that might be associated with coreceptor switching, nadir CD4 T cell count³⁷ and the interval since the last nadir viral RNA load (Fig. 8). Lower nadir CD4 T cell counts were predictive of coreceptor switching (Fig. 8A) in agreement with prior results.³⁷ Subjects with coreceptor switching tended to have a longer interval since the nadir viral load, but the difference was not significant (Fig. 8B).

Extreme divergence or rescue of a latent virus from prior super-(or dual-) infection

During these studies, we noted two examples of *env* sequences at the second (or last) sample time point that were

FIG. 6. (A) Mean entry via CCR5 in $\log_{10}$ RLU for all *env* clones from subjects who maintained CCR5 or CXCR4 use (NS) or subjects who switched to R5X4 or X4 (S). p value of paired, two-tailed T -test is shown, which indicates significantly lower entry function via CCR5 for *env* clones from subjects with coreceptor switching. (B) The same data, but plotted for sample time point 1 (first) and sample time point 2 (second) for both NS and S subjects. NS, nonswitch; S, switch. NS, non-switch controls when column label; ns, non-significant when referring to statistical test shown in Figure panel

extremely divergent from any prior sequences. A phylogenetic tree of sequences from one subject with four sample time points is shown in Figure 9. Three closely related R5X4 *env* clones (26.2, 26.15, and 26.41, red shading) were 28% divergent from sequences from the prior sample time 104 days earlier (yellow shading), and share unique V3 regions. Clones 26.6 and 26.8 (red arrows) were recombinants that shared regions of close homology with both the minor and major strains. No other subject showed evidence of super-(or dual-) infection, and these highly divergent sequences were excluded from other data calculations.

Discussion

Our results show that HIV-1 isolates from chronically infected, ART-experienced subjects with multiple drug-resistant mutations have both a great diversity of viruses (many with inferred diminished RC) and rapid turnover of virus populations from which variants with either enhanced entry function via CCR5 or gain of entry function via CXCR4 can be drawn. While we expected^{66–68} that *env* sequences from subjects with recent coreceptor switching would show greater divergence

FIG. 7. (A) Average V1/V2 length for *env* sequences from subjects who maintained R5 viruses at both sample time points compared to subjects who switched from R5 to R5X4 or X4 viruses at the second sample time point. *p* value from paired, two-tailed *T*-test. (B) Change in V1/V2 length between the first and second sample time point from the same *env* sequences presented in (A). The trend toward a shorter V1/V2 region following coreceptor switching was not significant by the Mann–Whitney nonparametric test.

from the most recent common R5 ancestor than sequences from control subjects without coreceptor switching, this was not observed. Instead, the results in Figures 2–5 show equivalent diversity and divergence for all *env* sequences independent of whether or not coreceptor switching had occurred between the sample intervals. The finding of equivalent diversity and divergence was sustained even when we limited analysis to the consensus R5 *env* sequences before coreceptor switching and the most robust R5X4 or X4 *env* sequences after switching (Fig. 5A). The only exception to this finding was the expected greater divergence in V3 sequences (Fig. 5B) associated with the R5 to R5X4 phenotypic switch. It should be emphasized that all sequence data were generated from *env* clones previously demonstrated to mediate virus entry via one or more coreceptors, so any defective *env* sequences that might be expected from plasma samples⁶⁹ had already been excluded from the sequence analysis.

The high diversity observed in this study was reminiscent of the recent report of Rothenberger *et al.*,⁷⁰ where extensive diversity was found in rebound viruses after a short, treatment-interruption trial. Mean diversity ranged from 0.7% to 5.3% and maximum inpatient diversity ranged from 2.2% to 9.0%

FIG. 8. (A) Nadir CD4 T cell counts for subjects with R5 viruses at both sample time points compared with those viruses that switched from R5 to R5X4 or maintained R5X4 tropism. *p* value calculated from paired, two-tailed *T*-test. (B) Months since last nadir viral load measurement for the same subjects. *T*-test indicated that the difference was NS. NS, nonsignificant.

in their study, and was evident by 2–3 weeks after cessation of ART. All of the subjects in our study were viremic at the time of both plasma samples (Table 1), and mean diversity ranged from 3% to greater than 20% (Fig. 3), reflecting the time of infection and/or the presence of dual- or superinfecting strains.⁷¹ There have been relatively few longitudinal studies of envelope evolution during coreceptor switching where HIV-1 tropism was determined by functional assays rather than by genotypic predictors. Nonetheless, several recent studies yielded results that are similar to ours. Mild *et al.*⁷² examined longitudinal V1–V3 *env* sequences from eight treatment-naïve subjects and concluded that predicted X4 variants evolved at a higher rate than R5 populations. This conclusion is not supported by our results, where four subjects with coreceptor switching had more divergent R5X4 or X4 *env* sequences and the other four subjects had less divergent R5X4 *env* sequences than R5 sequences (excluding the dual- or superinfected subject). However, our subjects were treatment experienced and the added selection pressure of ART may have contributed to our results. In addition, we evaluated entire gp160 sequences, not just V1–V3 sequences. Sede *et al.*⁷³ examined C2–V3–C3 *env* sequences from 19 subjects with samples collected yearly and inferred coreceptor use with the Geno2pheno tool with a 10% false-positive cutoff. They observed considerable diversity in the short sequences from most subjects, and predicted X4 variants

