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654

655

656 **legends**

657 Fig. 1. Amino acid sequences deduced from the nucleotide sequences of env-encoding

658 regions of proviral DNA isolated at the baseline (A), and indicated passages (p5, p6 and

659 p16) from HIV-1_{BaL} variants selected in the presence of KD-247 and (B) the passage

660 control (p16).The amino acid sequences of the envelope proteins of a clone of HIV-1_{BaL}

661 at baseline are shown at the top as a reference. Identity to the sequence at individual

662 amino acid positions is indicated by dots. The numbers of clones with the given amino

663 acid substitutions among a total of 61 clones (A) and 11–20 clones (B) are listed.

664

665 Fig. 2. The gp120 mutation profile of HIV-1_{BaL} evasion variants from KD-247 *in vitro*.

666 The ratio of the PNGS insertion in the V2 region and mutations in the C2 and V3

667 regions in gp120 of HIV-1_{BaL} variants were plotted for each passage. The y-axis

668 indicates the percentage of PNGS insertions or mutations in the tested clones and the

669 x-axis shows the concentration of KD-247 ($\mu g\ ml^{-1}$).

670

671 Fig. 3. Schematic representation of recombinant HIV-1_{BaL} env genes used for analysis

672 of the genetic basis for resistance to KD-247.

673 Mutated env genes were amplified from passaged HIV-1_{BaL} virus-infected PM1/CCR5
674 cells in the absence or presence of KD-247. The recombinant env genes were
675 constructed by replacing each region of passaged control with corresponding sequence
676 of escaped variant of HIV-1_{BaL} or by site-directed mutagenesis. The locations and
677 numbers of specific amino acids, based on the HXB2 sequence, are shown above the
678 reference HIV-1_{BaL} sequence.

679

680 Fig. 4. Sensitivities of HIV-1 strains pseudotyped with recombinant HIV-1_{BaL} env genes
681 to KD-247, 2D7, rsCD4 and CCR5 inhibitor.

682 KD-247, 2D7 (anti-CCR5 MAb), rsCD4 and maraviroc (CCR5 inhibitor) were
683 pre-incubated with 300 $TCID_{50}$ of each HIV-1_{BaL} pseudotype virus for 30 min, then
684 added to TZM-bl target cells. Inhibitory effects were determined by measuring
685 β-galactosidase activity on day 2 of culture.

686

687 Fig. 5. Viral infectivity of HIV-1 infectious clones with recombinant HIV-1_{BaL} mutant
688 env genes.

689 HIV-1 infectious clones with the env gene sequences listed in Figure 3 were prepared as
690 described in the Materials and Methods. PM1/CCR5 cells were exposed to the
691 infectious clones [input p24 amount; (A) 2 ng and (B, C) 10 ng] and cultured for 6 days
692 in the presence or absence of KD-247. The replication of the infectious clones with
693 mutant Env was monitored by measuring the amounts of p24 Gag protein produced in
694 the culture supernatants in the absence (A) or presence (B) of KD-247. The clones with
695 a PNGS and various other mutations were also monitored in the absence of KD-247 (C).

696

697 Fig. 6. The gp120 mutation profile of KD-247 HIV-1_{BaL} evasion variants for 14
698 additional passages without KD-247.

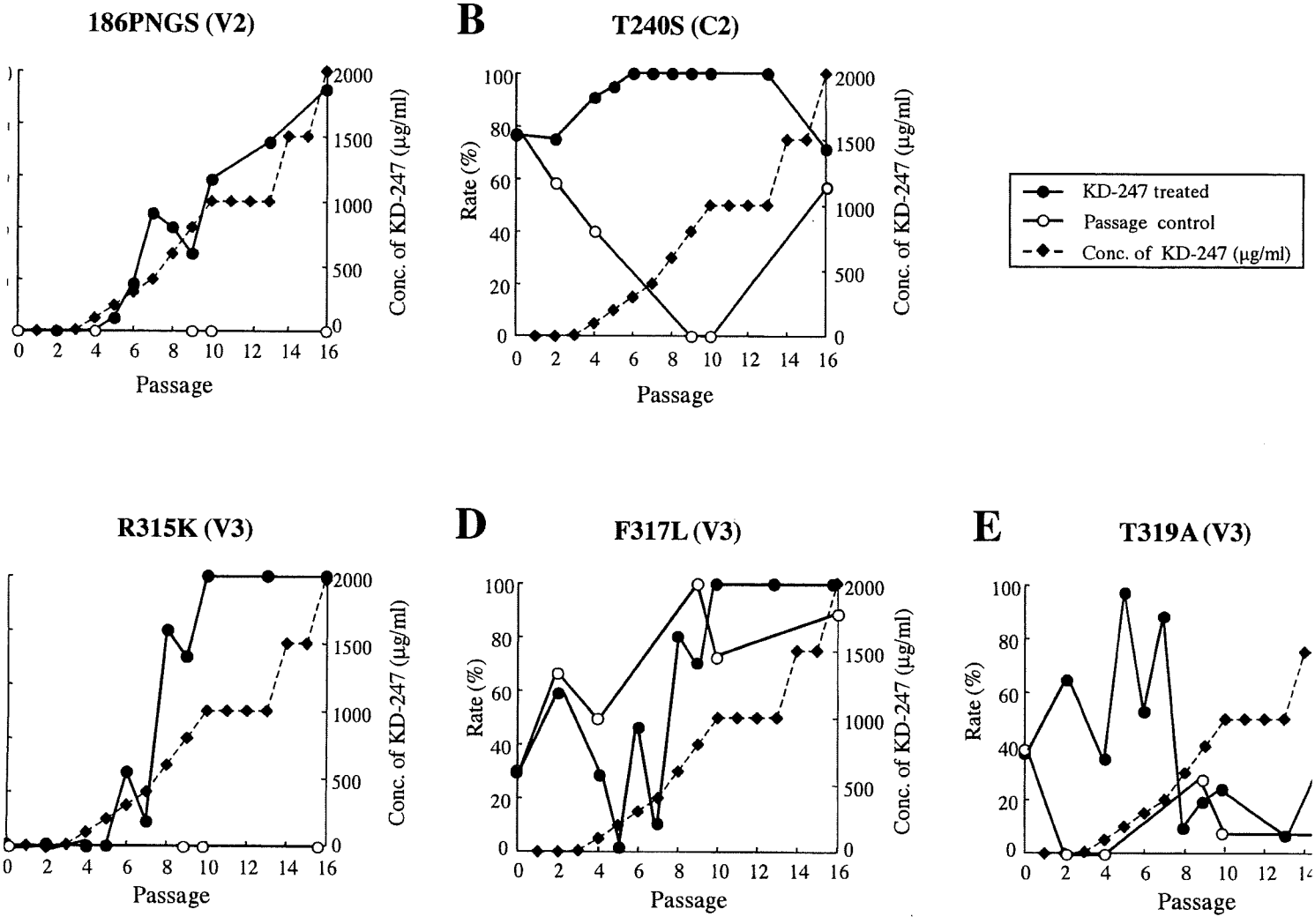
699 The ratio of the PNGS insertion in the V2 region and mutations in the C2 and V3 region
700 of HIV-1_{BaL} evasion variants in gp120 were plotted for 16 passages in the presence of
701 KD-247 and an additional 14 passages in the absence of the MAb. The y-axis shows the
702 percentage of PNGS insertions or mutations in the tested clones. The x-axis shows the
703 concentration of KD-247.

