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Coreceptor Tropism in Human Immunodeficiency Virus Type 1 Subtype D: High Prevalence of CXCR4 Tropism and Heterogeneous Composition of Viral Populations▽

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In human immunodeficiency virus type 1 (HIV-1) subtype B, CXCR4 coreceptor use ranges from ~20% in early infection to ~50% in advanced disease. Coreceptor use by non-subtype B HIV is less well characterized. We studied coreceptor tropism of subtype A and D HIV-1 collected from 68 pregnant, antiretroviral drug-naive Ugandan women (HIVNET 012 trial). None of 33 subtype A or 10 A/D-recombinant viruses used the CXCR4 coreceptor. In contrast, nine (36%) of 25 subtype D viruses used both CXCR4 and CCR5 coreceptors. Clonal analyses of the nine subtype D samples with dual or mixed tropism revealed heterogeneous viral populations comprised of X4-, R5-, and dual-tropic HIV-1 variants. In five of the six samples with dual-tropic strains, V3 loop sequences of dual-tropic clones were identical to those of cocirculating R5-tropic clones, indicating the presence of CXCR4 tropism determinants outside of the V3 loop. These dual-tropic variants with R5-tropic-like V3 loops, which we designated "dual-R," use CCR5 much more efficiently than CXCR4, in contrast to dual-tropic clones with X4-tropic-like V3 loops ("dual-X"). These observations have implications for pathogenesis and treatment of subtype D-infected individuals, for the association between V3 sequence and coreceptor tropism phenotype, and for understanding potential mechanisms of evolution from exclusive CCR5 use to efficient CXCR4 use by subtype D HIV-1.

Human immunodeficiency virus type 1 (HIV-1) infection requires interactions between the viral envelope (Env) surface glycoprotein (gp120), the cellular receptor (CD4), and a coreceptor (e.g., CCR5 and/or CXCR4) (36). CCR5, the most commonly used coreceptor, is present on primary T cells and macrophages. In contrast, CXCR4 is expressed on many cell types, including thymocytes, primary T cells, and macrophages (13). CXCR4-using viruses can induce formation of syncytia when cultured on the CXCR4-bearing MT2 cell line (syncytium-inducing, SI viruses) (3, 8, 21, 33, 48). SI or CXCR4-using viruses are typically found in individuals with advanced disease (2, 9, 16, 18, 19). However, it is not clear whether CXCR4 use precedes and causes more rapid disease progression or is merely the consequence of a change in target cell availability. The recent development of HIV-1 entry inhibitors that target CCR5 has heightened interest in coreceptor usage (44).

Several surveys of coreceptor tropism were reported recently. Brumme et al. (6) studied almost 1,000 antiretroviral drug naive HIV-1-infected patients. CXCR4-using virus was detected in 18% of those individuals, more than 99% of which were also able to use CCR5 and were thus categorized as dual- or mixed-tropic (DM). CXCR4 use was associated with decreased survival in univariate, but not multivariate, analyses. There was a statistically nonsignificant trend toward increased CXCR4 use in non-subtype B viruses (7 of 13 [54%] for non-subtype B versus 143 of 675 [21%] for subtype B; C. J. Brumme and P. R. Harrigan, unpublished data). Moyle et al. (37) evaluated predictive factors for coreceptor use among 402 antiretroviral drug naive and 161 antiretroviral drug experienced individuals. Twenty percent of the study subjects had CXCR4-using virus. The frequency of CXCR4-using virus was similar among the subjects with subtype B versus non-subtype B (mostly subtype A and C) infection and also among antiretroviral drug naive versus antiretroviral drug experienced subjects. Melby et al. (34) and Wilkin et al. (55) reported that among highly treatment-experienced patients (n = 724 and 391, respectively), 50% were CCR5-tropic, 46 to 48% were DM, and 2 to 4% were CXCR4. Demarest et al. (15) studied over 400 individuals and found CXCR4-using virus in 18% of subjects (37 of 113 [33%] for antiretroviral drug experienced versus 36 of 299 [12%] for antiretroviral drug naive). CXCR4 use was associated with a lower CD4 cell count in all five studies. Finally, Coakley et al. (12) reported tropism results for 1,428 treatment-naive and 2,560 treated patients; 15% of the naive patients and 44% of the experienced patients harbored virus capable of using CXCR4. Among the naive patients, 23% were infected with subtype C HIV-1; in these patients significantly less (5.7%) had CXCR4-using virus. These studies indicate that ca. 18 to 20% of individuals with subtype B infection harbor CXCR4-using virus and that the frequency of CXCR4 use is higher in individuals with more advanced disease.

Previous reports of SI (and therefore most likely CXCR4-using) virus prevalence in individuals with non-subtype B HIV-1 infection are limited, particularly by sample size. Although some studies have found no difference in phenotype among different subtypes (10, 47, 56), others have reported a

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trend for an unequal distribution of SI versus non-syncytium-inducing (NSI) viruses among different subtypes (1, 5, 17, 35, 39, 49–52, 57). One study (5) found that subtype D and CRF01_AE viruses were predominantly CXCR4-using (SI), whereas subtype A and C viruses were predominantly CCR5-using (non-SI, NSI). The data on the effect of antiretroviral therapy on tropism of non-B subtypes is similarly limited, although one study of subtype C HIV-1-infected patients from Zimbabwe indicated that antiretroviral drug treatment was associated with increased CXCR4 use (30).

In Uganda, most HIV-1 infections are caused by subtypes A and D (26, 42). Inter-subtype recombination involving subtypes A and D is also frequently observed (20). The HIVNET 012 trial in Uganda evaluated the efficacy of single-dose nevirapine for the prevention of mother-to-child transmission of HIV-1 (24, 27). This trial provided an opportunity to compare the coreceptor tropism of subtype A, D, and A/D recombinant HIV-1 among antiretroviral drug-naive women with known viral loads and CD4 cell counts.

