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Evolution of CXCR4 usage in subtype C HIV-1 in treated patients

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ABSTRACT

Envelope coreceptor tropism is a phenotypic characteristic of HIV-1 which may be estimated by genotypic algorithms and confirmed through phenotypic assays using virus isolates or recombinant viruses. Viruses can use either CCR5 (R5) or CXCR4 (X4) to enter host cells. Most studies have focused on subtype B HIV infection, although subtype C HIV-1 currently accounts for about 50% of infections globally. Previously, we identified an unexpectedly high frequency of subtype C X4-using virus among treated patients. The clinical isolates were frequently dual mixed (used both CCR5 and CXCR4) and it was not possible to distinguish between mixed infection with exclusively R5 and X4 virus and dual tropic viruses that could use either receptor (R5/X4). Therefore, we generated biological clones from 18 isolates obtained from 12 patients through limiting dilution and identified X4 and X4R5 viruses. Analysis of the biologic clones demonstrated that X4 and R5/X4 coreceptor tropism is associated with length polymorphisms, net charge and structural features of the V3 loop. Phylogenetic analyses confirm that emerging X4-usage in subtype C arises through the initial outgrowth of viruses that are R5/X4 sharing 99% or more *env* homology with R5 virus. Single nucleoside changes resulting in an amino acid net charge increase in the V3 loop as well as 2 amino acid insertions accounted for most of the X4 and R5X4 clonal isolates. Further characterizations of subtype C envelope are warranted to implement efficient genotyping algorithms to identify exclusive R5 use and better understand tropism and pathogenesis among Subtype C HIV-1 isolates.

KEYWORDS: HIV-1, envelope, tropism

INTRODUCTION

The envelope glycoprotein (*env*) of HIV-1 mediates viral entry through CD4 and CCR5 (R5) or CXCR4 (X4) chemokine receptors, as co-receptors facilitate viral binding, attachment and entry. Viruses that use R5 are commonly identified in early infection [1] and are associated with infection of macrophages, genital shedding and transmission. In contrast, X4 using viruses are lymphotropic and are prevalent later in infection with advanced immunodeficiency. The transition from exclusively R5 virus to X4 virus, "co-receptor switching", has been observed in studies of serial isolates from untreated individuals, and is associated with CD4 cell decline [2]. Recently, cross-sectional studies of plasma virus using a recombinant chemokine receptor tropism assay provide evidence of increasing prevalence of X4 tropic viruses among individuals with more advanced disease [3-6]. Studies of tropism following treatment with antiretroviral therapy (ART) suggest an increase in X4 virus after exposure to ART [3].

HIV-1 *env* mediates infection of host cells by a binding interaction between the first and second variable regions (V1 and V2) to CD4 molecules.

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Conformational change in the third variable loop (V3 loop) promotes an interaction with the chemokine co-receptor(s), generally either CCR5 or CXCR4 [7]. Env tropism has been predicted by the sequence of the V3 loop, with a strong association between basic amino acids, particularly at positions 11 and 25 of the V3 loop and X4-usage in subtype B [8]. N-linked glycosylation sites in V1, V2 and V3 regions of gp120 as well as mutations within V1/V2 have also been implicated as determinants of tropism [9-11].

These envelope determinants of chemokine co-receptor use have been most extensively studied in subtype B viruses. However, other HIV-1 subtypes such as A, C, D, and CRF_01AE also switch from R5 to X4 tropism in association with disease progression [12-15]. Although initial studies of subtype C suggested that R5 using viruses predominated [16, 17], more recent studies have identified examples of subtype C HIV-1 viruses that use X4 [18-20].

Viral entry by R5 using viruses can now be blocked by a new class of antiretroviral drugs that block CCR5 receptor mediated binding of HIV-1 [5, 21]. Susceptibility to R5 entry inhibitors is currently measured by a standardized, commercial recombinant envelope assay, Trofile (Monogram Biosciences, San Francisco, California, USA) [22]. Plasma viremia in the recombinant assays may be classified as exclusively using R5, exclusively using X4, or dual/mixed (D/M), a mixture of R5 and X4-using viruses. The aim of the current study is to identify the envelope tropism among subtype C HIV-1 isolates, distinguishing between true dual-tropic (R5X4) virus and mixtures of R5 and X4 viruses. We initially isolated 10 X4-using viral isolates from treated patients in Zimbabwe [23], of which 8 were dual/mixed. We approached this through limiting dilution biological cloning of infectious virus from low-passage isolates, and co-receptor (entry) assays in human cell lines engineered to express CD4 and either CCR5 or CXC4 co-receptors.

METHODS

Patients

Subjects were among patients receiving antiretroviral (ARV) drug therapy through The Centre, an association based treatment program in Harare, Zimbabwe. Blood samples and clinical histories, were obtained at clinic visits to monitor ARV therapy with CD4 cell counts, virus load testing, viral culture and genotypic drug resistance testing. Blood samples and clinical histories from patients seeking care through the clinic were unlinked from personal identifiers and the studies conducted under an exempt protocol approved by the human subjects panel at Stanford University.

Virus isolation

Initial virus isolates were obtained by co-culturing $5\text{-}10\text{x}10^6$ patient PBMCs with $5\text{x}10^6$ HIV-negative donor PBMCs. Virus growth was monitored by p24 ELISA; positive cultures were centrifuged to remove cells and the supernatant was frozen. Cultures were assessed for syncytium formation by co-cultivation with MT2 cells [24, 25]. Cell cultures were inspected every 3 days for the presence of syncytia for a total of 21 days.