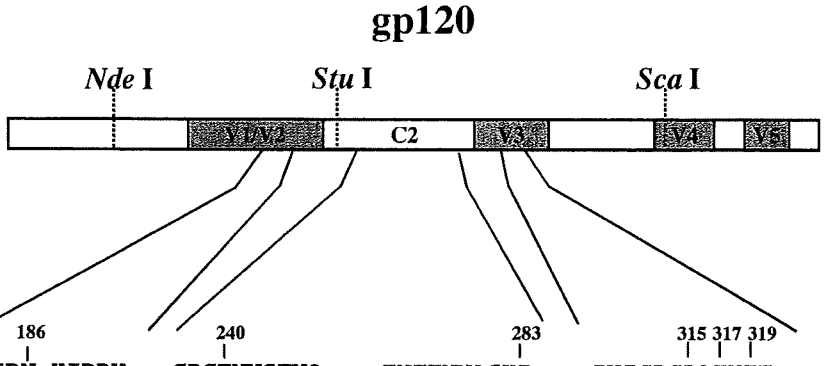
A

virus	V2	C2		V3
	186	240	283	315 317 319
	VPIDN-KIDRY	GPCTNVSTVQ	ENFTNNASKI	IHIGPGRAFYTT
8/61	.....-.....	....	....	....
6/61	.....-.....	...S......	....	....
5/61	.....-.....	...S......	.........T	.........L.A.
4/61	.....-.....	....	A..AD.....	....
3/61	.....-.....	....	....	.........L.A.
3/61	.....-.....	...S......	A..AD.....	.........L.A.
3/61	.....-.....	...S......	.........T	..........A.
3/61	.....-.....	...S......	A..AD.....	....
3/61	.....-.....	...S......	.........T	....
2/61	.....-.....	...S......	....	..........A.
2/61	.....-.....	...S......	....	.N......L...
2/61	.....-.....	...S......	A..AD.....	..........A.
1/61	.....-.....	...S......	....D.....	....
1/61	..V..-.....	....	....	....
1/61	.....-.....	...S......	....S....T	..........A.
1/61	.....-.....	...S......	A..A......	.........L...
1/61	.....-.....	...S......	A...D.....	....
1/61	.....-.....	....	.........T	.N......L...
1/61	.....-.....	....	....	.........L...
1/61	.....-.....	...S......	A...D.....	..........A.
1/61	.....-.....	...S......	....	.N......L...
1/61	.....-.....	....	.........T	M...........
1/61	.....-.....	...S......	....	.........L...
1/61	.....-.....	....	.........T	....
1/61	.....-.....	...S......	A..AD....T	....
1/61	.....-....K	...S......	A..AD.....	....
1/61	....KSNN...	...S......	A..AD.....	..........A.
1/61	.....-.....	..RS......	A..AD....T	..........A.
1/61	.....-.....	...S......	A..AD.....	T.........A.