In addition to analyzing tropism of viral populations from this cohort, we also characterized coreceptor tropism and the *env* sequences of individual HIV-1 clones from the samples. This allowed us to analyze the genetic determinants of coreceptor usage. The third variable (V3) region of HIV-1 Env is known to be a critical determinant of coreceptor tropism. Changes in the V3 region associated with CXCR4 use include the presence of positively charged amino acids at positions 11 and 25 (14, 22, 25), an increase in total net charge (23), and a reduction in the number of potential N-linked glycosylation sites in the V3 loop (11, 40, 41). Algorithms have been described for predicting coreceptor use based on V3 sequences, such as the 11/25 rule and the position-specific scoring matrix (PSSM) (29). Our results include a comparative analysis of how these methods perform in predicting phenotypic tropism from the V3 sequences of subtype D samples.

MATERIALS AND METHODS

Plasma samples, study subjects (HIVNET 012), and coreceptor tropism testing. We analyzed HIV-1 tropism using plasma samples from antiretroviral drug-naive Ugandan women who subsequently received single-dose nevirapine in the HIVNET 012 trial. The HIVNET 012 study protocol and use of trial samples for HIV analyses were approved by the institutional review boards in both the United States and Uganda. Informed consent was obtained from all women prior to enrollment into the HIVNET 012 study protocol. Viral load assays and CD4 cell counts were performed as previously described (24). *env* genes were successfully amplified from 69 women, and coreceptor usage was evaluated, using the Trofile assay (54). Samples were classified as CCR5-using (R5), CXCR4-using (X4), or dual/mixed (DM; indicating dual-tropic and/or mixed-tropic virus) based on a luciferase activity above background and a significant reduction in relative light units (RLU) in the presence of high concentrations of a CCR5 or CXCR4 antagonist.

***env* sequencing and subtype analyses.** The sequences of patient-derived *env* genes (gp160) were determined on a population basis by using *env* expression vector pools constructed for phenotyping using conventional dye-deoxy chain-terminator chemistry (ABI, Foster City, CA). A set of 16 primers was used to generate overlapping and redundant sequences from both DNA strands. Due to the frequent occurrence of mixtures of viruses containing insertions or deletions in the variable regions of gp120, most *env* population sequences contained regions of ambiguity. To assign *env* subtype to patient viral populations, we avoided these regions of length heterogeneity and used approximately 1,200 nucleotides of sequence corresponding to the constant region 5 (C5) of gp120 and the gp41 subunit of *env* to generate a multiple alignment including 94 sequences of known subtype downloaded from the Los Alamos National Laboratory database. Phylogenies were constructed by using both neighbor-joining and maximum-parsimony methods as implemented in PAUP* version 4.0b10 (46). HIV subtypes and recombinants were confirmed by using a combination of the Recombinant Identification Program (RIP) (45) and bootscanning analyses (http://sray.med.som.jhmi.edu/SCRoftware/simplot/) (43). RIP analyses were performed by using RIP 2.0 as implemented on the LANL website (http://hiv-web.lanl.gov), with a window size of 400 and gap stripping. Bootscanning (100 iterations) was performed with SimPlot 3.5.1 using our multiple alignment of 94 reference sequences, a window size 400, and a step size of 20.

Clonal analyses of DM viral populations. *env* clones were isolated from the nine subtype D samples that displayed DM-tropic phenotypes. Forty-eight *env* clones from each sample were prescreened for their ability to mediate infection of cells expressing CD4 and either CCR5 or CXCR4 coreceptor. A total of 226 clones (between 20 and 35 viable *env* clones from each sample, from a total of 48 screened each) were then tested in the Trofile assay to determine coreceptor tropism, and 133 of them (randomly chosen but representing all phenotypic patterns observed) were sequenced. Clonal sequences were submitted to GenBank (accession numbers EF575354 to EF575486). Phylogenetic analyses of complete gp160 sequences (~2,500 nucleotides) were performed by using neighbor-joining methods and bootstrap resampling (1,000 replicates) (31). The HXB2 reference sequence was used to root the topologies, and nodes with bootstrap support <50% were collapsed. V3 loop amino acid sequences from *env* clones and their association with coreceptor tropism were also determined. Phenotypic tropism assignments derived from the Trofile assay were compared to genotypic predictions from both the modified 11RK/25K rule (25) and the PSSM algorithm (29).

Statistical analyses. All statistical analyses were conducted by using SAS version 9.1, StatView version 5.01 (SAS Institute, Cary, NC), or Prism 5.0 (GraphPad Software, San Diego, CA). Exact binomial confidence intervals were calculated for the proportion of CCR5-using strains in subtype A, subtype D, and A/D recombinant samples. Student *t* tests were performed to test for differences in mean $\log_{10}$ viral load and mean CD4 cell count across subtypes. The Fisher exact test was performed to test associations of *env* subtype and coreceptor tropism. Two analyses were performed. The first analysis included only women with subtype A and subtype D infection (women infected with A/D recombinant strains were excluded). In the second analysis, women infected with A/D recombinant strains were grouped with women with subtype A infection. The nonparametric Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to analyze differences in V3 loop charge and length.

RESULTS

HIV-1 *env* subtypes. Gp160 *env* sequences were amplified from 69 antiretroviral drug-naive Ugandan women and transferred into an expression vector. A 1,200-bp sequence from the C5 region of gp120 and the gp41 subunit was used for subtyping. Analysis of the *env* sequence by four different techniques (see Materials and Methods) identified 33 subtype A, 25 subtype D, 10 heterogeneous A/D intersubtype recombinants, and 1 C/D recombinant among the 69 patients. The C/D recombinant sample was not included in subsequent analyses.