Biological cloning by limiting dilution

Peripheral blood mononuclear cells (PBMCs) were infected with 250 µl of virus stock from a clinical isolate. After 24 hours, cells were washed and suspended in 5 ml of RPMI-1640 (GIBCO), supplemented with 15% (v/v) Fetal Bovine Serum (FBS), antibiotics and IL-2. Limiting dilution cultures were performed as 12 replicates of 8 sequential two-fold dilutions in a 96 well plate containing $1\text{x}10^5$ uninfected PBMCs per well. After 7 days, the plate was assayed for p24 antigen production by ELISA (PerkinElmer, Waltham, MA) and supernatant transferred to MT-2 cells for syncytia assays, scored at 12 and 14 days after infection. Viruses from dilutions with four or fewer positive wells were transferred to flasks containing $5\text{x}10^6$ donor PBMCs and grown until p24 concentration was greater than 30 ng/ml. PBMCs were saved for DNA extraction using the blood and body fluid spin protocol of the QIAmp^R DNA Mini Kit (QIAGEN, Hilden, Germany).

PCR with Elongase enzyme

A 2619 bp fragment encompassing gp160 was amplified by nested PCR using genomic DNA, Elongase enzyme (Invitrogen, Carlsbad, CA) and two pairs of primers at 10 pmoles each: (Env5853

(5' - TAGAGCCCTGCAACCATCCAGGAAGT CAGCCTA - 3') and Nef9023 (5' - CATTGGTC TTAAAGGCACCTGAGGT - 3') for the first round and ENF (5' - AAAGAGCAGAAGACA GTGGCAATGAGAGTGATGG – 3') and ENR (5'-AGTACATTTTGACCACTTGCCCCCCAT-3') for the second round. The PCR reaction was amplified on a thermocycler using the following program: 94 C, 3 min [1x]; 94 C, 1 min, 57 C, 1 min, 70 C, 5 min [40x]; 70 C, 10 min [1x]. For the second round, 35 cycles were used instead of 40.

PCR with Platinum Taq

A 650 bp fragment encompassing the C2-V5 regions of envelope glycoprotein was amplified by nested PCR using 5 ul genomic DNA and two pairs of primers (50 pmoles each): KK1 and ED14 [26] in the first round and ES7 and ES8 [27] in the second round. Each 100 ul reaction was run on a PCR thermocycler: 94 C, 2 min [1x]; 94 C, 15 sec, 55 C, 20 sec, 72 C, 2 min [40x]; 72 C, 10 min [1x]. The second round was the same except for 30 cycles.

Sequencing

Sequencing reactions were performed with Big Dye terminators (ABI Biosystems, Foster City, CA) and 2.5 pmole of primer with 2 µl of purified PCR product. The following primers were used: ENF, ENV100 (5' - GACATGGTGGATCAAAT GCATGAGG - 3'), ENV105R (5' - CCTCATGC ATTTGATCCACCATGTC - 3'), MB4, ZV3, and MB3 [27]. Sequencing reactions were purified using Centri-Sep 8 Strips (Princeton Separations, Freehold, NJ). The purified sequences were then run on the ABI Prism^{TM} 377 DNA Sequencer. Alternatively, PCR product was sequenced by MClabs (South San Francisco, CA) using the same primers.

Sequence analysis

AutoAssembler and SeqMan were used to assemble sequences and obtain a single consensus sequence for the Stanford CFAR sequences and MClab sequences, respectively. Assembled sequences were aligned with BioEdit and manually adjusted to account for insertions/deletions. DNAdist and Neighbor within Phylip were used to calculate genetic distances and create phylogenic trees for evolutionary analysis. SynSCAN was used to determine the dN/dS ratio for pairs of sequences.

p24 GHOST assay

GHOST cells (parental, X4 and R5) were diluted with DMEM (10% v/v FBS, 1% w/v Pen/Strep/ glutamine, 500 ug/ml G418, 0.25% 100 µg/ml hygromycin), and the R5 and X4 cells were diluted using DMEM with the addition of 1 µg/ml puromycin. Virus (100 µl) was added to each of the three different GHOST cell types plated at $1x10^4$ cells per well in a 96 well plate. After 24 hrs, the virus was washed off and replaced with the appropriate media. GHOST cells were also observed at day 3 and day 5 under a light microscope for syncytia and physical state. At day 7, 20 µl of supernatant was transferred to a p24 plate at 1:100 dilutions for p24 ELISA. Cells were trypsinized and replated at 7 days.

TZM tropism assay

For each virus tested, 4 wells were plated with $5x10^4$ TZM-bl cells [28] in 1 ml per well in a 24 well plate (No inhibitor, TAK779, AMD3100, Both Inhibitors). The plate was then incubated overnight. After removing most of the media in each well, 50 µl DMEM (10% v/v FBS and Pen/Strep/Glututamine) containing 10 µg DEAE-Dextran was added to the wells without inhibitor. For the wells with inhibitor, 50 µl media containing 10 µg DEAE-Dextran and 1 µM inhibitor (TAK779 and/or AMD3100) was added. After the cells were incubated with inhibitor for 30 min, 200 µl virus was added (10 ng of p24) to each well. Wells without added virus were used to determine the background. The plate was incubated for 2 hr, then 0.75 ml DMEM (+/- 1 µM inhibitor) was added. The cells were incubated for 2 days.

To determine the infection of the cells after 2 days, 300 µg luciferin was added to each well and the emitted photons were captured with a charge-coupled device attached to a Hamamatsu camera. The total relative light units (RLU) were then calculated for each well by IgorPro software. Wells with RLUs 10-fold above background were considered positive.

RESULTS

Study population and the tropism of virus isolates

Virus isolates collected from 12 subjects in 2001 were studied, 9 that formed syncytia (SI) and 3 non-syncytia inducing (NSI) isolates. Sequential isolates were available from 5 subjects in 2003

and a third time point, 2004, from one individual. Age, gender, CD4, HIV RNA level and reported antiretroviral treatment (ART) at each time point for the 12 subjects are shown in Table 1. Coreceptor tropism assays of the 2001 isolates in GHOST cells indicated 3 were R5-using, 2 were X4-using and 7 Dual/Mixed (DM) isolates. The DM isolates suggest the presence of either a mixture of CXCR4 (X4) and CCR5 (X5) using viruses, or dual tropic (R5/X4) virus which could use either co-receptor.