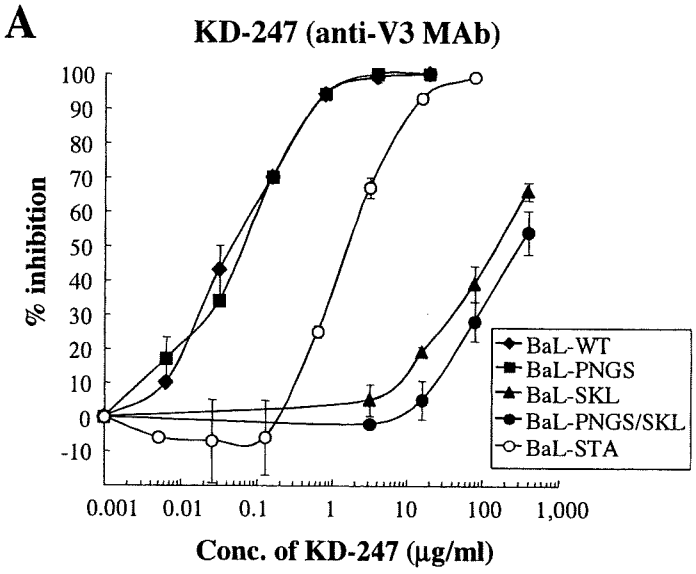
B

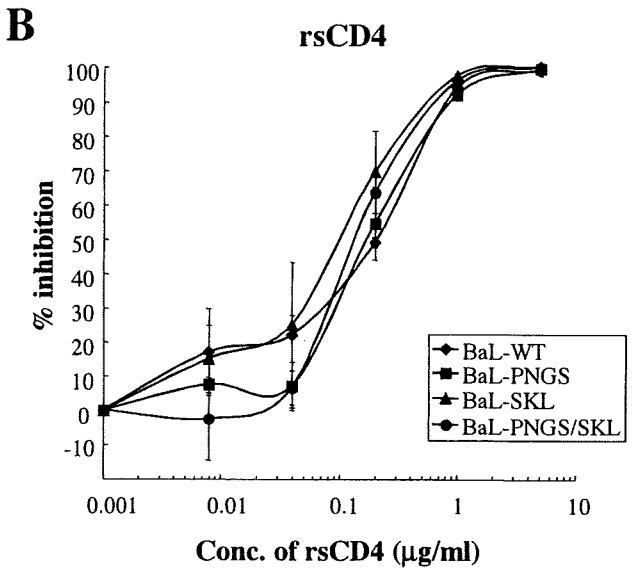
		V2	C2		V3
		186	240	283	315 317 319
		VPIDN-KIDRY	GPCTNVSTVQ	ENFTNNASKI	IHIGPGRAFYTT
7 selection					
00) p5	15/20	.....-.....	...S.....	.....T	.....A.
00) p5	1/20	.....-.....	...S...I..	.....T	.....A.
00) p5	1/20	.....-.....	...S.....	.....T	.N.....L...
00) p5	1/20	.....-.....	...S..N...	.....T	.....A.
00) p5	1/20	.....NSNN..	...S.....	.....T	.....A.
00) p5	1/20	.....-.....	...S.....	.....T	.N.....
00) p6	3/11	.....-.....	...S.....	.....	.....K.L...
00) p6	2/11	.....-.....	...S.....	.....T	.....A.
00) p6	2/11	.....NSNN..	...S.....	.....	.....A.
00) p6	1/11	.....-.....	...S.....	.....	.....L...
00) p6	1/11	.....-.....	...S.....	..S.....T	.N....K.L...
00) p6	1/11	.....-.....	...S.....	.....T	.N.....
00) p6	1/11	.....-.....	...S.....	.....	.....L.A.
000) p16	4/14	.....NSNN..	...S.....	.....	.....K.L.A.
000) p16	3/14	.....NSNN..	.....	.....	.....K.L.A.
000) p16	2/14	.....NSNN..	...S.....	.....	.....K.L...
000) p16	2/14	.....NSNN..	...S.....	.....	.....DK.L...
000) p16	1/14	.....NSNN..	...S.....	.....T	.....K.L...
000) p16	1/14	.....NSNN..	.....	.....	.....KTL...
000) p16	1/14	.....-.....	...S.....	.....	.....K.L...
e control					
) p16	4/13	.....-.....	.....	.....	.N.....L...
) p16	3/13	.....-.....	...S.....	.....	.N.....L...
) p16	1/13	.....-.....	...S..G...	.....	.N.....L...
) p16	1/13	.....-.....	...S.....	.....	.....
) p16	1/13	.....-.N...	...S.....	.....	.....L...
) p16	1/13	.....-.....	.....	.....	.....L...
) p16	1/13	.....-.....	.....	.....	.....L.A.
) p16	1/13	.....-.....	...S..R...	.....	.....L...

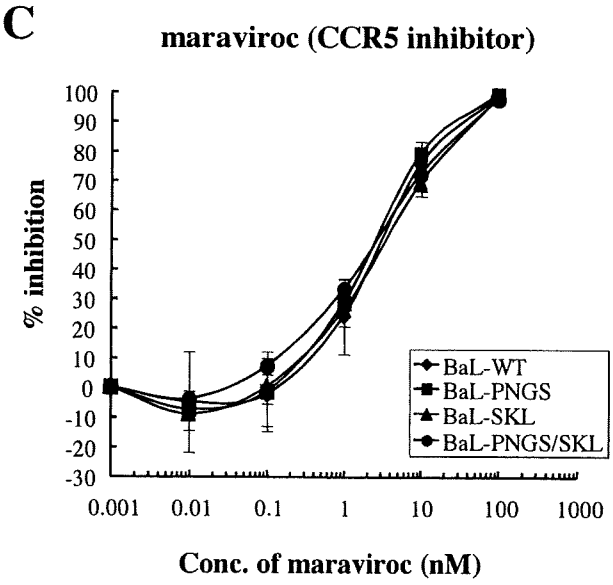

186PNGS (V2)
B
T240S (C2)
R315K (V3)
D
F317L (V3)
E
T319A (V3)
Rate (%)
Conc. of KD-247 (μg/ml)
Passage
KD-247 treated
Passage control
Conc. of KD-247 (μg/ml)