FIG. 9. Neighbor-joining phylogenetic tree from dual/superinfected subject. The tree includes 30 *env* sequences from subject 3, and 102 and 290 randomly chosen subtype B sequences (see Materials and Methods section). Major and minor clades found in this individual are shown in yellow and red highlighted regions, respectively. Two recombinant *env* sequences containing regions derived from both the major and minor clades are in red type and indicated with arrows.

were interspersed among different R5 clades. Bunnik *et al.*²⁵ compared V3 sequences obtained by deep sequencing to phenotypic or genotypic assays for coreceptor use in longitudinal samples from eight individuals, and found X4-predicted V3 sequences as minor populations 3–6 months before phenotypic detection of X4-variants. As in our studies, there were major differences between subjects in both the diversity of R5 and X4 variants and the diversification between samples.

Three findings differentiated samples from those subjects with coreceptor switching compared to those with maintained R5 virus. Entry function of *env* clones via CCR5 was significantly lower in the coreceptor switch group (Fig. 6) before

and after phenotypic switch from R5 to R5X4 virus. This observation supports the earlier hypothesis that loss of entry fitness is one factor leading to the emergence of R5X4 or X4 variants.^{3,74} The V1–V2 length was longer in the *env* clones from the subjects with coreceptor switching (Fig. 7), although this trend was more evident in the sample before switching than in the subsequent sample after switching. Changes in V1–V2 sequences have been observed previously to impact coreceptor use,^{8,9,75} and longer V1–V2 length has been associated with CXCR4 use in two prior studies.^{76,77} V1–V2 length tends to increase with longer duration of infection.⁶³ However, longer V1–V2 regions may impair CD4 binding,⁷⁸

which may need to increase in HIV-1 *envs* with poor entry via both CCR5 and CXCR4. Nadir CD4 T cell counts were significantly lower in subjects with coreceptor switching than those without (Fig. 8) even though CD4 T cell counts at the time of sample collection were similar (Table 1), in agreement with the prior study³⁷ of these same subjects. This observation supports the paucity of available target cells as one factor favoring coreceptor switching^{79–81} if one assumes that CD4⁺, CXCR4⁺ target cells remain, while susceptible CD4⁺, CCR5⁺ target cells are depleted.⁸² However, low nadir CD4⁺ T cell counts also correlate with immune dysfunction and poorer responses to ART,^{43,83} so the direct contribution of lower target cell numbers to coreceptor switching is confounded by other potential selective forces.

Several observations made in this study are worth noting even though they did not achieve statistical significance. First, the number of potential N-linked glycosylation sites did not differentiate R5 and R5X4 or X4 *env* sequences in these subjects. Second, CCR5 entry fitness tended to increase as PR/RT RC decreased in agreement with Mohri *et al.*⁶⁵ Third, the duration since the last viral load nadir tended to be longer in those subjects with coreceptor switching, suggesting that a longer period of viremia as opposed to the current viral load might be important. These observations will require further studies to confirm. In addition, we searched the *env* sequences for evidence of recombination contributing to coreceptor switching.^{84,85} *Env* clones from one subject infected from a single source and the individual with dual/superinfection showed evidence of recombination, and these clones were unique in that they shared a consensus V3 region with many R5 clones in the first instance and the majority strain in the second.

The low replicative and entry fitness of many variants may explain the rapid turnover of *env* genotypes. This low fitness landscape may present an opportunity for many divergent viruses to emerge, but a distinct fitness winner was rarely observed. In only one subject did we observe duplicate *env* sequences at one sample point, and the high level of divergence meant that few closely related sequences were observed at both sample time points. The two subjects with the longest duration of diagnosis and R5X4 viruses at both sample time points had the highest PR/RT RC scores but still had rapid turnover of *env* sequences and high diversity, suggesting that PR/RT replication fitness alone does not lead to a dominant HIV-1 *env* sequence.

Virus evolution is driven by a constant interplay between mutation and recombination of the viral genome and selective pressure exerted by many factors, including the host immune system, ART, and the need to maintain replication and entry fitness.^{3,48,72,86–91} In the setting of chronic infection with poor or intermittent responses to ART, diminished PR/RT RC, poorer entry fitness via CCR5 (for those subjects with coreceptor switching), and diminished immune responses were indicated by lower nadir CD4⁺ T cell counts, perhaps it should not be surprising that coreceptor switching is more prevalent at equivalent CD4⁺ T cell levels than in treatment-naïve subjects.^{32,37}

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