Coreceptor tropism. Phenotypic analysis of HIV-1 coreceptor tropism was performed for the 68 subtype A, D, and recombinant A/D samples by using the Trofile assay. All 68 samples produced moderate to high-level luciferase expression upon infection of CCR5-expressing cells (Fig. 1, RLU range, ca. 10^4 to 10^6). None of the subtype A or A/D samples was able to infect CXCR4-expressing cells, indicating that they were R5-tropic viruses. However, 9 of the 25 (36%) subtype D samples produced luciferase activity (RLU range, ca. 200 to 10^5) upon infection of CXCR4-expressing cells. Infection of CXCR4-expressing cells by these nine samples was effectively inhibited by a CXCR4 antagonist, confirming specificity of infection (data not shown). Thus, 9 of 25 subtype D samples were DM-tropic, and the remaining 16 subtype D samples were R5-tropic.

Association of tropism with HIV-1 viral load and CD4 cell count. Among the 68 women studied, the median $\log_{10}$ viral load was 4.67, and the median CD4 cell count was 356 (Table

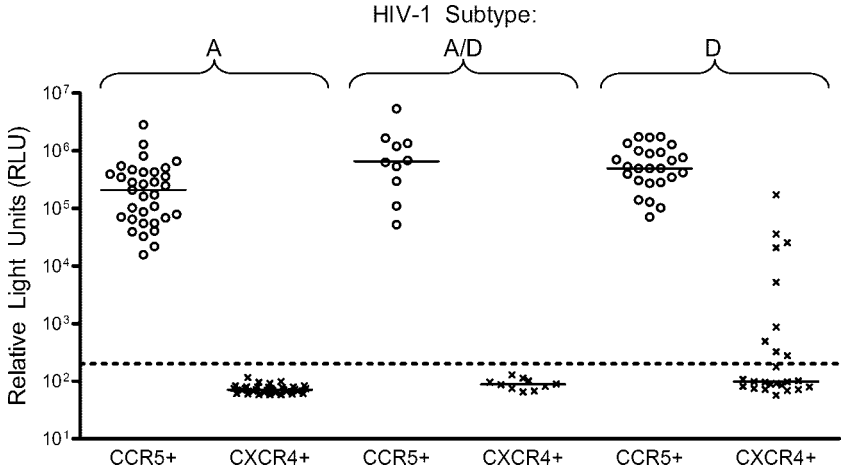

FIG. 1. Coreceptor use of viral *env* populations. Infection of CCR5-and CXCR4-expressing cells is indicated by open circles and crosses, respectively. Infection was measured by luciferase activity (in RLU) produced in cell culture. Samples are grouped by subtype (A, D, and A/D recombinant; $n = 33$, 25, and 10, respectively). The horizontal dotted line indicates 200 RLU, the threshold for defining CXCR4 use in this study. The median RLU of each group is indicated by horizontal bars.

1). Baseline $\log_{10}$ viral load was similar among the three subtype groups ($P = 0.347$, analysis of variance test). The CD4 cell count was significantly higher among women with A/D viruses than among women with either subtype A or D viruses ($P = 0.006$, Student t test). Among all 68 women, those who were infected with CXCR4-using viruses had a higher mean baseline $\log_{10}$ viral load and lower mean baseline CD4 cell count than women infected with viruses able to use CCR5 only (for $\log_{10}$ viral load, 5.1 versus 4.7 [$P = 0.044$]; for CD4 cell count, 220 versus 425 [$P = 0.006$, Student t test]). Among women infected with subtype D virus, those who were infected with CXCR4-using viruses had significantly lower mean baseline CD4 cell count than women infected with viruses able to use CCR5 only (220 versus 392 [$P = 0.008$]), and a trend for higher viral load ($\log_{10}$ viral load, 5.1 versus 4.7 [$P = 0.093$, Student t test]).

Coreceptor use of *env* clones from DM-tropic subtype D viral populations. The nine subtype D samples with DM tropism were further analyzed to determine the coreceptor tropism of individual *env* clones. The composition of DM *env* populations was determined by assaying the tropism of 226 individual *env* clones isolated from the nine samples with DM-tropic subtype D HIV-1. Tropism results for both viral populations and *env* clones are summarized in Table 2. In the viral populations the luciferase activity in CXCR4-expressing cells ranged from 276 to 171,826 RLU, whereas in CCR5-expressing cells it ranged from 128,877 to 1,758,256 RLU. Different patterns of tropism were observed in the *env* clones from these samples. Three samples contained mixtures of R5- and X4-tropic clones (DM1-DM3); four samples contained mixtures of R5-tropic, X4-tropic, and dual-tropic clones (DM4 to DM7); and two samples had mixtures of R5-tropic and dual-tropic clones (DM8 and DM9). Three of nine samples with lower RLU (<500) on CXCR4-expressing cells were comprised of R5-tropic clones with either X4-tropic (DM2) or dual-tropic clones (DM9) or with both dual-tropic and X4-tropic clones (DM7). The percentage of CXCR4 using clones (combining X4-tropic and dual-tropic) varied from 3 to 70% in the nine samples analyzed. *env* clones that used CXCR4 exclusively were identified in seven of nine samples, whereas dual-tropic clones were identified in six of nine samples.