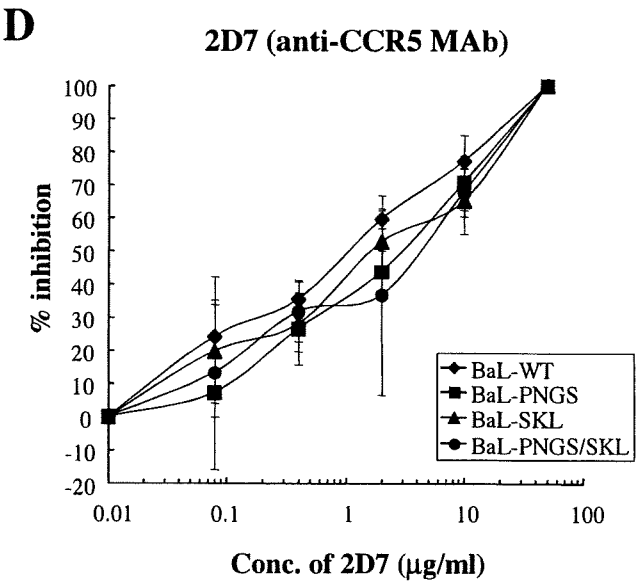


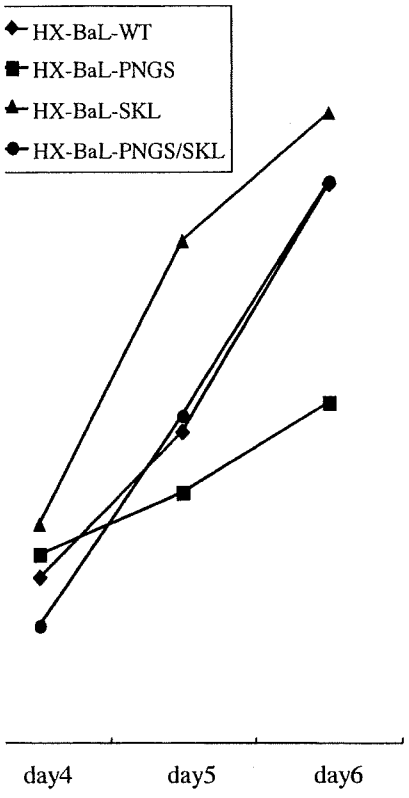
	186	240	283	315 317 319	Insertion and/or mutations
WT	VPIDN-KIDRY	GPCTNVSTVQ	ENFTNNASKI	IHIGPGRAFYTT	
STA	.....-.....	...S.....	.....T	.....A.	T240S+I283T+T319A
PNGS/SKL	.....NSNN..	...S.....	.....	.....K.L...	186PNGS+T240S+R315K+
PNGS	.....NSNN..	.....	.....	.....	186PNGS
SKL	.....-.....	...S.....	.....	.....K.L...	T240S+R315K+F317L
S	.....-.....	...S.....	.....	.....	T240S
K	.....-.....	.....	.....	.....K.....	R315K
L	.....-.....	.....	.....	.....L...	F317L
PNGS/S	.....NSNN..	...S.....	.....	.....	186PNGS+T240S
PNGS/K	.....NSNN..	.....	.....	.....K.....	186PNGS+R315K
PNGS/L	.....NSNN..	.....	.....	.....L...	186PNGS+F317L
Q	....QNSNN..	.....	.....	.....	186Q
Q/K	....QNSNN..	.....	.....	.....K.....	186Q+R315K


A
KD-247 (anti-V3 MAb)
% inhibition
100
90
80
70
60
50
40
30
20
10
0
-10
0.001
0.01
0.1
1
10
100
1,000
Conc. of KD-247 (μg/ml)
BaL-WT
BaL-PNGS
BaL-SKL
BaL-PNGS/SKL
BaL-STA

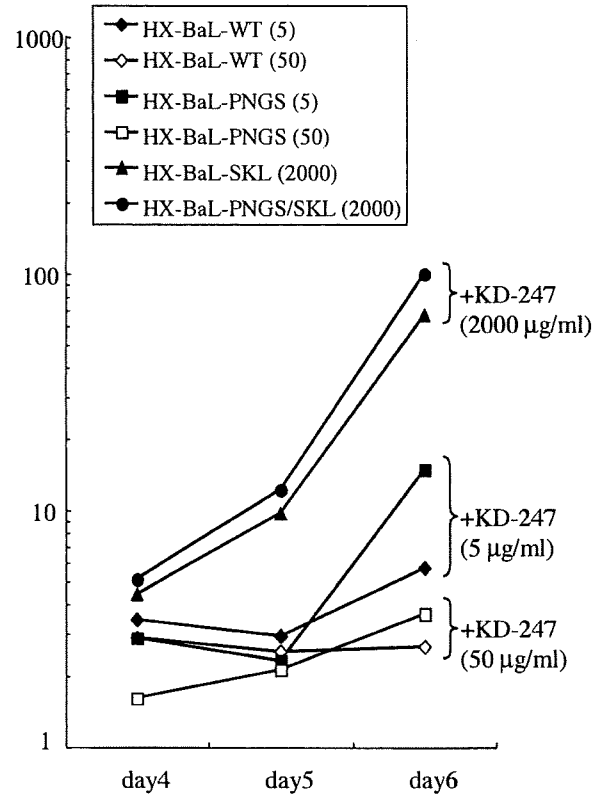

B
rsCD4
% inhibition
100
90
80
70
60
50
40
30
20
10
0
-10
0.001
0.01
0.1
1
10
Conc. of rsCD4 (μg/ml)
BaL-WT
BaL-PNGS
BaL-SKL
BaL-PNGS/SKL


C
maraviroc (CCR5 inhibitor)
% inhibition
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
0.001
0.01
0.1
1
10
100
1000
Conc. of maraviroc (nM)
BaL-WT
BaL-PNGS
BaL-SKL
BaL-PNGS/SKL

