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SCIENCE DIRECT

Virology 334 (2005) 299–305

VIROLOGY

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Differential evolution of cell-associated virus in blood and genital tract of HIV-infected females undergoing HAART

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Received 29 September 2004; returned to author for revision 17 November 2004; accepted 26 January 2005

Abstract

PBMC and vaginal cell (VC) viruses were studied from 5 HIV-infected females for the presence of drug-resistance and non-drug resistance associated mutations. A 1318-bp fragment of polymerase gene was amplified from PBMC and VC proviral DNA. Four of the 5 PBMC viruses exhibited drug resistance-associated mutations in reverse transcriptase and protease genes, whereas only 2 VC viruses contained drug resistance-associated mutations. However, all 5 females showed non-drug resistance-associated mutations both in PBMC and VC virus suggesting continuous evolution of the virus in these compartments. The emergence of drug resistance was slower in PBMC and VC viruses than that observed in the cell-free plasma (P) and vaginal secretion (VS) viruses. Phylogenetic analysis revealed that VC virus was closer to PBMC virus than either cell-free viruses (P and VS) suggesting comparable evolution among cell-associated viruses.

Keywords: HIV; HAART; Cell-associated virus; Virus compartmentalization; Phylogenetics

Introduction

The human immunodeficiency virus type-1 (HIV-1) is predominantly spread by heterosexual transmission with the genital mucosa serving as a site of the initial contact. Therefore, the study of the virus genotype in male and female genital compartments is critical to the development of vaccines and treatment strategies. Furthermore, study of the virus genotype in the female genital compartment may also offer strategies to block not only the heterosexual transmission but also mother-to-child transmission. Several previously reported studies have shown diverse virus evolution in different anatomical compartments (Kemal et al., 2003; Wong et al., 1997; Zhu et al., 1996) and the reason for this may be attributed to the immune and/or drug pressure leading into development of immune escape and drug resistant virus. The emergence of drug resistant virus can be explained on the basis of diverse pharmacokinetic properties (Kepler and Perelson, 1998; Wong et al., 1997) and poor bioavailability of a particular drug in different compartments (Lafeuillade et al., 2002). Earlier studies have mostly demonstrated differential evolution based on mutations in *pol* gene in the virus collected from plasma and cervicovaginal fluid in HIV-infected females undergoing highly active antiretroviral therapy (De Pasquale et al., 2003; Tirado et al., 2004). However, there is no report available on drug-resistance and non-drug resistance-associated mutations in *pol* of cell-associated

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doi:10.1016/j.virol.2005.01.030

Table 1
Demographic, clinical and virologic profiles of study subjects

Patient ID	Age/risk factor[a]	Year HIV Diag.	AIDS Diag.	CD4 count cell/μl[b]	Plasma Viral load[c]	Vaginal Viral load[d]	Anti-retroviral drug history[e]	Current treatment
012	46/H	1994	yes	22	>750,000	96,781	2001: d4T, 3TC, IDV, SQV	d4T + 3TC + LPV/RTV
36E	39/H	1993	no	1088	15,749	450	1990: AZT, ddC, ddI, d4T, 3TC, RTV, IDV, NFV	d4T + 3TC + NFV
41J	39/H	1996	yes	206	24,022	918	1996: AZT, ddC, ddI, d4T, 3TC, IDV, NFV, SQV	EFV +3TC + d4T
43L	40/H	1987	yes	64	>750,000	NA	2000: 3TC, ddI, d4T, EFV	3TC, d4T, EFV
004	51/H	1997	yes	382	42,322	11,798	1997: 3TC, d4T, RTV, NFV, AZT	APV + EFV + ddI

[a] Risk Factor H = Heterosexual.
[b] Percent CD4 T cell counts were determined by flowcytometry and Absolute CD4 T cell counts were calculated by multiplying the CD4 T cell percentage with absolute lymphocyte count.
[c] Plasma viral load (VL) was determined by Amplicor assay (Roche Diagnostic).
[d] Vaginal viral RNA load was determined by Amplicor assay (Roche Diagnostic). Since vaginal secretion was diluted 8.5 times, therefore Viral load in vaginal fluid is expressed as assay value × 8.5.
[e] Antiretroviral used in the study. d4T, stavudine; 3TC, lamiduvine; IDV, indinavir; AZT, zidovudine; NFV, nelfinavir; ddC, zalcitabine; ddI, didanosine; RTV, ritonavir; SQV, saquinavir; EFV, efavirenz; LPV, lopinavir.

virus in blood and vaginal compartments. In this study, we examined 5 HIV-infected females for drug resistance pattern in peripheral blood mononuclear cells (PBMC) and vaginal cells (VC) for the emergence of drug-resistance and also mutation in other regions of protease and reverse transcriptase.