***env* clone sequences from DM-tropic subtype D viral populations.** To evaluate genetic characteristics of subtype D *env* clones and the potential association of specific sequence determinants with coreceptor tropism, we examined full-length *env* (gp160) sequences obtained from 133 of the 226 clones

TABLE 1. HIV viral load, CD4 cell count, and coreceptor tropism among 68 Ugandan women in the HIVNET 012 trial

env subtype	No. of samples	Parameter (mean ± SE)		Coreceptor tropism (no. [%])	
		Viral load ($\log_{10}$)	$CD4^+$ cells (cells/μl)	R5-tropic[a]	DM-tropic
A	33	4.60 ± 0.13	398 ± 35	33 (100)	0 (0)
D	25	4.81 ± 0.12	330 ± 32	16 (64)	9 (36)[b]
A/D	10	4.81 ± 0.13	565 ± 89	10 (100)	0 (0)

[a] Exact binomial 95% confidence intervals for % R5-tropic: subtype A, 89 to 100%; subtype D, 43 to 82%; A/D recombinants, 69 to 100%.

[b] Fisher's exact test between groups: subtype A versus subtype D, $P = 0.0002$; and subtype D versus the combined group of subtype A and A/D recombinants, $P = 0.00004$.

TABLE 2. Analysis of DM-tropic subtype D *env* clones

Sample	RLU[a] produced from *env* pools		No. of viable clones	No. of clones (%) with indicated tropism		
	$CCR5+$ cells	$CXCR4^+$ cells		R5	X4	Dual
DM1	492,847	35,675	20	6 (30)	14 (70)	
DM2	1,353,460	276	32	31 (97)	1 (3)	
DM3	128,877	20,795	20	13 (65)	7 (35)	
DM4	305,798	25,305	28	17 (61)	3 (11)	8 (28)
DM5	281,906	171,826	25	11 (44)	9 (36)	5 (20)
DM6	939,088	5,232	20	12 (60)	2 (10)	6 (30)
DM7	1,758,256	491	20	18 (90)	1 (5)	1 (5)
DM8	394,877	870	35	15 (43)		20 (57)
DM9	1,004,996	324	26	20 (77)		6 (23)

[a] Luciferase activity is an indicator of virus entry.

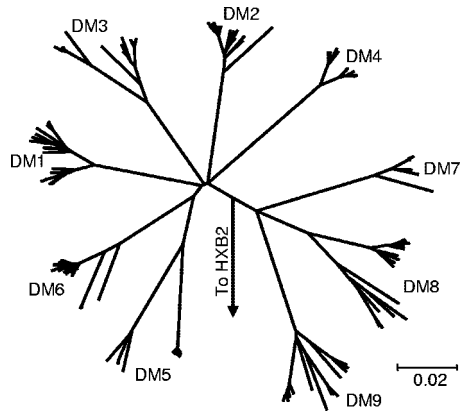

FIG. 2. Phylogenetic analysis of *env* clones (gp16D) from DM-tropic subtype D samples. A 1,000-replicate bootstrap resampling of the data revealed >99% support for all patient-nodes.

from nine DM samples (11 to 16 clones per patient). Phylogenetic analysis revealed that clones from each sample formed monophyletic clades that were distinct from those of clones from other samples (Fig. 2). Most of the samples contained two or more reciprocally monophyletic clusters with strong bootstrap support (Fig. 3). In most cases, sequences of X4-tropic clones were distinct from those of R5-tropic clones and segregated into distinct intrapatient clusters (green branches in Fig. 3A and B). In contrast, sequences from dual-tropic clones were not monophyletic (light blue and purple branches in Fig. 3B and C). Specifically, sequences from dual-tropic clones clustered with R5-tropic clones in three samples (DM5, DM6, and DM7, Fig. 3B), with X4-tropic clones in one sample (DM4, Fig. 3B) and were dispersed across phylogenetically distinct clusters in two samples (DM8, and DM9, Fig. 3C).

Performance of common genotypic algorithms for predicting coreceptor tropism. We also evaluated the ability of published methods to predict tropism based on V3 amino acid sequences. Translation of the V3 sequences from the 133 clones described above produced a data set of 61 unique V3 amino acid sequences (Table 3). V3 loop sequences from X4-tropic *env* clones were distinct from those from R5-tropic clones derived from the seven patients harboring both X4- and R5-tropic strains (DM1-DM7). Among the six samples that had dual-tropic clones (DM4 to DM9), five samples had dual-tropic clones with V3 loop sequences that were identical to the V3 loop sequences of R5-tropic clones. Those R5-tropic-like dual-tropic clones were designated dual-R-tropic (dual-R). Phylogenetic analyses of the complete gp160 nucleotide sequences of the dual-R clones showed that these clones clustered with the R5-tropic clones (Fig. 3). In contrast, the other dual-tropic clones (those with distinct V3 loop sequences, referred to as dual-X) clustered either with X4-tropic clones or R5-tropic clones (Fig. 3).

The presence of a positively charged lysine or arginine residue at positions 11 and/or lysine at 25 in the V3 loop has been associated with X4 tropism in other studies (25). We evaluated the ability of this "11KR/25K rule" to predict CXCR4 usage among 61 unique V3 loop amino acid sequences (Table 3). Overall, the concordance between 11KR/25K rule predictions and phenotypes was 61%. The proportion of all viruses that were able to use CXCR4 that were predicted to be X4-tropic was 44%; the proportion of all viruses that only use CCR5 that were predicted to be R5-tropic was 74%. The tropism of at least one clone from seven of nine samples was incorrectly predicted using this method. We also tested the original, unmodified 11/25 rule that includes an arginine at position 25, but the overall concordance of this algorithm was worse (48%).

