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Differential Evolution of Cell-Free and Cell-Associated HIV-1 in Blood and Vaginal Tract

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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. Highly active anti-retroviral therapy (HAART) may present different dynamics in different body compartments and may lead to the emergence of distinct drug resistant strains.

Several 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. Earlier studies have mostly demonstrated differential evolution based on mutations in *pol* gene in the virus collected from plasma and cercovaginal fluid in HIV-infected females undergoing highly active antiretroviral therapy (Tirado et al., 2004; De Pasquale et al., 2003). The emergence of drug resistant virus can be explained on the basis of diverse pharmacokinetic properties (Wong et al., 1997; Kepler & Perelson, 1998) and poor bioavailability of a particular drug in different compartments (Lafeuillade et al., 2002).

In this study we compared the role of HAART in the selection and evolution of drug resistance mutations in cell-free and cell-associated HIV-1 from blood and vaginal samples of five HIV-infected females undergoing HAART. Their demographic, clinical and virologic profiles are presented in Table 1. *Pol* sequences were also used for phylogenetic analysis comparing the RNA and proviral form of HIV-1 in the blood and genital compartment.

METHODS

Sample preparation: Blood and vaginal samples were collected from 5 HIV-infected females receiving highly active anti-retroviral treatment (HAART). PBMC was separated from blood by spinning on Ficoll-Hypaque. Vaginal samples were collected by using Dacron tipped applicators and taking swabs of the cervicovaginal area. Swabs were washed in 2ml of R-10 to release the virus and loosen cells. Vaginal cells were separated by centrifugation followed by washing in RPMI-1640.

Nucleic acid extraction, amplification and sequencing: RNA of cell-free virus from plasma and vaginal secretions was isolated using the QIAgen RNA kit. Proviral DNA was isolated from PBMC and vaginal cells using the QIAgen Blood DNA kit. For virus from plasma and vaginal secretions, a 1,318 base pair fragment of *pol* gene was amplified using the TruGene Genotyping Kit (Bayer Healthcare) according to manufacturer's instructions. For DNA samples a modified PCR protocol was used. Bi-directional sequences of protease and part of the reverse transcriptase were detected using the OpenGene System. Sequence data were processed with the GeneObjects software.

Sequence analysis: Drug resistance associated mutations were determined and drug resistance reports were generated using GuideLines 8.0 (updated 04/2003).

Additionally, sequences were analyzed for relatedness with each other. Sequences were aligned with BioEdit version 5.0.9 and Clustal X. Clustal X was set to perform multiple sequence alignments using the default penalties, IUB DNA weigh matrix and unweighted transitions. Phylogenetic reconstructions were generated by the neighbour-joining (NJ) method and trees visualized using the TreeView program. The distance matrices were calculated using the BioEdit program.

Table 1. Demographic, clinical and virologic profiles of study subjects

Patient ID	Age/risk factor	Year HIV Diag.	AIDS Diag.	CD4 count cell/ul	Plasma Viral load	Vaginal Viral load	Anti-retroviral drug history	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

Table 2. Distribution of drug resistance pattern in protease and reverse transcriptase of cell-associated virus from blood and vagina

Patient	Compartment analyzed	Amino Acid Position and Identification
REVERSE TRANSCRIPTASE		41 44 62 65 67 69 70 74 75 77 98 100 101 103 106 108 135 136 138 151 181 184 188 190 208 210 215 219 225 230 236 M E A K D T K L V F A L K K V V Y F V Q Y M Y G H L T K P M P
012	Plasma	. . . . . V . . . . I . . . .
	PBMC	. . . . . K . . . .
	Vag. Secr.	. . . . . V . . . . Y . . . .
	Vag. Cell	. . . . . L K . . . .
36E	Plasma	. . . . N D R . . . . . N . . . . . V . . . . . Q H . .
	PBMC	. . A . ND D R . . . . Q . . .
	Vag. Secr.	. . . . . S . E . . . . . Y . . . .
	Vag. Cell	. . . . .
41J	Plasma	. . . . . V . . . . .
	PBMC	. . . . . L . . . . MV . . . . .
	Vag. Secr.	. . . . . V . . . . .
	Vag. Cell	. . . K . . . . . A . E . . . . .
43L	Plasma	. . . . ND . . . . . KQ N . . . . . C I . A . . . . .
	PBMC	. . . . ND . . . . . N . . . . . YC MVI . AG . . . . HP . .
	Vag. Secr.	. . . . N . . . . . N . . . XYLI . . . . .
	Vag. Cell	. . . . . I S F . . E . . I . . I . C . . A . W . N P .
004	Plasma	. . . . . KTQP N . . . . IV . . V . . . . Y . . . .
	PBMC	. . A . . . . . L . N . V . F IV . . V . . . . YS . P . .
	Vag. Secr.	. . . . . KTQP N . IV . . IV . . V . . . . Y . . . .
	Vag. Cell	. . . . . V . F . Q . . . . . P . .
PROTEASE		10 20 24 30 32 33 36 46 47 48 50 54 63 71 73 77 82 84 88 90 L K L D V L M M I G I I L A G V V I N L
012	Plasma	. . . . . P . . . . .
	PBMC	. K . . . . . P . . . . .
	Vag. Secr.	. . . . . P . . . . .
	Vag. Cell	. K . . . . . P . . . . .
36E	Plasma	F . . . . VL . L . . . . P T . . A . . M
	PBMC	L . . . . . ML . . . . P TA . . . . .
	Vag. Secr.	. R . . . . . I . . . V . V P . . . A . . .
	Vag. Cell	. . . . . I . . . . . P A . . . .
41J	Plasma	IV . . . . .
	PBMC	IV . . . . . L . . . . .
	Vag. Secr.	IV . . . . .
	Vag. Cell	V . . . . . L . . . . .
43L	Plasma	. . . . . P . . . . .
	PBMC	L K . . . . . P . . . . .
	Vag. Secr.	. . . N . . . . . P . . . . . ML
	Vag. Cell	L K . N . . V . . . . . P V . I . . D .
004	Plasma	V . . . . I I IM . . . P V . . . V . M
	PBMC	V . . . . I I . . . . P V . . . V N M
	Vag. Secr.	V . . . . I I IM . . . P V . . . V . M
	Vag. Cell	. . . . . I . . . . . P A . . . . .