Results and discussion

Demographic, clinical and virologic profile of all the patients have been described previously (Tirado et al., 2004) except for the patient 004. The demographic, clinical and virologic profiles of these patients are shown in Table 1.

Table 2
Comparison of drug resistance-associated mutations in reverse transcriptase and protease in PBMC and VC virus of HIV-infected females[a]

Patient	Virus	Amino acid position and identification												
REVERSE TRANSCRIPTASE		41	44	62	65	67	69	70	74	75	77	98	100	101
		M	E	A	K	D	T	K	L	V	F	A	L	K
012	PBMC	–	–	–	–	–	–	–	–	–	–	–	–	*K*
	VC	–	–	–	–	–	–	–	–	–	–	–	*L*	K
36E	PBMC	–	–	*A*	–	**N*D***	**D**	**R**	–	–	–	–	–	–
	VC	–	–	–	–	–	–	–	–	–	–	–	–	–
41J	PBMC	–	–	–	–	–	–	–	–	–	–	–	*L*	–
	VC	–	–	–	*K*	–	–	–	–	–	–	*A*	–	–
43L	PBMC	–	–	–	–	**N*D***	–	–	–	–	–	–	–	–
	VC	–	–	–	–	–	–	–	**I**	**S**	*F*	–	–	**E**[c]
004	PBMC	–	–	*A*	–	–	–	–	–	–	–	–	*L*	–
	VC	–	–	–	–	–	–	–	–	–	–	–	–	–
PROTEASE		10	20	24	30	32	33	36	46	47	48	50	54	63
		L	K	L	D	V	L	M	M	I	G	I	I	L
012	PBMC	–	*K*	–	–	–	–	–	–	–	–	–	–	**P**
	VC	–	*K*	–	–	–	–	–	–	–	–	–	–	**P**
36E	PBMC	*L*	–	–	–	–	–	–	***M*L**	–	–	–	–	**P**
	VC	–	–	–	–	–	–	–	–	–	–	–	–	**P**
41J	PBMC	**IV**	–	–	–	–	–	–	–	–	–	–	–	*L*
	VC	**V**	–	–	–	–	–	–	–	–	–	–	–	*L*
43L	PBMC	*L*	*K*	–	–	–	–	–	–	–	–	–	–	**P**
	VC	*L*	*K*	–	**N**	–	–	**V**	–	–	–	–	–	**P**
004	PBMC	**V**	–	–	–	–	–	**I**	–	–	–	–	–	**P**
	VC	–	–	–	–	–	–	–	–	–	–	–	–	**P**

[a] The mutations at amino acid level are shown in bold, whereas silent mutations are shown in italics. The sequence showing more than one amino acid at a particular location represents different quasispecies of the virus. The sequence from HIV-infected females were aligned with representative sequence from HIV-LAI (on the top). Dash denotes parent sequence used for comparison.
[b] Unexpected amino acid polymorphism at a drug resistance site. It has been associated with in vitro drug resistance in clade C viruses (Loemba et al., 2002).
[c] K101E has been found to be associated with drug resistance to NNRTIs (Loemba et al., 2002; Lyons et al., 2005).