We also evaluated the predictive value of the PSSM algorithm (Table 3) (29). This algorithm uses precalculated matrices derived from aligned sequences with known tropism. An overall score determines the tropism prediction and is calculated by summing the assigned value for amino acids at specific positions. The results from this method were similar to those from the 11KR/25K rule, with an overall concordance of 59%. The proportion of all viruses that were able to use CXCR4 that were predicted to be X4-tropic was 67%, better than that of the 11KR/25K rule. However, the proportion of all viruses that only use CCR5 that were predicted to be R5-tropic was slightly lower (53%). The PSSM correctly predicted CXCR4 usage for all of the X4-tropic clones but only 31% of the dual-tropic clones. The PSSM algorithm incorrectly predicted the tropism of at least one clone from each sample. Improvements to the accuracy of PSSM-based predictions may require subtype-specific training data sets (28).

In general, the X4-tropic clones had a higher net charge than R5-tropic clones (median charge +5 versus +3, respectively; $P < 0.05$) (Fig. 4). As expected, dual-tropic clones segregated based on whether their V3 loop sequences were X4-tropic-like or R5-tropic-like. However, there was overlap in the range of net charge among X4-tropic, dual-tropic and R5-tropic clones. X4-tropic clones also tended to have slightly longer V3 loops compared to R5-tropic clones (data not shown).

Loss of the potential N-linked glycosylation site g15 (amino acids 6 to 8, Asn-X-Thr/Ser-X, where X≠Pro) in V3 has previously been shown to be associated with CXCR4 use in subtype B HIV-1 (11, 41). Predictably, all 14 X4-tropic clones tested lacked this site, while all but 1 of the 34 R5-tropic clones tested (a clone from sample DM8) possessed the site. All nine dual-R-tropic clones possessed the g15 site, whereas two of four dual-X-tropic clones with X4-tropic-like V3 regions had lost the site. Our data suggest that the g15 N-linked glycosylation site is conserved in R5-tropic variants, but not in X4-tropic variants and is variably present in dual-tropic variants.

***env* sequences outside of the V3 loop contribute to CXCR4 use.** As described above, we identified dual-tropic clones that clustered with R5-tropic clones from the same sample and had V3 loop sequences identical to those in the R5-tropic clones (dual-R-tropic). These findings reveal that determinants for CXCR4 use by these dual-R-tropic clones must reside in *env* but outside of the V3 loop. We compared the infectivity of the dual-R-tropic and dual-X-tropic clones to R5-tropic and X4-tropic clones (Fig. 5). In CCR5-expressing cells, the infectivity of the dual-R-tropic clones was similar to that of the R5-tropic clones (Fig. 5A), and was higher than that of most dual-X-tropic clones that had distinct V3 loop sequences. In CXCR4-expressing cells, the infectivity of the dual-R-tropic clones was lower than that of other CXCR4-using clones (X4-tropic and dual-X; Fig. 5B). Taken together, these data suggest that determinants in *env* that are outside of the V3 region can confer CXCR4-tropism without compromising CCR5 coreceptor use