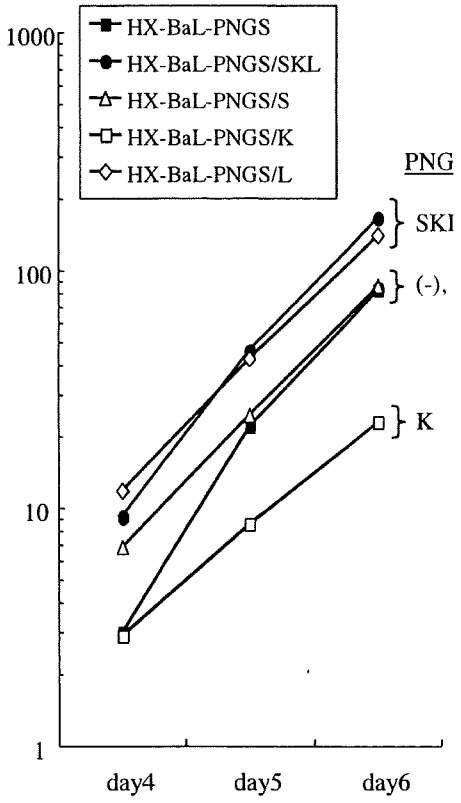

D
2D7 (anti-CCR5 MAb)
% inhibition
100
90
80
70
60
50
40
30
20
10
0
-10
-20
0.01
0.1
1
10
100
Conc. of 2D7 (μg/ml)
BaL-WT
BaL-PNGS
BaL-SKL
BaL-PNGS/SKL

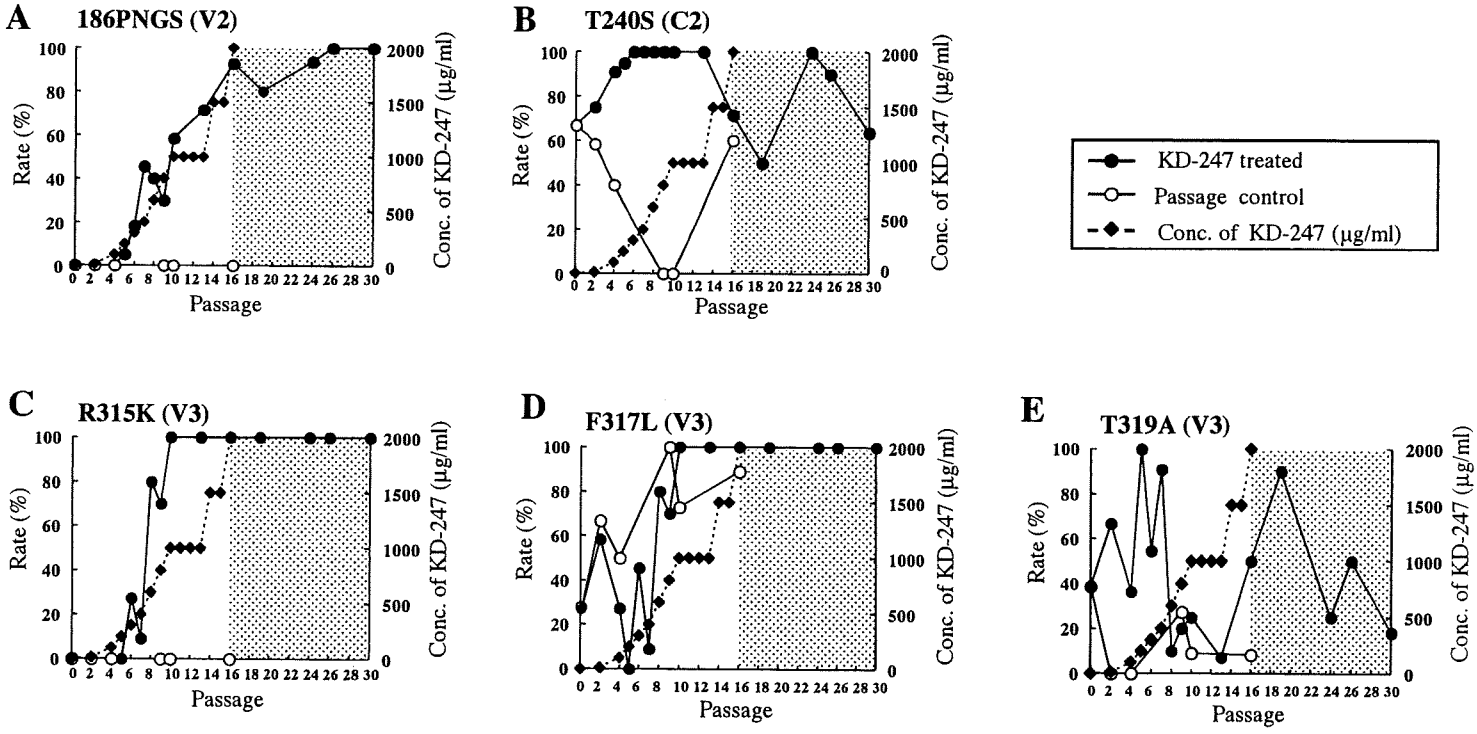

HX-BaL-WT
HX-BaL-PNGS
HX-BaL-SKL
HX-BaL-PNGS/SKL
day4
day5
day6
Post infection

B


HX-BaL-WT (5)
HX-BaL-WT (50)
HX-BaL-PNGS (5)
HX-BaL-PNGS (50)
HX-BaL-SKL (2000)
HX-BaL-PNGS/SKL (2000)
1000
100
10
1
+KD-247
(2000 μg/ml)
+KD-247
(5 μg/ml)
+KD-247
(50 μg/ml)
day4
day5
day6
Post infection