Dualtropic (X4R5) virus from dual/mixed viral isolates

To determine whether the SI isolates that displayed "dual/mixed tropism" were truly dual tropic, or a mixture of R5 and X4 viruses, limiting dilution culture of peripheral mononuclear blood cells (PBMC) infected with the original isolates were performed to isolate viable, infectious virus resulting from infection at Poisson distribution where a single infectious virus was expanded. From the 9 SI viruses and 3 NSI isolates collected in 2001, 3-8 limiting dilution biologic clonal isolates were expanded and tested for coreceptor use by TZM coreceptor assays. All of the clones from the 3 R5 isolates used exclusively R5 and all of the clones from the two X4 isolates yielded only X4 clones. Of the 7 DM/X4 isolates, only one (TC25) was a mixture of R5 and X4 viruses. Three DM/X4 isolates yielded only X4 tropic viruses and two sets of clones used both X4 and R5.

The V3 to V5 region of *env* (approx. 650 bp) was sequenced for all the clones and aligned with the original isolate sequence. As shown in the

Table 1. Cohort characteristics of patients from The Centre.

Participant ID (yr)	Age at enrollment	CD4+ cells per cu mm	HIV-1 RNA level ($\log_{10}$ copies/mL)	Current antiretroviral therapy	MT-2 assay	Isolate coreceptor tropism
TC02_(01)[1]	36	9	5.07	AZT+3TC+IDV	NSI	R5
TC02_(03)		81	3.94	Not Available	SI	Dual/Mix
TC03_(01)	46	81	5.02	DDI+AZT+HYD	SI	Dual/Mix
TC04_(01)	43	1	4.53	ABC+APV+EFV	SI	Dual/Mix
TC08_(01)	36	34	3.15	D4T+3TC+SQV	SI	X4
TC08_(03)		81	<2.6	D4T+3TC+EFV	SI	X4
TC08_(04)		167	4.04	Not available	SI	X4
TC13_(01)	60	3	2.58	AZT+3TC+SQV	SI	Dual/Mix
TC22_(01)	40	214	4.23	IDV+NVP+SQV	SI	Dual/Mix
TC23_(01)	48	255	5.02	AZT+DDC+SQV	SI	Dual/Mix
TC23_(03)		25	5.47	AZT+DDC+SQV	SI	Dual/Mix
TC25_(01)	33	1	5.97	SQV+RTV+EFV	SI	Dual/Mix
TC28_(01)	31	71	5.17	CBV+SQV	SI	X4
TC30_(01)	37	42	5.04	CBV	SI	Dual/Mix
TC31_(01)	40	13	4.48	D4T+3TC+APV	NSI	R5
TC31_(03)		15	5.07	EFV+IDV+LPV	NSI	R5
TC35_(01)	33	158	3.26	CBV+NFV	NSI	R5
TC35_(03)		155	4.19	AZT+3TC+NFV	NSI	R5

[1]Samples collected in 2001 are designated (01), 2003 (03) and 2004 (04).

phylogenetic tree, all clones clustered with their respective isolate sequence (Figure 1). Intraclone variability was determined using the program DNAdist. Clones from the 3 R5 isolates had an average genetic distance of 0.0161 (range: 0.0087 - 0.0246). Among clones from the nine X4/dual isolates, the average genetic distance was 0.020 (range: 0 - 0.0449).