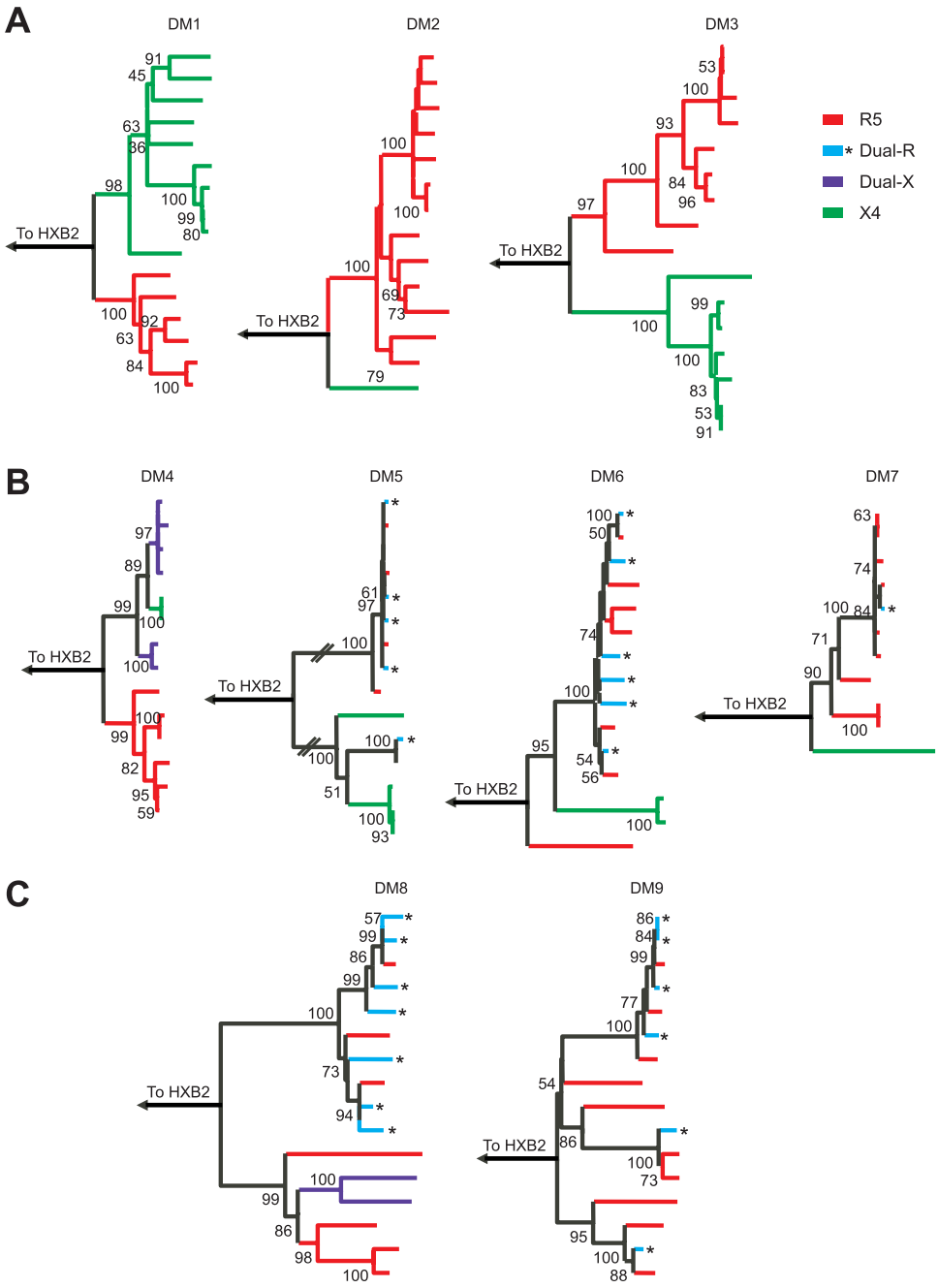

FIG. 3. Topologies of clones (gp160) from DM-tropic subtype D *env* populations. Different colors are used to indicate the tropism of individual *env* clones: red, R5-tropic clones; light blue with asterisk, dual-R-tropic clones (V3 region is R5-tropic-like [see the text]); purple, dual-X-tropic clones (V3 region is divergent or X4-tropic-like [see the text]); green, X4-tropic clones. (A) Samples with pure R5-tropic and pure X4-tropic viruses (no dual-X/dual-R-tropic clones); (B) samples with pure R5-tropic, pure X4-tropic, and dual-X/dual-R-tropic viruses; (C) samples with pure R5-tropic and dual-X/dual-R-tropic clones (no X4-tropic clones).

and that efficient CXCR4 use requires the acquisition of changes in V3 which compromise CCR5 use.

DISCUSSION

By analyzing HIV-1 from antiretroviral drug-naive Ugandan women, we found that a higher portion of subtype D viruses used the CXCR4 coreceptor compared to subtype A viruses. Among women included in this substudy, without regard to subtype or within subtype D only, CD4 cell counts were lower in women with CXCR4-using DM strains than in women with viruses that used CCR5 exclusively. These data, along with the association between CXCR4 use and rapid disease progression, are consistent with previous studies that suggest that subtype D strains are more pathogenic. In subjects from Tanzania or Kenya, individuals with subtype D HIV-1 infection progressed more rapidly to AIDS and

TABLE 3. V3 sequences, phenotypic tropism, and predicted tropism of *env* clones

Sample	No. of clones[a]	V3 loop amino acid sequence[b]	V3 loop characteristics			Trofile phenotype[d]	Predictions	
			Length	Net charge	PNGS[c]		11KR/25K	PSSM
DM1	3	CTRPYNNTIQ**S**TRIGPGQAFYTSG**R**IIGDIRKAHC	35	4	1	R5	R5	X4
	1	.....Q...	35	3	1	R5	R5	X4
	2	.....Q.....R...	35	3	1	R5	R5	X4
	1	.....EIRR.R.S..Q....H.HLK.T....Q.Y.	35	4	0	X4	X4	X4
	1	.S...NIRR.R.S..Q....H.HLK.T....Q.Y.	35	5	0	X4	X4	X4
	5	.S...ESKR.R.S..Q...LH.HLK.T....Q.Y.	35	4	0	X4	X4	X4
	1	.....DIRR.R.S..Q...LH.HLK.T....Q.Y.	35	4	0	X4	X4	X4
	1	.....DIRR.R.S..Q...LH.HLK.T.....Y.	35	5	0	X4	X4	X4
	1	.....DIKR.R.S..Q....H.HLK.T.....Y.	35	5	0	X4	X4	X4
DM2	6	CSRPYNNTRQ**G**THIGPGQ--ALFTTT**R**IVGDIRQAHC	35	3	1	R5	R5	X4
	3	.L.....--.....I.....	35	3	1	R5	R5	X4
	1	.T.....--.....	35	3	1	R5	R5	X4
	1	.....--....A.....	35	3	1	R5	R5	X4
	2	.....--..Y.A.....	35	3	1	R5	R5	X4
	1	.T..NY...KAI.T....GQ.VY.AAK...N.....	37	5	0	X4	X4	X4
DM3	8	CTRPYNNTRQ**S**THIGPGQSLFTT**K**VI-GDIRQAYC	34	3	1	R5	X4	X4
	1	.....A.....-.....H.	34	3	1	R5	X4	X4
	6	.....RSEIRR.S..L....V.Q.T-KN.K..Q.	34	6	0	X4	X4	X4
	1	.....RSGI.R.S..L...IV.Q..TTT.....R.	35	5	0	X4	X4	X4
DM4	3	CTRPYNNTRT**G**VHVG---PGRAYWTQ**N**IVGNIRHAHC	34	4	1	R5	R5	X4
	3	.....Q.I...---.....	34	4	1	R5	R5	X4
	6	.....S...K.....HVG....F.....	37	5	0	Dual-X	R5	X4
	2	.....S...K.....HVG....F.....	37	5	0	X4	R5	X4
DM5	4	CTRPYNNTRQ**G**VHLGPGRAYYTYAT**R**IIG-NIRRAYC	36	6	1	R5	R5	X4
	5	.....-.....	36	6	1	Dual-R	R5	X4
	1	.S...K.I...IYT....GQAY.T.NLQ.RKV.P.H.	37	7	0	Dual-X	R5	X4
	1	.S...K.I...IYT....GQAY.TINLK.KQV.P.H.	37	7	0	X4	R5	X4
	4	.S...K.I...IYT....GQAY.T.NLQ.RKV.P.H.	37	7	0	X4	R5	X4
DM6	3	CTRPYNNTRR**G**THIGPGQAYFTT**K**IIGDIRQAHC	34	4	1	R5	X4	R5
	6	.....	34	4	1	Dual-R	X4	R5
	1	.....N.....Y.	34	3	1	R5	R5	R5
	1	.....V.....	34	4	1	R5	X4	R5
	1	.A.....	34	4	1	R5	X4	R5
	1	.....E.....	34	2	1	R5	R5	R5
	2	.....R.I....P..L..V....N.T.YKG....	34	5	1	X4	R5	X4
DM7	6	CIRPYNNTRQ**S**THIGPGQALYTT**R**IIG-DIR-QAHC	34	3	1	R5	R5	R5
	1	.....-...-....	34	3	1	Dual-R	R5	R5
	2	.T.....-...-....	34	3	1	R5	R5	R5
	1	.T.....-...-..Y.	34	3	1	R5	R5	R5
	1	.T...TY...R.P..L.....N..VGVKGRP..	36	6	0	X4	X4	X4
DM8	1	CTRPNNNTRQ**S**TPIGPGQALYTTR**R**IIGDIRHAYC	35	4	1	R5	R5	R5
	2	.....	35	4	1	Dual-R	R5	R5
	1	.....Q...	35	4	1	R5	R5	R5
	2	.....Q...	35	4	1	Dual-R	R5	R5
	1	.I.....Q...	35	4	1	R5	R5	R5
	3	.I.....Q...	35	4	1	Dual-R	R5	R5
	1	.....HL.....F..TK.....Q.H.	35	3	1	R5	X4	R5
	1	.I....D...G.HL.....F..TK.....Q.H.	35	3	0	R5	X4	X4
	1	.I.....G.HL.....F..AK.....K.H.	35	4	1	R5	X4	X4
	1	.I..S.....G.HL.....F..AK.....K.H.	35	4	1	R5	X4	X4
	1	.I.....KGIHV...R..F..TK.....K.H.	35	6	1	Dual-X	X4	X4
	1	.I.....KGIHL...R..F..T.....R.H.	35	6	1	Dual-X	R5	R5
DM9	3	CTRPNNNTRQ**G**VHLGPGQALFTT-**D**VIGDIRQAYC	34	1	1	R5	R5	R5
	1	.....-....N.....	34	2	1	R5	R5	R5
	4	.....-.....	34	1	1	Dual-R	R5	R5
	2	.....K.....-.I.....H.	34	2	1	R5	R5	R5
	1	.....K.....-.I.....H.	34	2	1	Dual-R	R5	R5
	1	.....-N.....H.	34	2	1	R5	R5	R5
	2	.....Y..KEI.....H.	35	2	1	R5	R5	R5
	1	.....Y..KEI.....H.	35	2	1	Dual-R	R5	R5
	1	.....S.....Y..TGI.....H.	35	1	1	R5	R5	R5