The PBMC virus showed more frequent drug resistance-associated mutations as 4 of the 5 individuals showed mutations associated with resistance to one or more drugs, whereas only 2 *(41J and 43L)* of the vaginal cell (VC) virus showed mutations in the region targeted by antiretrovirals (Table 2). This is in sharp contrast with our earlier findings wherein we reported that all 4 plasma (P) and vaginal secretion (VS) viruses showed one or more mutations in the protease and/or reverse transcriptase genes that was associated with drug resistance (Tirado et al., 2004). In addition the 5th individual (004) also showed P and VS virus containing more than one drug-resistance-associated mutation (results not shown). The most frequent mutations in the cell-associated virus included L63P in the protease that was identified in both PBMC and VC virus from 4 individuals. However, this mutation has been shown to be not directly associated with drug resistance (D'Aquila et al., 2003). Therefore, even in the presence of this mutation, the virus was still considered wild type. Patient 43L exhibited maximum mutations wherein PBMC virus had 6 drug resistance-associated mutations in reverse transcriptase, and the VC virus had 9 and 5 mutations in reverse transcriptase and protease genes, respectively. In our earlier study, the plasma virus from this patient also showed 6 mutations in the reverse transcriptase (Tirado et al., 2004), but the pattern was different than that observed in the PBMC virus suggesting differential evolution of drug resistance pattern in plasma and PBMC virus. Likewise, VC virus also showed differential pattern of evolution of the drug resistance pattern than that seen in the VS virus. Based on our finding with 5 individuals, it can be suggested that viral evolution follows different course in cell-free and cell-associated state even in the same anatomical compartments. This diverse kinetics of emergence of drug-resistance pattern can be attributed to different pharmacokinetic properties of the antiretrovirals in various compartments (Wong et al., 1997). We also noticed that the emergence of drug-resistance mutation(s) was slower in cell associated compartments compared to that in the cell-free viruses. The donor 012 showed wild type PBMC and VC virus in this study whereas plasma and VF virus from the same donor had shown 2 drug resistance-associated mutations, reported in the previous study (Tirado et al., 2004). We do not know definite reason for the absence of drug-resistance mutation(s) in the cell-associated virus. However, the sample was collected only few months after initiation of antiretroviral treatment and the emergence drug-resistance mutation in the cellular compartments might have been slower than those seen in cell-free compartments. This phenomenon has also been observed in our previous report where the emergence

103	106	108	115	116	118	151	181	184	188	190	208	210	215	219	225	230	236
K	V	V	Y	F	V	Q	Y	M	Y	G	H	L	T	K	P	M	P
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	**Q**	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	***M*V**	–	–	–	–	–	–	–	–	–
E[b]	–	–	–	**–**	–	–	–	–	–	–	–	–	–	–	–	–	–
N	–	–	–	*F*	–	–	***Y*C**	***M*VI**	–	**A*G***	–	–	–	–	**H*P***	–	–
–	–	**I**	–	–	**I**	–	**C**	–	–	**A**	–	**W**	–	**N**	*P*	–	–
N	–	*V*	–	*F*	**I*V***	–	–	**V**	–	–	–	–	**Y/S**	–	*P*	–	–
–	–	*V*	–	*F*	–	***Q***	–	–	–	–	–	–	–	–	*P*	–	–

71	73	77	82	84	88	90
A	G	V	V	I	N	L
–	–	–	–	–	–	–
–	–	–	–	–	–	–
T*A*	–	–	–	–	–	–
A	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
V	–	**I**	–	–	**D**	–
V	–	–	–	**V**	*N*	**M**
A	–	–	–	–	–	–

of drug resistance-associate mutation was different in two different anatomical compartments (Tirado et al., 2004). Other 2 patients (36E and 004) with wild type VC virus in this study were shown to contain 9 and 13 drug resistance mutations in VS virus in the previously reported study (Tirado et al., 2004), again suggesting slow emergence of the drug-resistance associated mutations in cellular virus.

On the other hand, the PBMC and VC virus from all 5 individuals showed other mutations in reverse transcriptase and protease that were not associated with emergence of drug resistance. These mutations are shown in Table 3. Even in the cases where PBMC and VC viruses showed wild phenotype, there were numerous other mutations that were not associated with drug resistance. The VC virus from donor 43L exhibited 9 mutations followed by PBMC viruses from patient 41J and 004, and VC virus from patient 012, each containing 7 mutations in the reverse transcriptase. Likewise, protease was also target of mutations with 43L VC and 36E PBMC virus exhibited 6 mutations followed by 012 VC virus that had 5 mutations. In reverse transcriptase, there were two regions which had mutations in most patients. The 122E-K change in reverse transcriptase was present in 3 PBMC and 2 VC viruses. Furthermore, both PBMC and VC virus in patient 41J had silent mutation at this position. The 211R-K mutation in the reverse transcriptase was also present in both viruses from patient 012 and 41J and also in VC virus from patient 43L. Likewise, all PBMC and VC virus exhibited mutation at amino acid position 37 in the protease gene. The regions encompassing positions 122

Table 3
Comparison of non-drug resistance-associated mutations in reverse transcriptase and protease in PBMC and VC virus of HIV-infected females[a]