C


HX-BaL-PNGS
HX-BaL-PNGS/SKL
HX-BaL-PNGS/S
HX-BaL-PNGS/K
HX-BaL-PNGS/L
1000
100
10
1
PNG
SKI
(-),
K
day4
day5
day6
Post infection


A
186PNGS (V2)
B
T240S (C2)
C
R315K (V3)
D
F317L (V3)
E
T319A (V3)
Rate (%)
Conc. of KD-247 (μg/ml)
Passage
KD-247 treated
Passage control
Conc. of KD-247 (μg/ml)

e 1. Neutralization sensitivities of passaged variants to KD-247.

	passage No.	KD-247 conc. (μg ml^{-1})	IC_{50} (μg ml^{-1}) [a]
Baseline virus	p0	0	0.32 ± 0.20
Passage control	p10	0	0.09 ± 0.04
KD-247 selection	p2	10	0.54 ± 0.19
	p5	200	5.68 ± 1.48
	p6	300	> 100
	p7	400	> 100
	p10	1000	> 100
	p16	2000	> 100

[a] TZM-bl cells (2×10^4 cells $well^{-1}$) were exposed to 300 $TCID_{50}$ of passage control (p10) or KD-247 selected variants (p2, p5, p6, p7, p10, p16) in the presence of various concentrations of KD-247 in 96-well flat-bottom microculture plates and incubated for 48 h. The IC_{50} values were determined using a chemiluminescent assay for β-galactosidase detection. Data shown represents the means ± 1 standard deviation from the results of three independent experiments.

e 2. Anti-HIV1 activities of KD-247 and the CCR5 inhibitor, maraviroc.

	IC_{50} ± SD of maraviroc (nM) [a]	IC_{50} ± SD of KD-247 (μg ml^{-1}) [a]
HX-BaL-WT	2.0 ± 0.72	0.092 ± 0.028
HX-BaL-PNGS	1.2 ± 0.28	0.047 ± 0.028
HX-BaL-Q	1.9 ± 1.3	0.12 ± 0.047
HX-BaL-S	1.8 ± 0.72	0.087 ± 0.021
HX-BaL-L	2.6 ± 0.33	0.036 ± 0.012
HX-BaL-STA	2.5 ± 1.7	4.6 ± 0.71
HX-BaL-PNGS/SKL	2.4 ± 0.29	214 ± 84
HX-BaL-K	1.6 ± 0.35	285 ± 76
HX-BaL-PNGS/K	2.7 ± 0.52	582 ± 59
HX-BaL-Q/K	1.6 ± 0.32	276 ± 31

b * ** ***

[a] TZM-bl cells (2×10^4 cells $well^{-1}$) were exposed to 300 $TCID_{50}$ of the infectious clones with wild-type or mutant Env in the presence of various concentrations of maraviroc or KD-247, and incubated for 48 h. IC_{50} values were determined using a chemiluminescent assay for β-galactosidase detection. All assays were conducted in duplicate or triplicate and the data shown represent means ± 1 standard deviation from the results of three independent experiments.

[b] *P* values < 0.05 were considered statistically significant (Student's t test). **P*=0.006, ** *P*=0.007, *** *P*=0.89.

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HIV Type 1 Subtype Diversity and Drug Resistance among HIV Type 1-Infected Kenyan Patients Initiating Antiretroviral Therapy

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Abstract

The treatment of HIV-1 infection with antiretroviral drugs has greatly improved the survival of those who are infected. However, HIV-1 diversity and drug resistance are major challenges in patient management, especially in resource-poor countries. To evaluate HIV-1 genetic diversity and drug resistance-associated mutations among drug-naive patients in Kenya prior to antiretroviral therapy (ART), a genetic analysis of HIV-1 *pol*-RT and *env-gp41* was performed on samples collected from 53 (18 males and 35 females) consenting patients between April and June 2005. The average age, baseline $CD4^+$ T cell counts, and viral loads were 38 (range, 24–62) years, 475 (range, 203–799) cells/mm^3, and 4.7 (range, 3.4–5.9) log_{10} copies/ml, respectively. Phylogenetic analysis revealed that 40 samples (75.5%) were concordant subtypes for the two genes and 13 (24.5%) were discordant, suggesting possible recombination and/or dual infections. Prevalent subtypes included A1/A1(*pol*-RT/*env-gp41*), 31 (58.5%); D/D, 9 (16.9%); A1/C, 2 (3.8%); A1/D, 4 (7.5%); G/A1, 2 (3.8%); A1/A2, 1 (1.9%); C/A1, 2 (3.8%); D/A1, 1(1.9%); and D/A2, 1 (1.9%). Major reverse transcriptase inhibitor (RTI) resistance-associated mutations were found in four patients (7.5%). Of these patients, three had nucleoside RTI resistance mutations, such as M184V, K65R, D67N, K70R, and K219Q. Nonnucleoside RTI resistance-associated mutations K103N and Y181C were detected in three patients and one patient, respectively. Multiple drug resistance mutations were observed in this drug-naive population. With increasing numbers of patients that require treatment and the rapid upscaling of ART in Kenya, HIV-1 drug resistance testing is recommended before starting treatment in order to achieve better clinical outcomes.