[a] The number of clones with the indicated V3 loop sequence; in all, 61 unique sequences were identified.
[b] Positions 11 and 25 are shown in boldface and underlined.
[c] PNGS, potential N-linked glycosylation site.
[d] Dual-tropic clones are divided into two groups based on their V3 sequences. Dual-tropic clones with V3 sequences that are identical to R5-tropic clones from the same patient are referred to as dual-R, whereas those with V3 sequences that resemble those of X4-tropic clones are referred to as dual-X.

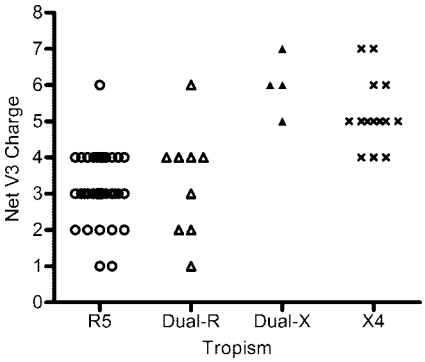

FIG. 4. Net V3 charge of *env* clones. R5-tropic clones are indicated by open circles (median net V3 charge +3), dual-R clones are indicated by open triangles (median +4), dual-X clones are indicated by filled triangles (median +6), and X4-tropic clones are indicated by crosses (median +5).

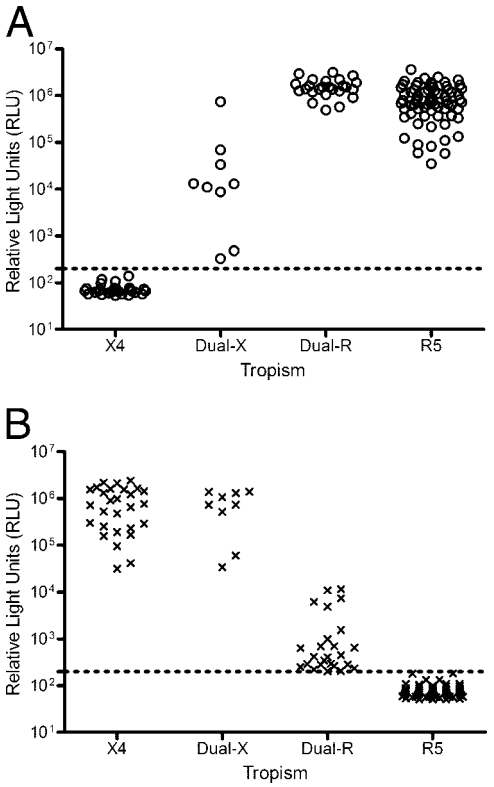

FIG. 5. Infectivity of X4-, R5-, dual-X- and dual-R-tropic *env* clones. Infectivity was measured by luciferase activity (in RLU) produced in cell culture. (A) Infectivity in CCR5-expressing cells; (B) infectivity in CXCR4-expressing cells. Dual-R-tropic clones (V3 loop is R5-tropic-like) and dual-X-tropic clones (V3 loop is divergent or X4-tropic-like) are graphed separately. The horizontal dotted line indicates 200 RLU, the threshold for defining CCR5 or CXCR4 use in the present study. In CCR5-expressing cells (A), the median RLU were as follows: X4-tropic, 66; dual-X-tropic, 12,868; dual-R-tropic, 1,514,000; and R5-tropic, 775,516. In CXCR4-expressing cells (B), the median RLU were as follows: X4-tropic, 745,039; dual-X-tropic, 740,747; dual-R-tropic, 415; and R5-tropic, 69.