Patient	Virus	Amino acid position and identification														
REVERSE TRANSCRIPTASE		42	43	45	47	51	53	60	66	80	82	83	87	89	90	93
		E	K	G	I	G	E	V	K	L	K	R	F	E	V	G
012	PBMC	*E*	–	–	–	–	–	–	–	–	*K*	–	*F*	–	*V*	–
	VC	*E*	–	–	*I*	G	–	–	–	–	*K*	–	*F*	–	*V*	–
36E	PBMC	–	–	*G*	–	–	–	**I**	*K*	–	–	–	–	–	–	–
	VC	–	–	–	–	–	–	–	–	–	*K*	–	*F*	–	–	–
41J	PBMC	–	–	–	–	–	–	–	–	*L*	*K*	**K**	–	–	–	–
	VC	–	**R**	–	–	–	–	–	–	*L*	*K*	**K**	–	–	–	–
43L	PBMC	–	–	–	–	–	–	–	–	–	–	–	–	*E*	–	*G*
	VC	–	–	–	–	–	*E*	–	–	*L*	–	–	–	–	–	–
004	PBMC	–	–	–	–	–	–	–	–	–	–	**K**	–	–	–	–
	VC	–	–	*G*	–	–	–	–	–	–	–	**K**	–	–	–	–
		162	163	167	170	172	173	177	178	179	180	182	186	196	197	198
		S	S	I	P	R	L	D	I	V	I	Q	D	G	Q	H
012	PBMC	*S*	–	–	*P*	–	–	–	–	*V*	*I*	–	*D*	–	*Q*	–
	VC	–	–	–	*P*	–	–	–	–	*V*	–	–	*D*	–	*Q*	–
36E	PBMC	–	–	–	*P*	–	–	–	–	–	–	–	*D*	–	–	*H*
	VC	–	–	–	–	**K**	–	–	–	–	–	–	–	–	–	–
41J	PBMC	–	–	–	–	–	**K**	–	–	–	–	–	–	***G*V**	–	–
	VC	–	–	–	–	–	**K**	–	–	–	–	–	–	–	–	–
43L	PBMC	*S*	–	–	*P*	–	–	–	–	–	*I*	–	*D*	–	*Q*	–
	VC		–	*I*	–	–	**Q**	**N**	–	–	–	*Q*	–	–	*Q*	–
004	PBMC	**C**	*S*	–	–	–	–	–	**L**	–	–	–	–	–	–	*H*
	VC	**C**	**T**	–	–	–	–	**E**	–	–	–	–	–	–	–	*H*
PROTEASE		5	11	12	13	14	15	17	18	25	31	32	33	35	37	38
		L	V	T	I	K	I	G	Q	D	T	V	L	E	S	L
012	PBMC	*L*	–	–	–	–	–	–	–	–	–	–	–	D	**E**	–
	VC	*L*	–	–	–	*K*	***I*V**	–	–	–	–	–	–	D	**E**	–
36E	PBMC	–	–	**P**	–	**R**	–	–	*Q*	–	–	–	**V*L***	–	**ND**	*L*
	VC	–	–	–	–	*K*	–	–	*Q*	–	–	*V*	**I**	–	**T**	–
41J	PBMC	–	–	–	***I*V**	–	–	–	–	–	–	–	–	–	**N**	–
	VC	–	–	–	–	–	–	–	–	–	–	–	–	–	**N**	–
43L	PBMC	–	–	–	–	–	–	–	–	–	*T*	–	–	–	**N**	–
	VC	–	*V*	–	–	–	–	*G*	–	–	–	–	–	D	**E**	–
004	PBMC	–	–	–	–	*K*	–	–	*Q*	*D*	–	–	**I**	D	**N**	–
	VC	–	–	–	–	*K*	–	–	–	–	–	–	**I**	–	**T**	–

[a] The mutations at amino acid level are shown in bold, whereas silent mutations are shown in italics. The sequence showing more than one amino acid at a particular location represents different quasispecies of the virus. The sequence from HIV-infected females were aligned with representative sequence from HIV-LAI (on the top). Dash denotes parent sequence used for comparison.