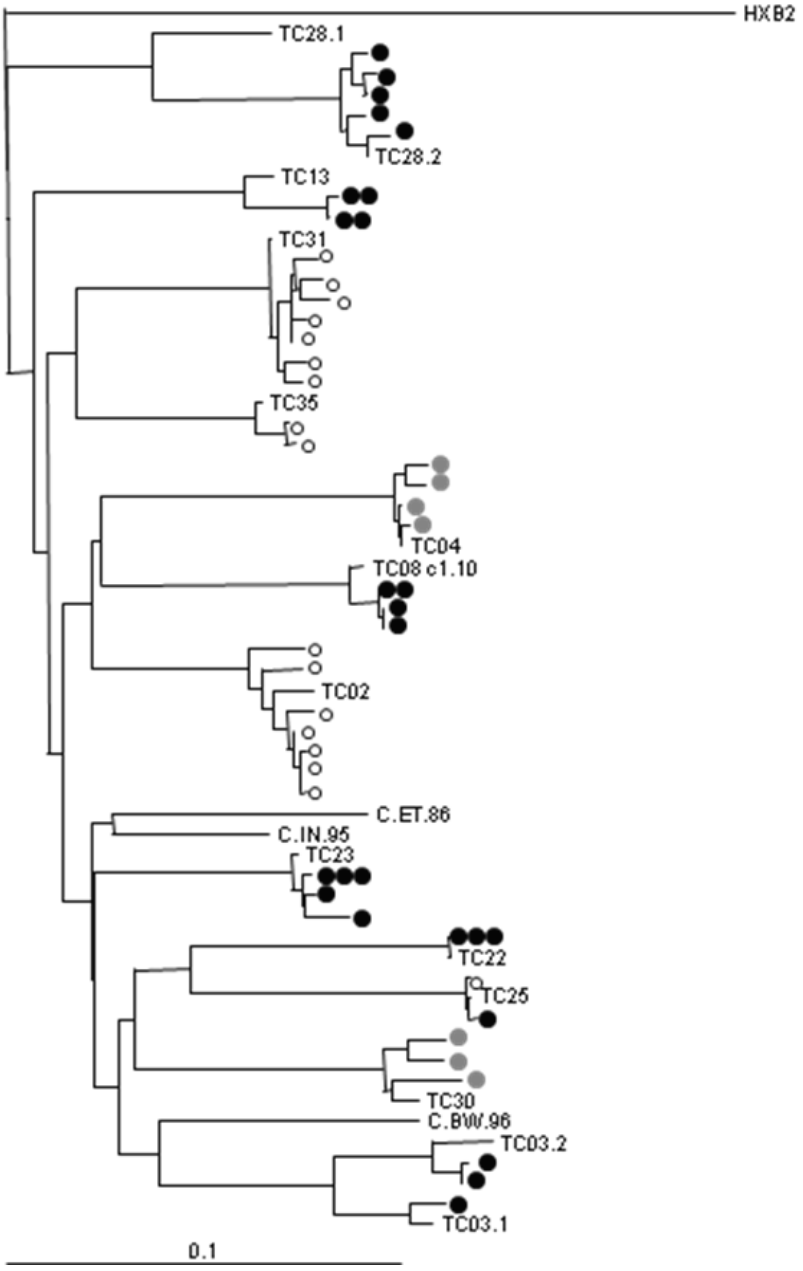

Figure 1. Neighbor joining phylogenetic tree of isolates and clones from 2001 using *env* sequences containing V3 through V5. Sequences of the 650 bp fragment of V3 through V5 were manually aligned in BioEdit and gaps were removed before making tree. HBX2 (Subtype B) was used as the outgroup and three Subtype C sequences were included for reference (C.ET.86, C.IN.95, and C.BW.96). Isolate sequence is labeled with sample name and corresponding biological clones are marked with circles. Closed black circles represent X4 viruses, closed grey circles represent X4R5 viruses and open circles represent R5 viruses.

Evolution of SubC gp120

Biologic clones were obtained from viral isolates after 2 years (2003) from 5 subjects, 2 women and 3 men aged 35-56 years (Table 1). A third time point was obtained from one subject (TC08) in 2004. In total, 49 biological clones (2 to 8 per isolate) were obtained from the 5 patients (Table 3). The V1 to V5 region containing 1500 base pairs of the gp120 region of *env* from each clone was amplified, sequenced and aligned. All sequences from the same individual clustered together (Figure 2).

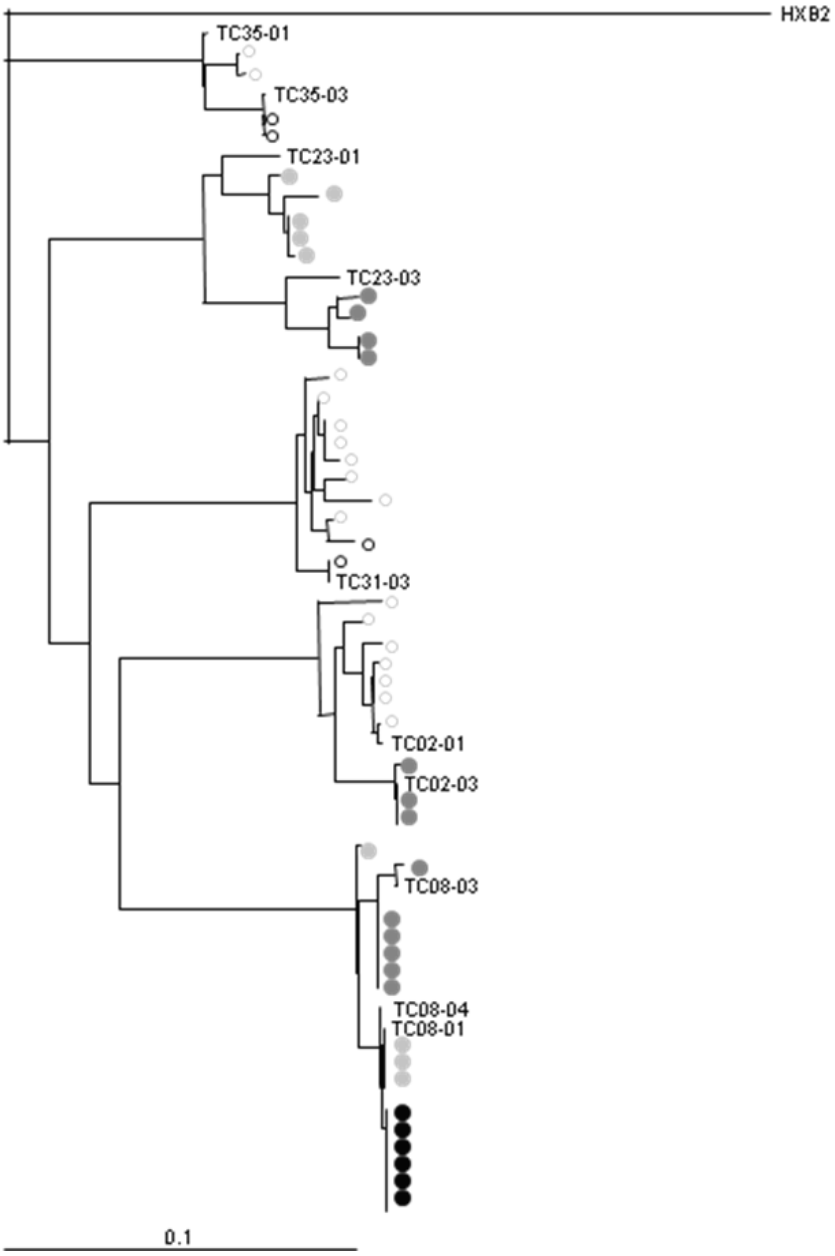

Figure 2. Neighbor joining phylogenetic tree of clones from 2001, 2003 and 2004 using *env* sequences containing 2619bp (V1 through V5). HXB2 (Subtype B) is the outgroup. The isolate sequences are labeled with the sample name, clones are represented by circles. Closed circles are X4 using clones and open circles are R5 using clones. Lightest colored circles are samples from 2001, mid grey circles are from 2003 and black circles are from 2004.