death than those with subtype A infection (4, 53). A similar trend was observed in a preliminary study of subjects in Uganda (32). We cannot rule out the possibility that the women with CXCR4-using strains had more advanced HIV-1 disease and that a switch to DM tropism was a consequence rather than a cause of disease progression. However, we consider this unlikely, since the women in this cohort were geographically and epidemiologically homogeneous. In a separate study of a large, cross-sectional data set, we also found a high rate of CXCR4 use in subtype D HIV-1. In that study, the portion of samples that used CXCR4 was similar for subtype B (20%) and subtypes A, C, F, G, CRF01_AE and CRF02_AG (9 to 18%) but was much higher for subtype D (ca. 60%; W. Huang, unpublished data). Further studies are needed to evaluate the relationship between HIV-1 subtype, coreceptor tropism, and disease progression.

Our analysis of HIV *env* clones revealed significant variability in the composition of the viral populations from DM-tropic subtype D samples. Samples from individual patients contained various mixtures of R5-tropic, X4-tropic, and dual-tropic viruses. Clones that used CXCR4 exclusively were present in most DM samples tested. Furthermore, two different types of dual-tropic *env* clones were identified in the virus populations: (i) "dual-X" clones that used CXCR4 efficiently and CCR5 less efficiently (these clones had V3 loop sequences that resembled those from X4-tropic clones from the same sample) and (ii) "dual-R" clones that used CCR5 efficiently and CXCR4 less efficiently (these clones had V3 loop sequences similar or identical to those from R5-tropic clones from the same sample). Identification of these genetically and phenotypically distinct categories of dual-tropic HIV-1 variants demonstrates that some determinants for CXCR4-usage reside outside of the V3 loop. These determinants may play an important evolutionary role as HIV-1 variants evolve from R5-tropic to X4-tropic. We speculate that the dual-R-tropic variants represent early progenitors of CXCR4-using variants that arise later and use the CXCR4 coreceptor efficiently. We hypothesize that mutations within the V3 region that are necessary to confer efficient CXCR4 use are highly detrimental to the fitness of CCR5-using strains and are under strong negative selective pressure (Fig. 6). Consequently, in the absence of compensatory changes, the genetic barrier for coreceptor switching via mutations in the V3 region is high. In contrast, variants that acquire mutations outside of the V3 region that confer inefficient CXCR4 use without compromising CCR5 use may have an opportunity to enter and replicate in cells that express CXCR4. These variants may be able to tolerate additional mutations within V3, leading to more efficient CXCR4 use, even though CCR5 use is compromised. This could provide an evolutionary path for coreceptor switching with a lower genetic barrier. The data reported previously by Carrillo et al. (7) and Pastore et al. (38) are consistent with this model. Additional studies are needed to further develop and test this hypothesis and to test whether the findings from the present study are generalizable to subtypes other than D. It is possible that the genotypic changes outside of the V3 region, which are associated with the dual-R phenotype, exist more commonly as polymorphisms in subtype D compared to other subtypes.

As drugs that target the CCR5 and CXCR4 coreceptors reach the clinic, it may be important to evaluate HIV tropism in patients prior to initiating treatment. Subtype-based differences in the prevalence of viruses with different tropism may influence how these new drugs are used in differ-

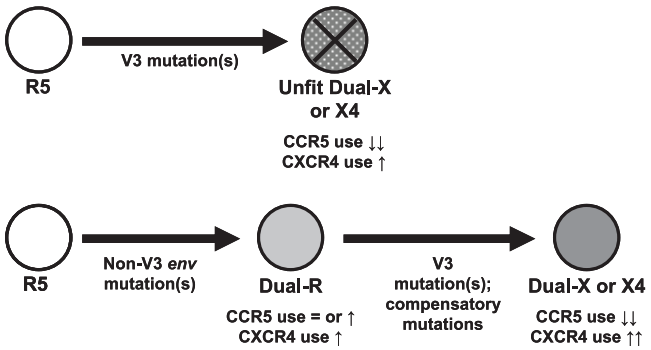

FIG. 6. Model for evolution of coreceptor tropism. R5-tropic viruses are represented in white, dual-R viruses in light gray, and dual-X/X4 viruses in dark gray. The top panel shows the introduction of mutations associated with CXCR4 tropism in the V3 loop of an R5 virus results in X4-tropic or dual-X progeny that have a large fitness disadvantage relative to the parental strain (indicated by an X). The bottom panel shows how the acquisition of non-V3 *env* mutations which confer low-level CXCR4 use, while maintaining efficient CCR5 use (dual-R viruses), leads to strains that may have a fitness advantage and correspond to precursors of dual-X or X4 viruses. See the Discussion for details.

ent parts of the world. In addition, when a patient harbors dualtropic HIV-1 variants, the efficiency with which these variants utilize the CXCR4 coreceptor may influence the patient's response to CCR5 antagonists.

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