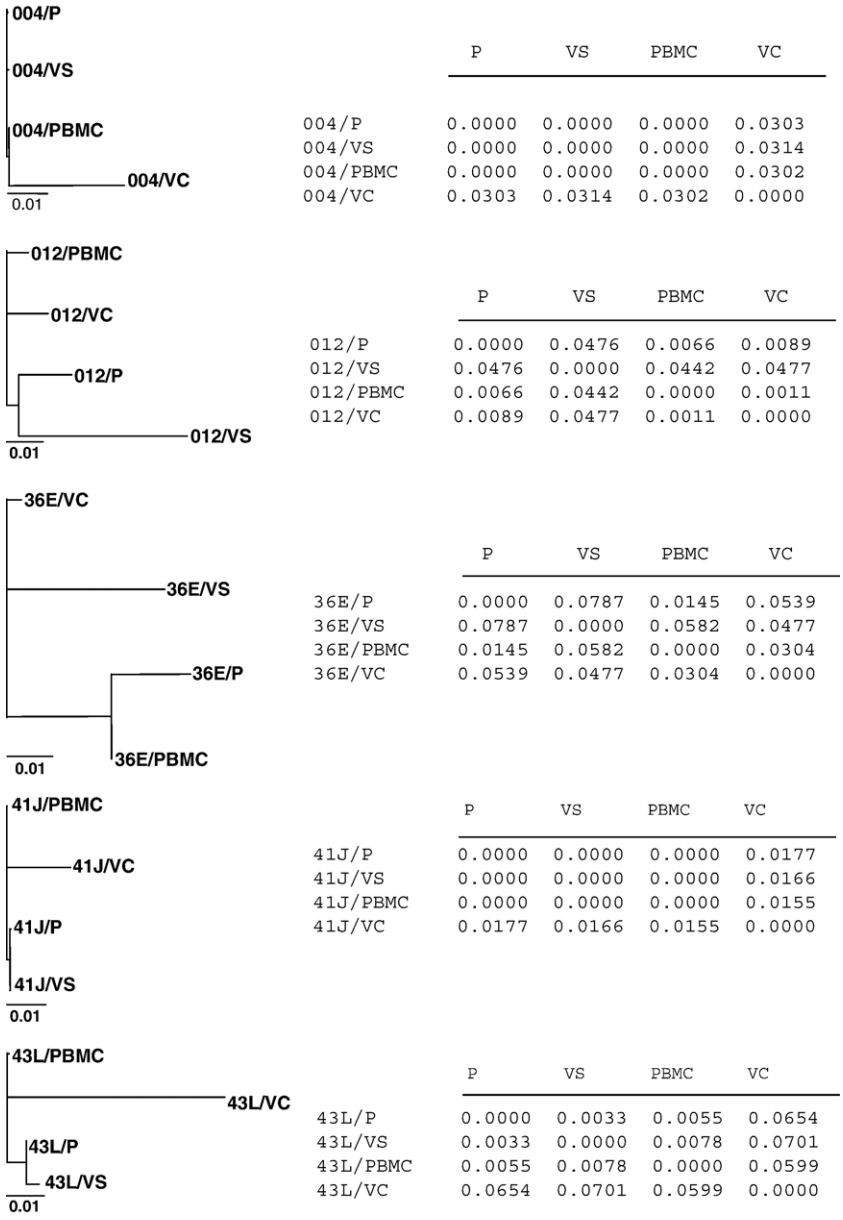
and 211 in reverse transcriptase and 37 in protease genes have been identified as CTL epitopes (Altfeld et al., 2001; Kaul et al., 2001; van der Burg et al., 1997). These results suggest that immune pressure in tandem with drug pressure leads to the development of variant viruses, and a mutation in CTL epitope region will help in the development of CTL-escape virus. In addition, there were numerous silent mutations in the reverse transcriptase and protease genes suggesting that virus evolution was a continuous phenomenon.