Introduction

GENETIC VARIATION IS INHERENT TO ALL RNA VIRUSES, but it has been extensively characterized for human immunodeficiency virus type 1 (HIV-1). The genetic diversity of HIV-1 originates from rapid viral turnover in an infected individual and a high rate of incorrect nucleotide substitutions during HIV reverse transcription in the absence of proofreading mechanisms.[1,2] HIV-1 continuously evolves, overcoming barriers to transmission, avoiding different immune responses, and resisting various antiretroviral regimens.[3–6] Though vaccination is one of the potential options for curtailing the epidemic, the diversity of HIV-1 presents an extraordinary challenge to drug and vaccine development.[7–9]

Antiretroviral therapy (ART) using nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs) has sharply reduced HIV transmission, morbidity, and mortality in developed countries, but it has created the long-term specter of drug resistance. Widespread use of ART in these countries has resulted in an increased prevalence of drug-resistant variants, ranging from 10% to 20% among drug-naive patients.[10–12] Intervention through such programs as the World Health Organization's (WHO's) 3 by 5 plan to treat 3 million people by the end of 2005 and the President's Emergency Plan for AIDS Relief has significantly promoted access to ART in low-income and middle-income countries.[13–15]

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As access to ART rapidly increases in these resource-limited countries, the prevalence of HIV-1 drug-resistant strains among drug-naive patients is also expected to increase. Studies conducted among drug-naive individuals in Cameroon found increased drug resistance, from 0% in 2002 before the start of the WHO's 3 by 5 initiative to 9.8% in 2004, when the availability of ART to those in need was 13.5%.[16–18] In Mozambique, where ART was available to 32% of the estimated population in need in 2004, the prevalence of mutations conferring resistance to both NRTIs and NNRTIs among drug-naive patients was reported to be 5.9%.[19] In Botswana, the prevalence was 0% in 2001 before the country's ART program began.[20] Currently, all patients meeting country-based criteria for ART receive treatment and drug resistance among drug-naive patients has been projected to rise to 15% by the end of 2009.[21] In Uganda, Tanzania, and Malawi, major RTI-resistant HIV-1 mutants were rarely found among newly diagnosed patients.[22–24]

In Kenya, the current standard first-line therapy consists of two NRTIs, stavudine (d4T) and lamivudine (3TC), plus one NNRTI, either nevirapine (NVP) or efavirenz (EFV). PI-containing regimens are not yet widely available. The rapid upscaling of ART was accompanied by increased availability to those in need, increasing to 17% by the end of 2005.[25,26] By that time, the WHO's ART guidelines for surveying and monitoring HIV drug resistance in resource-poor countries[27–29] had not been fully implemented. In addition, the use of single dose NVP among HIV-infected antenatal clinic attendees influenced the need for laboratory monitoring.[30–33] Kenyan HIV-1 vertically infected children were reported to have acquired drug resistance mutations.[34] However, the magnitude of drug resistance in adults has not been determined. The current study was conducted to determine HIV-1 diversity and RTI resistance-associated mutations among HIV-1-infected drug-naive adults in Kenya where ART is being rapidly scaled up.

Materials and Methods

Study population and samples

Individuals who were 18 years of age or older and who presented themselves to the clinic for treatment were considered for recruitment after giving informed consent. Demographic data, such as age and gender, together with the ART and/or single dose NVP history for each individual were obtained using a self-reporting questionnaire. Patients who reported prior exposure to ART and/or single dose NVP for prevention of mother-to-child transmission (PMTCT) of HIV, together with those who declined to consent were excluded from the study. A total of 87 patients (50 females and 37 males) from Nairobi were sequentially enrolled between April and June 2005. Five milliliters of blood was collected from each participant and tested for anti-HIV-1 antibodies using Unigold (Trinity Biotech, NY) and Determine (Abbott, IL). HIV-1 antibody positivity was further confirmed by enzyme-linked immunosorbent assay (ELISA) (Enzygnost, Dade-Behring, Marburg, Germany). Ethical clearance was obtained from the National Ethics Committee through the Kenya Medical Research Institute (KEMRI).

$CD4^+$ T cell counts and HIV-1 RNA quantification

Baseline $CD4^+$ T cell counts were performed using a FACSCalibur flow cytometer (Becton-Dickinson, NJ) equipped with automated acquisition and analysis software. Individual test results were reviewed to confirm the accuracy of the automated software analysis. Baseline viral loads were determined using Nuclisens EasyQ (Biomérieux, Marcy l'Etoile, France), with a lower limit of quantitation of 50 (1.69 $\log_{10}$) copies/ml of plasma, according to the manufacturer's instructions.

PCR and sequencing

Peripheral blood mononuclear cells (PBMCs), from confirmed ELISA-positive samples, were obtained by Ficoll-Hypaque density gradient centrifugation. Proviral DNA was extracted from the uncultured PBMCs using DNAzol (GIBCO BRL, Life Technologies) lysis and ethanol precipitation. Nested polymerase chain reaction (PCR) was performed using AmpliTaq Gold (Roche Molecular Systems, Branchburg, NJ). A segment of HIV-1 *env-gp41* corresponding to nucleotides 7850–8310 of HIV-1_{HXB2} was amplified using the primers gp40F1 (5'-TCTTAGGAGCAGCAGGAAGCACTATGGG-3') and gp41R1 (5'-AACGACAAAGGTGAGTATCCCTGCCTAA-3') in the first round and gp46F2 (5'-ACAATTATTGTCT GGTATAGTGCAACAGCA-3') and gp47R2 (5'-TTAAACC TATCAAGCCTCCTACTATCATTA-3') in the second round (www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/1998/III/GP41RENU.pdf). A segment of the HIV-1 RT gene corresponding to nucleotides 2265–3180 of HIV-1_{HXB2} was amplified using the primers RT18 (5'-GGAAACCAAAAAT GATAGGGGGAATTGGAGG-3') and KS104 (5'- TGACTT GCCCAATTTAGTTTTCCCACTAA-3') in the first round and KS101 (5'-GTAGGACCTACACCTGTTCAACATAATTG GAAG-3') and KS102 (5'-CCCATCCAAAGAAATGGAGG AGGTTCTTTCTGATG-3') in the second round. Amplification was achieved using 1 cycle of 95°C for 10 min and 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. The amplicons were sequenced as previously described.[22,34,35]