TABLE 2. Deduced amino acid sequence of HIV-1 Protease (top) and Reverse Transcriptase (bottom) genes in cell-associated and cell-free blood and vaginal samples. Only those codons associated with drug resistance are shown. When more than one amino acid is shown it represents a quasispecies of the virus. Coding changes in amino acids are shown in bold whereas silent mutations are shown in italics. The sequence from HIV-infected females were aligned with representative sequence from HIV-LAI (on top).

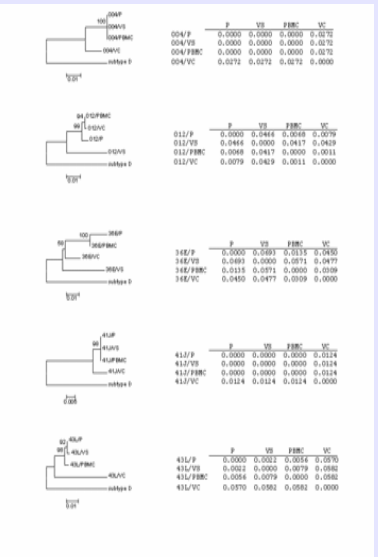
FIGURE 1. Phylogenetic reconstruction and nucleotide distance of viruses from 4 different compartments of 5 HIV-infected females. The *pol* sequences were aligned using ClustalX and BioEdit. The BioEdit program was also used to calculate distance matrices. Phylogenetic trees were generated with ClustalX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

RESULTS AND DISCUSSION

The most frequent mutations in blood were L10IFV (P=3/5; PBMC=1/5), M46IL (P=2/5; PBMC=1/5), L90M (P=2/5; PBMC=0/5), K103N (P=3/5; PBMC=2/5), and M184V (P=4/5; PBMC=3/5). In the genital compartment the most frequent resistance mutations were L10IV (VS=2/5; VC=0), M36IV (VS=2/5; VC=0), L90M (VS=2/5; VC=0), M184V (V=3/5; VC=0), and T215Y (VS=3/5; VC=0). Mutation 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.

We found that 75% (9/12) of females showed discordant mutation patterns between vaginal secretions (VS) and plasma (P) virus. Treatment history showed resistant mutants remained detectable in the VS virus population for 2-4 years after cessation of drug. Analysis of P virus from a treatment naïve patient showed no resistance mutations, however VS virus showed mutation M184V. Additionally, *pol* sequences from cell-associated PBMC virus showed resistance mutations in 4 of 5 individuals whereas only 2 of 5 of the vaginal cell (VC) virus showed resistance mutations. Drug resistance mutation pattern shown by P virus was different from that of PBMC virus. It was also observed that VC virus presents a different drug resistance pattern compared to VS virus. Non-resistance associated mutations, many located at CTL epitopes suggest immune and drug selective pressure to the differential evolution of variant viruses. Cell-associated compartments were less likely to show drug resistance mutations compared to the cell-free compartments suggesting a delay in emergence of drug-resistance mutations. Analysis of *pol* sequences of PBMC, VC, P and VS viruses showed that VC virus is genetically more related to PBMC virus than to VS and P virus.

FIGURE 1. Phylogenetic comparison of sequences from cell-free and cell-associated HIV-1 from blood and vaginal samples



RESULTS AND DISCUSSION

Female 012 showed wild type PBMC and VC virus in this study whereas plasma and VF virus from the same female had shown 2 drug resistance-associated mutations, reported in a previous study (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.

Even in the cases where PBMC and VC viruses showed wild type drug resistance genotype, there were numerous other mutations that were not associated with drug resistance. 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 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).

We analyzed the sequences from cell-free and cell-associated virus from blood and vaginal samples for relatedness among compartments. The phylogenetic trees and distance matrices are shown in Figure-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). 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).

SUMMARY

Detection of drug-resistance mutations was less likely to occur in the cell-associated virus compared to the cell-free viruses.

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 of the vaginal cell (VC) virus showed mutations in the region targeted by antiretrovirals.

Numerous mutations located at known CTL epitopes in absence of drug resistance genotypes 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 3 of 5 individuals the VC virus was more closely related with PBMC virus (nucleotide distance 0.1-5.99) than the VS virus (1.66-7.01). Based on our findings it can be suggested that viral evolution follows different course in cell-free and cell-associated state even in the same anatomical compartments.

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

CONCLUSIONS

This study provides further insight into the kinetics of drug resistance-associated changes in protease, reverse transcriptase and the influence of HAART on the selection of certain viral forms. The presence of drug resistance mutants than could only be detected in the vaginal compartment suggests the vaginal tract may serve as a reservoir for drug resistant viruses and that it may contribute to the transmission of drug-resistance. The delayed emergence or elimination of drug resistant variants suggest differences in turnover rates and other local differences among which may included CTL escape mutants and pharmacokinetic factors. Understanding viral evolution in the genital compartment is critical for successful development of vaccines and treatment strategies that would effectively reach the vaginal tract and prevent the development of drug resistant viruses.

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