We analyzed the sequences from PBMC and VC virus, presented in this report, along with previously published sequences of P and VS viruses (Tirado et al., 2004). The phylogenetic trees and distance matrices are shown in Fig. 1. In 3 of 5 individuals the VC virus was more closely related with PBMC virus (nucleotide distance 0.1%–5.82%) than the VS virus (1.24%–5.82%). The difference between VC virus on one hand and PBMC and VS virus on the other were statistically significant in Wilcoxon Rank Sum Test ($P < 0.05$). Furthermore, the VS virus was identical to P virus in 2 patients (004 and 41J) whereas it showed 3.51 and 1.24% disparity with VC virus, respectively. In another patient (43L) the VS virus was again more closely associated with P virus than with the VC virus (0.22 vs. 5.82% distance). In only one patient (36E) the VS virus was more closely related with VC virus (4.77%) than P virus (6.93%). Overall, our results suggest that cell-associated viruses follow different evolutionary pathways compared to that of cell-free virus in the same anatomical compartment and cell-associated virus in female genital compartment are

97	104	109	112	117	119	122	123	125	134	135	139	141	146	147	154	156	160	161
P	K	L	G	S	P	E	D	R	S	I	T	G	Y	N	K	S	F	Q
–	–	*L*	–	–	*P*	**K**	–	*R*	–	–	*T*	*G*	–	–	–	–	*F*	–
–	–	*L*	–	–	*P*	**K**	–	*R*	***SG***	–	–	–	–	–	–	–	*F*	–
P	–	–	–	*S*	–	**K**	–	–	–	**T**	–	–	–	–	–	*S*	–	–
–	–	*L*	–	–	–	**K**	–	*R*	–	–	–	–	–	–	–	–	–	–
P	–	–	–	*S*	–	*E*	–	–	–	–	–	–	*Y*	–	–	–	–	*Q*
–	–	–	–	*S*	–	*E*	–	–	–	–	–	–	–	–	–	–	–	–
–	–	*L*	–	*S*	–	–	–	–	–	**T*I***	–	–	*Y*	–	–	–	–	–
–	–	*L*	*G*	–	–	–	**G**	–	–	–	–	–	–	–	–	–	*F*	–
–	*K*	*L*	–	–	–	**K**	–	–	–	**T**	–	–	–	*N*	*K*	–	–	–
–	–	*L*	–	–	–	**K**	–	–	–	**T**	–	–	–	–	–	–	–	–

200	203	204	207	209	211	213	214	218	220	221	223	227	231	233	238	241	245	246
T	E	E	Q	L	R	G	L	D	K	H	K	F	G	E	L	V	V	L
–	–	*E*	*Q*	*L*	**K**	–	**F**	–	*K*	–	–		–	*E*	–	–	**M**	–
–	–	*E*	*Q*	*L*	**K**	–	**F**	–	*K*	–	–	*F*	–	–	–	–	**KMQL**	*L*
–	–	*E*	–	–	–	–	–	*D*	–	–	*K*	–	–	–	–	*V*	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A	*E*	*E*	***Q*E**	*L*	**K**	*G*	**F**	–	–	–	–	–	–	–	–	–	–	–
A	*E*	*E*	–	–	**K**	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	*E*	–	*L*	–	–	–	–	–	–	–	–	*G*	–	–	–	–	–
***T*A**	**K**	*E*	**E**	–	**K**	*G*	**F**	–	–	**Q**	–	–	–	–	–	–	–	–
–	–	*E*	–	–	–	*G*	*L*	–	–	–	*K*	–	–	–	**KT**	–	–	–
–	–	*E*	–	–	–	–	*L*	–	–	–	*K*	–	–	–	–	–	–	–

41	57	58	60	61	64	65	68	69	72	76	79	83	92	93	95	98	99
R	R	Q	D	Q	I	E	G	H	I	L	P	N	Q	I	C	N	F
–	–	–	–	–	**M**	–	–	–	–	–	–	–	–	–	–	–	–
–	–	**E**	–	–	**M**	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	**Q**	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*N*	–
–	–	–	*D*	–	**V**	–	–	–	–	*L*	–	–	–	–	*C*	–	–
–	–	–	*D*	–	**V**	–	–	–	–	*L*	–	–	–	–	*C*	–	–
–	–	–	–	–	–	–	*G*	–	**T**	–	–	*N*	*Q*	–	–	–	–
–	**K**	–	**E**	E	–	*E*	–	–	–	–	–	–	–	**L**	–	–	–
K	–	–	–	–	–	–	–	–	–	–	*P*	–	–	**L**	–	*N*	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*N*	–



	P	VS	PBMC	VC
004/P	0.0000	0.0000	0.0000	0.0303
004/VS	0.0000	0.0000	0.0000	0.0314
004/PBMC	0.0000	0.0000	0.0000	0.0302
004/VC	0.0303	0.0314	0.0302	0.0000