Table 1. Baseline Characteristics of HIV-1-Infected Kenyan Patients before Initiating Antiretroviral Therapy

		Gender	
	All (n = *53*)	*Females* (n = *35*)	*Males* (n = *18*)
Age (years)			
Mean (range)	38 (24–62)	37.5[a] (24–57)	39.5[a] (27–62)
Viral load ($\log_{10}$ copies/ml)			
Mean (range)	4.7 (3.4–5.9)	4.7[b] (3.4–5.9)	4.8[b] (3.9–5.8)
$CD4^+$ T cell counts (cells/mm^3)			
Mean (range)	475 (203–799)	449[c] (203–780)	525[c] (233–799)
Range			
<300	6	5	1
301–400	13	10	3
401–500	12	8	4
>500	22	12	10

[a] $p = 0.45$ by paired t-test.
[b] $p = 0.41$ by paired t-test.
[c] $p = 0.28$ by paired t-test.

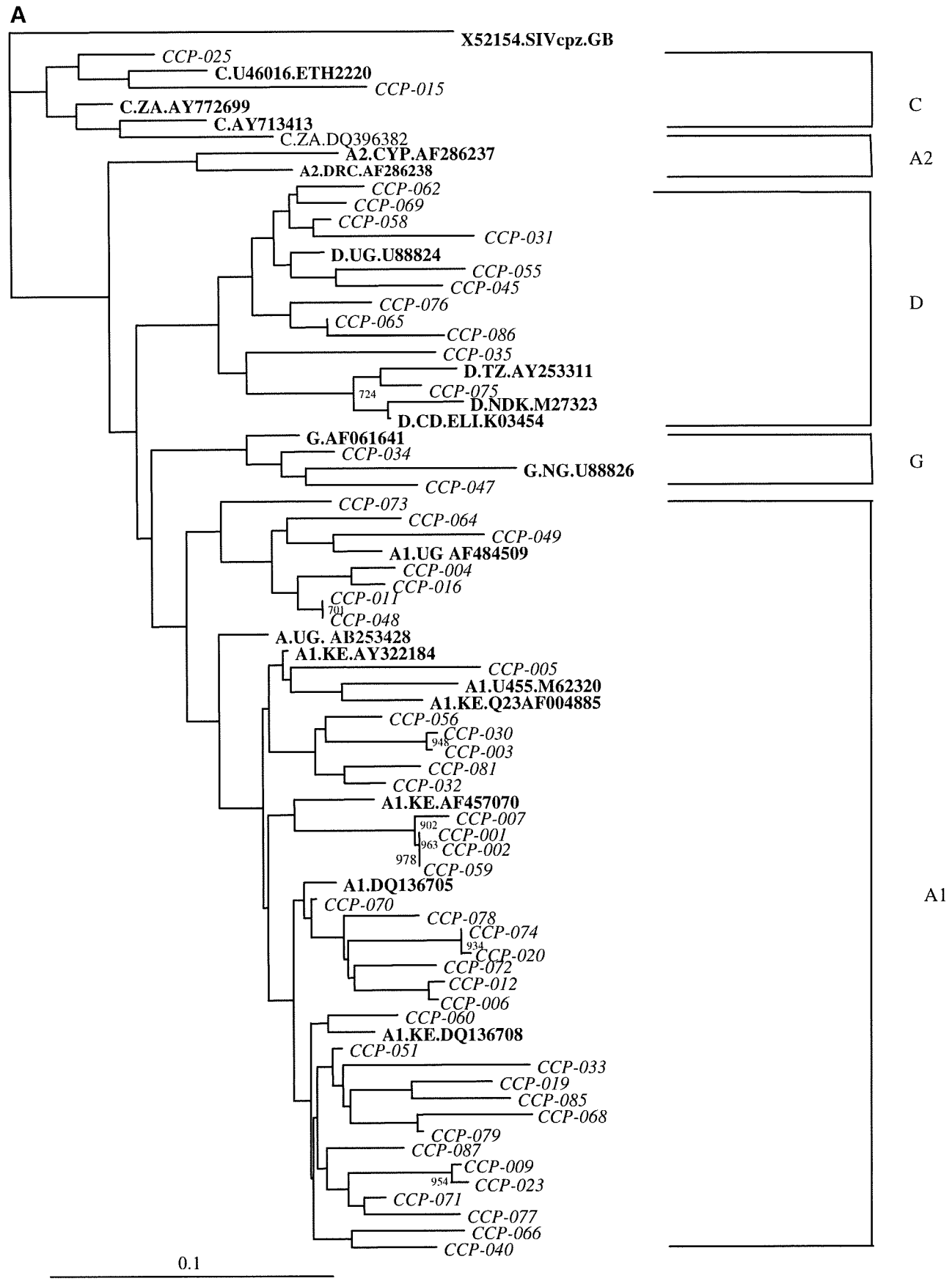

FIG. 1. Phylogenetic tree of the HIV-1 *pol-RT* (**A**) and *env-gp41* (**B**) regions. Patient samples (italics) were aligned and compared with reference sequences from the Los Alamos HIV database (boldface). Phylogenetic relationships were constructed using the neighbor-joining method and rooted with SIVcpzGAB. The bootstrap values of 1000 replicates above 70% are indicated next to the node. Brackets on the right indicate the subtype clusters.