To assess the differences among individual biologically cloned isolates from the same time point, and from clones derived from sequential isolates, genetic distance and Synonymous-Nonsynonymous Mutation Rates (dN/dS) between sequences were obtained using DNAdist and SynScan, respectively. Figure 3a illustrates the mean genetic distance between the gp120 (1500 base pairs) of clonal isolates from each viral isolate between two time points. Clones from TC02-01 and -03 (2001 and 2003, respectively), that switched from exclusive R5 usage to dual tropism over two years demonstrated the greatest nucleotide distance between time points, 0.051. A similar genetic distance of 0.047 was observed between TC23-01 and 03 as the clones demonstrated a change from exclusively using X4 to using both R5 and X4 coreceptors. In contrast, exclusively R5 tropic isolates (TC31 and TC35) had lower genetic distances between 2001 and 2003 clonal sequences of 0.0198 and 0.0309 respectively. The TC08 clonal isolates, which were uniformly X4 over three years, were highly conserved with intraclonal distances of 0.00735 and then 0.00785 from 2001 to 2003 and to 2004, respectively. The 6 biologic clones from 2004 were identical, suggesting one dominant virus.

In addition to the intra-patient nucleotide difference over time, the intra-clone nucleotide distance is illustrated in Figure 3b. For all five samples, the intraclone differences were less than the average nucleotide distance within patients over time. The clones from TC35 had the highest mean nucleotide distance (0.0246), followed by TC23 (0.0161). Clones from TC02 differed by 0.0087 on average, while the clones from 2 years later differed only by an average of 0.0012. Clones from TC35-03 in addition to TC08-04 demonstrated a monophyletic pattern with little difference among the biological clone's sequences.

To differentiate positive and negative (purifying) selection over time, the dN/dS between time points was examined for each of the five patients for the V1 through V5 regions as well as the smaller V1 through V3 region is shown in Figure 4. Four patients had dN/dS ratios less than 1 (0.19 to 0.71) indicating a predominance of negative or purifying selection for both V1-V5 and the smaller region of V1-V3. Two subjects who remained exclusively R5, TC31 and TC35 demonstrated the lowest ratios. In contrast, sequential clones from TC08 that were exclusively X4-using (from 2001, 2003 and 2004) had the highest dN/dS ratios.

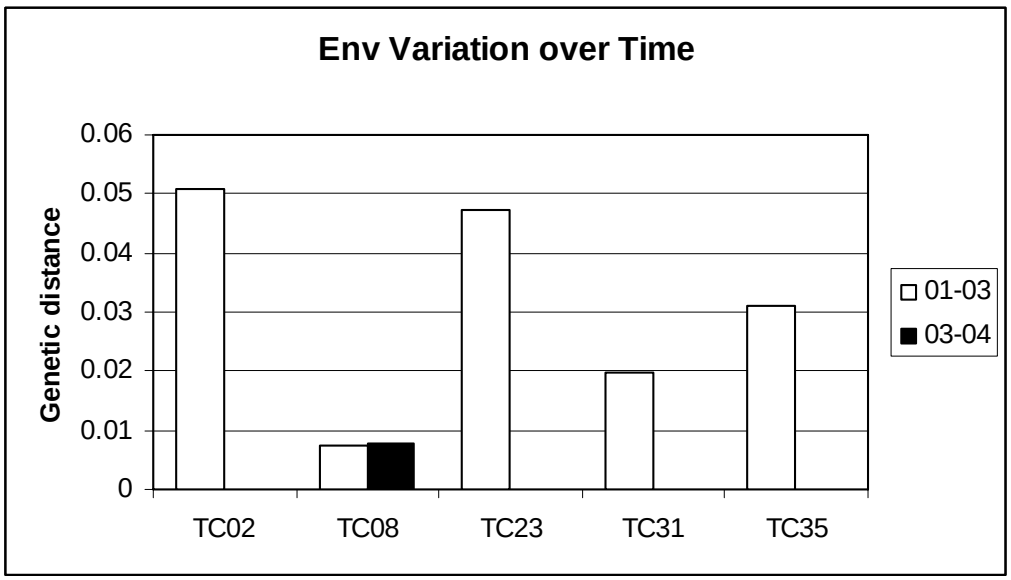

Figure 3a. Envelope variation over time. genetic distances calculated for V1 to V5 sequences with DNAdist program between clones from 2001 and 2003 (white bar) and 2003 to 2004 (black bar).