	P	VS	PBMC	VC
012/P	0.0000	0.0476	0.0066	0.0089
012/VS	0.0476	0.0000	0.0442	0.0477
012/PBMC	0.0066	0.0442	0.0000	0.0011
012/VC	0.0089	0.0477	0.0011	0.0000

	P	VS	PBMC	VC
36E/P	0.0000	0.0787	0.0145	0.0539
36E/VS	0.0787	0.0000	0.0582	0.0477
36E/PBMC	0.0145	0.0582	0.0000	0.0304
36E/VC	0.0539	0.0477	0.0304	0.0000

	P	VS	PBMC	VC
41J/P	0.0000	0.0000	0.0000	0.0177
41J/VS	0.0000	0.0000	0.0000	0.0166
41J/PBMC	0.0000	0.0000	0.0000	0.0155
41J/VC	0.0177	0.0166	0.0155	0.0000

	P	VS	PBMC	VC
43L/P	0.0000	0.0033	0.0055	0.0654
43L/VS	0.0033	0.0000	0.0078	0.0701
43L/PBMC	0.0055	0.0078	0.0000	0.0599
43L/VC	0.0654	0.0701	0.0599	0.0000

Fig. 1. Phylogenetic reconstruction and nucleotide distance of viruses from 4 different compartments of 5 HIV-infected females. The *pol* sequences were aligned using ClustalX and MEGA. The MEGA program was used to calculate distance matrices. Phylogenetic trees were generated with MEGA using neighbor-joining method and the trees were visualized using TreeView program. P = Plasma virus; VS = Vaginal secretion virus; PBMC = Peripheral blood mononuclear virus and VC = Vaginal cell virus.

closer to the cell-associated in blood than to the cell-free virus in the vaginal compartment.

Materials and methods

Patient population and sample preparation

Five HIV-infected females were enrolled for this study. An informed consent was obtained from each subject. PBMC was separated by spinning the blood on Ficoll-Hypaque. Vaginal samples were collected by using two Dacron-tipped applicators and taking swabs from cervico-vaginal area. The swabs were vigorously washed in 2 ml of R-10 (RPMI-1640, HEPES, and 10% human AB plasma) to release the virus and loosen cells before being removed from tubes. Vaginal cells were separated by centrifugation followed by two washings in RPMI-1640.

DNA extraction, PCR and sequencing

HIV-1 proviral DNA was isolated from PBMC and VC, using the QIAamp DNA Blood Kit (QIAGEN, Inc., California). A 1318 base pair fragment of the polymerase (*pol*) gene was amplified using a modification of the TruGene HIV-1 Genotyping Kit (Bayer Healthcare-Diag-

nostic Div.) protocol. The PCR condition included 94 °C for 3 min, followed by 20 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1.5 min, then followed by 15 cycles of 94 °C for 30 s, 60 °C for 30 s, and 70 °C for 2 min, with a final extension of 70 °C for 7 min. Sequencing reactions were performed according to manufacturer's instructions. Bi-directional sequences of the protease (*pro*) gene (codons 1–99) and most of the reverse transcriptase (*RT*) gene (codons 39–247) were detected with the OpenGene System (Bayer Healthcare-Diagnostic Div.). The resistance pattern was generated using Guidelines 8.0 (updated April 2003), provided by the supplier.

Computational analysis

The *pol* sequences from PBMC, VC, P and VS viruses were analyzed for their relatedness with each other. The sequences for the 1st 2 viruses were taken from this study and the P and VS virus from our previously reported study (Tirado et al., 2004). The *pol* sequences were aligned with BioEdit version 5.0.9 (Hall, 1999) and ClustalX (Thompson et al., 1997). The ClustalX program was set to perform multiple sequence alignments using the default penalties, IUB DNA weigh matrix and unweighted transitions. Sequence alignments were manually verified and edited if necessary. Phylogenetic reconstructions were generated by the Neighbour-Joining (NJ) method (Saitou and Nei, 1987), and the trees were visualized using the TreeView program. The distance matrices were calculated using MEGA version 2.1 (Kumar et al., 2001). Genetic distances between different compartment were statistically analyzed using Wilcoxon Rank Sum Test.

Acknowledgments

We thank Ms. Silkha Abreu and Dr. Diego Zavala-Pizarro for help with statistical analysis. The study was supported by National Institutes of Health grants DA015013, AA015045, G12RR03050, and GM08239.

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