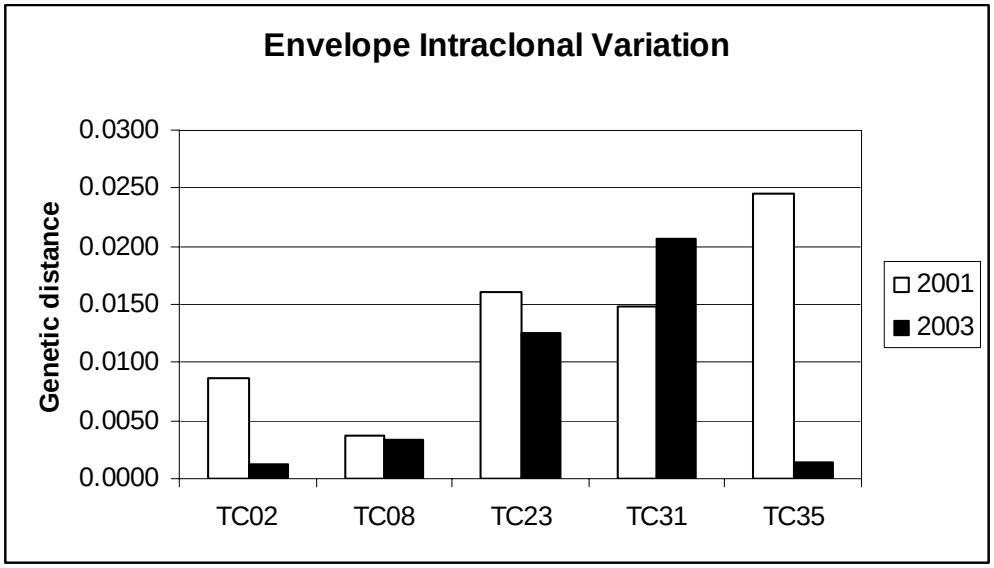

Figure 3b. Clonal Variation between clones within each time point. Genetic distances calculated for V1 to V5 sequences with DNAdist for clones from 2001 (white bar) and from 2003 (black bar).

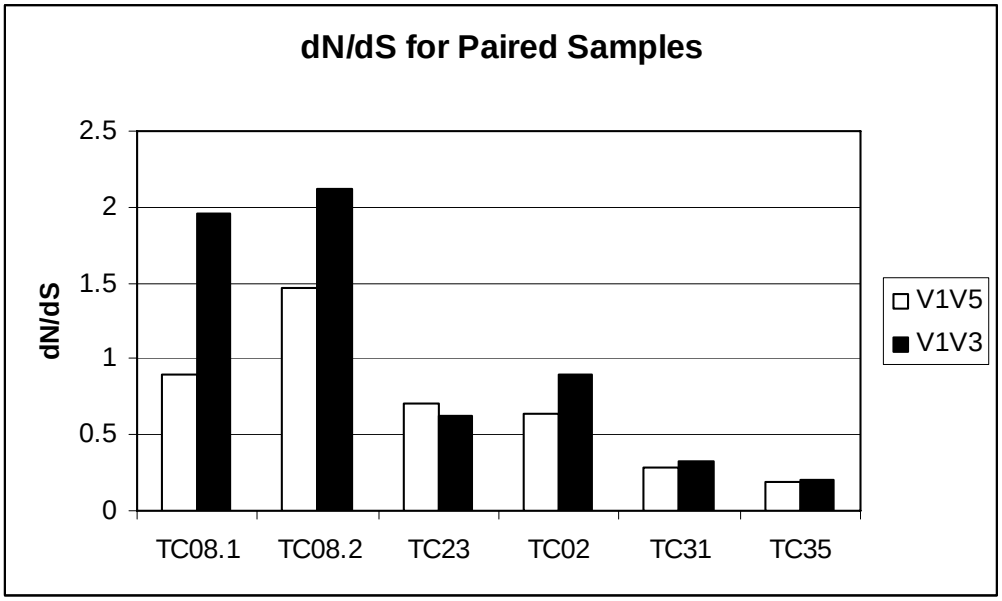

Figure 4. dN/dS for Paired Samples over Time. dN/dS was calculated using SynScan.for V1 to V5 sequences (white bars) and the shorter V1 to V3 sequence (black bars). TC08.1 is between sequences from 2001 to 2003 while TC08.2 is between sequences from 2003 to 2004.

Comparison of the clones from 2001 to 2003 in the V1 to V5 regions yielded a dN/dS of 0.9, while the clones from 2003 to 2004 had a ratio of 1.24 with strong evidence for positive selection as shown by dN/dS rations of 1.96 (2001 to 2003) and 2.12 (2003 to 2004) respectively for the V1 to V3 region while maintaining X4 tropism. There was very little variability between all the clones from the three years, so the few amino acid changes greatly contributed to the dN/dS ratios.

V3 Loop sequences and tropism from biological clones

A total of 75 biologic clones were obtained from 18 isolates by limiting dilution culture. V3 loop sequences from the unique biologic clones are illustrated in Table 2 and 3, where the aligned V3 sequences, the net charge, number of amino acids and tropism as determined by TZM assays are illustrated for 9 SI and 3 NSI isolates from 2001 (Table 2) and 3 SI and 2 NSI isolates from 2003 and 2004 (Table 3). Although net charge and positive charges at two distinct residues - positions 11 and 25 – of V3 have been linked to X4 tropism in subtype B [8], among the X4 and dualtropic clones, only TC13-01 demonstrated a positively charged arginine (R) at codon 11 of the V3 loop. Position 25 was more variable. Four isolates TC02-03, TC03-01, TC04-01 and TC30-01, had either an R or lysine (K) at position 25. One of 5 clones from TC23 also had lysine (K) at position 25. The remaining X4 and R5X4 clones had an increase in the number of positive amino acids, either in the crown region (GRGQ, GQRR, GHRH, GPGR) or adjacent to the crown between amino acids 19 and 30. A positive amino acid at position 24 is a predictor of X4-usage [29]; clones from 2 isolates had a positive charge at 24 but not at 11 or 25. The R5 clones were characterized by a consistent neutral amino acid motif (GPGQ) in the crown and there were no positively charged amino acid at either position 11 or 25.

Among 23 R5-using clones, there were 5 unique V3 loops, with an overall mean net charge of +5.0 (range: +4 to +6). Among 61 X4 and X4R5 biological clones, there were 13 unique V3 loops with a mean net charge of +7.2. The average X4R5 V3 loop net charge of +6.6 (range: +5 to +8) was lower than the mean of X4 net charge of +7.38 (range: +5 to +10), although not statistically significant ($p = 0.29$). However, the mean net charge between the consensus sequences of clones that are exclusively X4 and R5, +7.38 and +5, respectively was significant ($p = 0.003$).

Length polymorphisms in the V3 loop of envelope have also been associated with tropism [30, 31] and, here, envelope insertions and deletions were common among both X4 and X4R5 clones. Clones from 5 of the 9 2001 X4/DualMixed isolates (TC04, TC08, TC 22, TC23, and TC25) harbored two amino acid inserts (either GI or GV) proximate to the crown region, increasing the length of the V3 loop to 37 amino acids. The inserts were maintained in the two isolates with follow-up samples (TC08 and TC23). In addition, clones of TC03-01, with exclusively X4-usage had a single amino acid deletion at position 24 in the 3' end of the V3 region, resulting in 34 amino acids in the V3 loop. Finally, although the R5-using isolate TC02-01 had 35 amino acids and showed little heterogeneity among 7 clones, a subsequent isolate TC02-03 demonstrated an unusual five amino acid insert that increased the length of the V3 loop from 35 to 40 amino acids accompanied by the acquisition of new X4-usage [32]. Thus, clones from 8 of 10 isolates with X4/dual-mixed virus had a change in V3 loop length. In comparison, all of the R5 clones were 35 amino acids in length.

N-linked glycosylation patterns in the V1V2 and V3 regions

In addition to the net charge and specific V3 loop amino acid substitutions, the loss of an N-linked glycosylation patterns within the V3 loop is associated with X4 and dual tropism in subtype B viruses [9] and some subtype C viruses [31]. Among biological clones that use X4 and both coreceptors, only the five clones from TC28 did not contain a V3 loop glycosylation site. Pollakis *et al*. (2001) described a recombinant subtype B virus in which the addition of an N-linked glycosylation site in the V1V2 region of env with an R5 V3 loop resulted in dual tropism. Thus, we examined the N-glycosylation sites within the V1V2 regions of the clones with more than one time point. No clear pattern of loss or gain of glycosylation sites in the V1V2 region of envelope in relation to envelope tropism was observed.

DISCUSSION

Among subtype C viral isolates from Zimbabwe, we found a high frequency of isolates with X4 and dual/mixed (DM) tropism. From these initial isolates, limiting dilution culture identified viable, infectious viruses from "single hit" infection in limiting dilution cultures. The biologic clones derived from isolates can be phenotyped and

Table 2. Biological clone characteristics from 2001 isolates.

Isolate	Number of clones	V3 loop sequence	Charge	Length	Tropism
		1 11 25			
TC03	1	CTRPGNNTRTSIRI GPGQ TFYT-SKVIG-DIRQAHC[1]	5	34	X4
	2	CIRPGNNTSKSIRI GQRR PVYV-NKIIG-DIRQAHC	7	34	X4
TC04	3	CTRPSNNTRKSARIGV GPGQ AFYAIKKIIG-DIRQAHC	7	37	X4R5
	1	CTRPSNNTRKSARVGV GPGQ AFYAIKKIIG-DIRQAHC	7	37	X4R5
TC08	4	CTRPNNNTRKSVRIGI GRGQ AIYAKKAIIG-DIRQAHC	8	37	X4
	1	CTRPNNNTRKSVRIGI GRGQ AFYAKKAIIG-DIRQAHC	8	37	X4
TC13	4	CTRPNNNTRKRVRI GHRH LVYAHGEIIG-NIRQAHC	10	35	X4
TC22	3	CTRPGNKTRQSIRIGI GRGQ SFHATGAIIG-DIRKAYC	7	37	X4
TC23	1	CTRPNNNTRKSVRIGI GRGQ TFYATGKIVG-DIRQAHC	7	37	X4
	4	CTRPNNNTRKSVRIGI GRGQ TFYATGNIVG-DIRQAHC	6	37	X4
TC25	2	CTRPGNNTRRSVRI GPGQ SFHATGTIIG-DIRQAHC	6	35	R5
	1	CTRPGNNTRKSARIGI GPGQ SFRATKQIIG-DIRQAHC	7	37	X4
TC28	4	CGRPNNHRIKGLRI GPGR AFFAMGAIRGGEIRQAHC	8	36	X4
	1	CGRPNNHRIKGLRI GPGR AFFAMGAIGGGEIRQAHC	7	36	X4
TC30	3	CTRPGNNTRRAIGI GPGR TFYATDRIIG-DIRQAHC	5	35	X4R5
TC02	6	CTRPNNNTRKSVRI GPGQ TFYATNGIIG-DIRQAHC	5	35	R5
	1	CTRPNNNTRKSVRI GPGQ VFYATNGIIG-DIRQAHC	5	35	R5
TC31	8	CTRPGNNTRKSVRI GPGQ AFYATGGIIG-DIRQAHC	5	35	R5
TC35	2	CTRPGNNTRKSVRI GPGQ AFYATGEIIG-DIRQAHC	4	35	R5

[1]Positively charged residues lysine (K), arginine (R), and histidine (H) are underlined and negatively charged residues aspartate (D) and glutamate (E) are highlighted in grey.

Table 3. V3 loop sequence for clones at from sequential samples.

		1	11	25	Net charge	Tropism
Consensus		CTRPNNNTRK	SVRI--GRGQAFYA	T-----GA IIGDIRQAHC		
TC02	(6)[1]	CTRPNNNTRK	SVRI--GPGQTFYA	T-----NG IIGDIRQAHC[2]	+5	R5
	(1)	CTRPNNNTRK	SVRI--GPGQVFYA	T-----NG IIGDIRQAHC	+5	R5
TC02-03	(3)	CTRPNNNTRK	SIRL--GPGQAFYA	TRSYTPKR IIGDIRQAHC	+8	X4R5
TC23	(4)	CTRPNNNTRK	SVRIGIGRGQTFYA	T-----GK IVGDIRQAHC	+6	X4
	(1)	CTRPNNNTRK	SVRIGIGRGQTFYA	T-----GN IVGDIRQAHC	+6	X4
TC23-03	(4)	CTRPNNNTRK	NVRIGIGRGQTFYA	T-----GE IVGDIRKAHC	+6	X4R5
TC31	(8)	CTRPGNNTRK	SIRI--GPGQAFYA	T-----GG IIGDIRQAHC	+5	R5
TC31-03	(2)	CTRPGNNTRK	SIRI--GPGQAFYA	T-----GG IIGDIRQAHC	+5	R5
TC35	(2)	CTRPGNNTRK	SIRI--GPGQAFYA	T-----GE IIGDIRQAHC	+4	R5
TC35-03	(2)	CTRPGNNTRK	SIRI--GPGQAFYA	T-----GE IIGDIRQAHC	+4	R5
TC08	(4)	CTRPNNNTRK	SVRIGIGRGQAIYA	K-----KA IIGDIRQAHC	+8	X4
	(1)	CTRPNNNTRK	SVRIGIGRGQAFYA	K-----KA IIGDIRQAHC	+8	X4
TC08-03	(6)	CTRPNNNTRK	SVKIGIGRGQAIYA	K-----KA IIGDIRQAHC	+8	X4
TC08-04	(6)	CTRPNNNTRK	SVRIGIGRGQAIYA	K-----KA IIGDIRQAHC	+8	X4

[1]Number of clones with V3 loop sequence.
[2]Positively charged residues lysine (K), arginine (R), and histidine (H) are underlined, and negatively charged residues aspartate (D) and glutamate (E) are highlighted in grey.

accurately sequenced through the envelope region to identify the genotypic correlates of X4-usage. Through analysis of cultured isolates and biologic cloning of low passage isolates we sought to gain a greater understanding of the gp120 sequence of HIV-1 subtype C viruses, evolution within *env* and the relationship of V3 loop and N-linked glycosylations to chemokine receptor tropism.

Previous studies with small cohorts of Subtype C X4-using have shown a correlation between X4 usage and highly charged V3 loop consensus sequence and positively charged residues in the crown, or at positive residues at positions 11 and 25 [18, 31, 33, 34]. Correlation with the 11/25 amino acid rule for the V3 region [8] was observed in at least one clone from 6 of 13 isolates with X4/DM virus (46%). If position 24 is also included, the number of isolates associated with X4 tropism increases to 10 of 13 (77%). The three remaining X4/DM samples that did not have positively charged amino acids at positions 11, 24 or 25, did have a positively charged amino acid in the crown region, a net charge of above +6 or greater and an increase in the length of the V3 loop from 35 to 36 or 37 amino acids.

Charged residues in the crown region of Subtype C viruses and insertions in the V3 loop account for most of the X4 and dual-mixed viruses seen here, and differ from subtype B viruses. While both R5 and X4 Subtype B viruses often have a crown sequence of GPGR, subtype C R5 viruses usually have GPGQ at the crown (amino acids 15-18) of the V3 loop. Among the isolates we examined which were selected for X4-usage, X4 and X4R5 clones from 10 of 13 isolates contained at least one positively charged amino acid (R, lysine, K, arginine, or H, histidine) within the crown region. Five isolates contained a 2 amino acid insertion (GI or GV) and had an overall V3 length of 37 amino acids. Three of the clonal isolates were X4-using, and yet had a GPGQ crown and contained a two amino acid insert 5' to the crown of the V3 loop with the GI or GV inserted in an area that is pivotal for coreceptor binding [13]. As suggested by Coetzer *et al*. [31], a positive charge in the crown region may be a necessary step for switching to X4 use. The highly conserved GPGQ crown typical in subtype C R5 viruses may delay the rate of switching. However either an insertion that lengthens the loop to 37 amino acids, a positively charged amino acid in the crown sequence, or both, appear to be strongly associated with X4-usage among subtype C HIV-1 viruses.

The rate of overall evolutionary change of the *env* protein may provide an explanation for co-receptor switching (Figure 2). Comparison of the evolution of envelope of replication competent virus isolates over two years using multiple biologic clones obtained through limiting dilution cultures showed change in up to 5% of nucleic acid bases in gp120 over the course of two years. However amino acid changes were nearly uniformly less frequent and four of the five subjects with multiple time points demonstrated purifying (negative) selection (Figure 4). Only the isolates from a subject with exclusively X4 virus, showed continuous positive selection, though the absolute number of changes over time was low.

We examined envelope evolution and co-receptor use in selected viral isolates obtained from subtype C infected individuals where there was a high rate of X4 use. Biological cloning of virus isolates provides a unique view of HIV envelope function, since biologic clones are viable, infectious virus isolates, while molecular cloning may capture genes and viral sequences that are not replication competent. Cloning of the Dual/Mixed isolates revealed a high proportion of X4 and X4R5 viruses. This is in contrast to other findings of mostly R5 viruses among Dual/Mixed plasma samples from untreated patients [35]. On the other hand, the small numbers of selected patient isolates, the relatively short follow-up time and the limited number of biologic clones are each limiting factors in generalizing these observations. Nevertheless, evolutionary sequence and tropism analyses suggest that the predominance of dual/mixed R5/X4 viruses may be explained by changes in charge distribution and structural features of the V3 loop.

CONCLUSIONS

The phylogenetic analyses show that emerging X4-usage in subtype C HIV-1arises through the outgrowth of viruses that are X4/R5, although these viruses share 99% or more *env* homology with R5 virus. Key amino acid changes result in

an increase in net charge, particularly with in the crown region of the V3 loop, and 2 amino acid insertions within the loop accounted for most of the differences between R5 and X4-using clonal isolates. Other aspects of gp120 need to be investigated for affects on tropism, and much still has to be done in terms of characterizing viral coreceptor usage to gain a better understanding of tropism among Subtype C HIV-1 isolates.

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