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Diversity of HIV-1 RNA and DNA in breast milk from HIV-1-infected mothers

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Abstract

We compared human immunodeficiency virus type 1 (HIV-1) RNA and DNA populations in the different fractions of breast milk (lactoserum, lipid layer, cell pellet) and between right and left breasts in four HIV-1-infected mothers by analyzing the hypervariable env C2-V5 region. Phylogenetic analyses of the viral quasispecies revealed that RNA populations and DNA populations were clearly distinct and that viral RNA sequences were similar in lipid layer and lactoserum in the milk of 3 out of 4 mothers. Comparison of viral DNA between milk from right and left breast showed a differential distribution of variants in three mothers. In contrast, RNA variants detected from milk of the two breasts were mixed in 3 out of 4 mothers. This study suggests that each mammary gland is subjected to microenvironmental pressure that may differ from the contralateral breast.

Keywords: HIV-1 RNA and DNA populations; Breast milk fractions; Selection pressure; Compartmentalization

Introduction

Transmission of human immunodeficiency virus type 1 (HIV-1), both by sexual intercourse or by breast-feeding, implies a mucosal portal of entry. Breast milk of an HIV-1-infected mother contains both cell-free and cell-associated HIV-1, but very few studies have characterized virus populations in breast milk.

While HIV-1 transmission during early stages of lactation frequently involves cell-associated virus, transmission of cell-free virus is more frequently found at later stages of lactation (>9 months postpartum) and is associated with a lower viral envelope glycosylation (Koulinska et al., 2006a, 2006b). We previously suggested the existence of a compartmentalization of HIV-1 populations in peripheral blood and breast milk and, within breast milk, between free virus and provirus. Our observations showed also that breast milk transmission of HIV-1 involves variants that may not be predicted from viral populations in blood (Becquart et al., 2002). These results are in agreement with compartmentalization of HIV-1 previously described in semen and cervicovaginal secretions (Delwart et al., 1998; Ellerbrock et al., 2001; Overbaugh et al., 1996; Zhu et al., 1996). However, a recent study comparing HIV-1 RNA populations by a V1/V2 heteroduplex tracking assay concluded that HIV-1 V1/V2 variants in blood plasma and breast milk are similar (Henderson et al., 2004).

To date, studies of viral populations in breast milk have been mainly restricted to the lactoserum and cell fraction despite the fact that human breast milk contains 1 to 10% lipid (Jensen, 1999) that can preferentially entrap HIV-1 RNA (Ghosh et al., 2003; Hoffman et al., 2003).

In the present study, with the aim of determining whether HIV-1 distribution in the lactoserum is representative of the viral quasispecies found in all fractions of milk (lactoserum, lipid layer, cell pellet) and between the two breasts of HIV-1-infected

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mothers, the *env* C2-V5 region was sequenced and compared in breast milk samples collected from a previous study conducted in Kwa Zulu-Natal, South Africa (Willumsen et al., 2001). This genomic region includes the hypervariable V3 loop region which had been chosen in our previous study to identify the origin of cell-free and cell-associated virus in breast milk.

Results

RNA and DNA were extracted from milk samples collected from the left and right breast of 15 HIV-1-infected mothers naive of antiretroviral (ARV) treatment. HIV-1 DNA and RNA variants were successfully amplified in all fractions from breast milk of the two breasts of 4 out of 15 mothers (#01, #02, #13 and #15). Clinical and biological data of these four women are summarized in Table 1.

At least fifteen viral clones from each breast milk fraction were generated and sequenced. Sequence analyses showed that all viruses derived from breast milk of the 4 mothers belonged to *env* sequence subtype C. Each set clustered as monophyletic groups compared with those from other mothers in this study (data not shown). With the exception of the right breast of mother #02, the mean diversity of DNA variants from the cellular fraction was lower than the mean diversity of RNA variants detected in lactoserum and lipid fraction in each breast of all women (see Table 2).

Comparison of HIV-1 DNA and RNA diversity in milk samples from right versus left breast

Phylogenetic analyses by the neighbor joining method of the viral quasispecies in *env* C2-V5 region showed that major DNA variants were distinct from major RNA variants in mothers #01, #13 and #15. In contrast, in each breast, mother #02 exhibited DNA and RNA viral quasispecies that were intermingled within the different phylogenetic clusters.

Comparison of RNA variants between lactoserum and milk lipid fraction

Phylogenetic analyses revealed different distribution patterns according to breast and breast milk fractions (Fig. 1). In mothers #01 and #02, there was no significant clustering of the C2-V5 sequences of viral populations detected in the lipid fraction and the lactoserum. Overall, sequences of variants were mixed and only moderate clustering was observed. In mother #13, distinct viral populations in lipid fraction versus lactoserum were identified only in the right breast. In the left breast, RNA viral quasispecies from the 2 fractions interspersed. Contrastingly, in mother #15, the two viral populations detected in each breast were clearly distinct from each other, forming different clusters (homogeneous quasispecies) with significant bootstrap values (>70%).

Table 2
Comparison of the mean nucleotide distance (%) of DNA and RNA variants between right and left breasts[a]

Mothers	RNA		DNA	
	Left breast	Right breast	Left breast	Right breast
#01	4.5	5.4	1.5	1.2
#02	6.9	7.5	5.5	10.0
#13	5.0	3.9	1.6	2.6
#15	8.2	5.0	1.0	3.5

[a] Estimated by the Kimura two-Parameter method.

Comparison of RNA and DNA variants between left and right breast

In mothers #01, #13 and #15, DNA clones derived from the milk of the left breast were clearly distinct from those detected in the right breast. By contrast, in mother #02, HIV-1 DNA viral populations in the two breasts were mixed. Same DNA viral quasispecies were found in the cellular fraction in the milk of the two breasts but in a different proportion. In mother #15, the HIV-1 RNA populations from lactoserum and lipid fraction, distinct in the milk of each breast, were also clearly different between the two breasts. In mothers #01, #02 and #13, RNA variants detected from milk of the two breasts were mixed.

Prediction of X4− and R5− viral tropism of HIV-1 variants

We investigated whether HIV-1 quasispecies from left or right breast and from the different fractions of breast milk

Table 1
Biological characteristics of blood and breast milk samples from four women with a complete set of amplifiable breast milk samples

Mothers	Blood			Breast milk			
	CD4+ T cells × 10^6/l	CD8+ T cells × 10^6/l	CD4/CD8	Breast	Time of sampling, weeks post partum	Na/K	Viral load (copies/ml)
#01	104	356	0.29	Right	12 weeks	0.30	10,403
				Left		0.38	11,445
#02	88	338	0.26	Right	1 week	0.83	114,890
				Left		0.99	68,767
#13	ND	ND	ND	Right	1 week	6.07[a]	16,686
				Left		4.24[a]	104,639
#15	244	695	0.35	Right	1 week	3.38[a]	30,831
				Left		0.71	2893

ND=not determined.
[a] Severe mastitis is defined as a ratio of (Na/K>1).

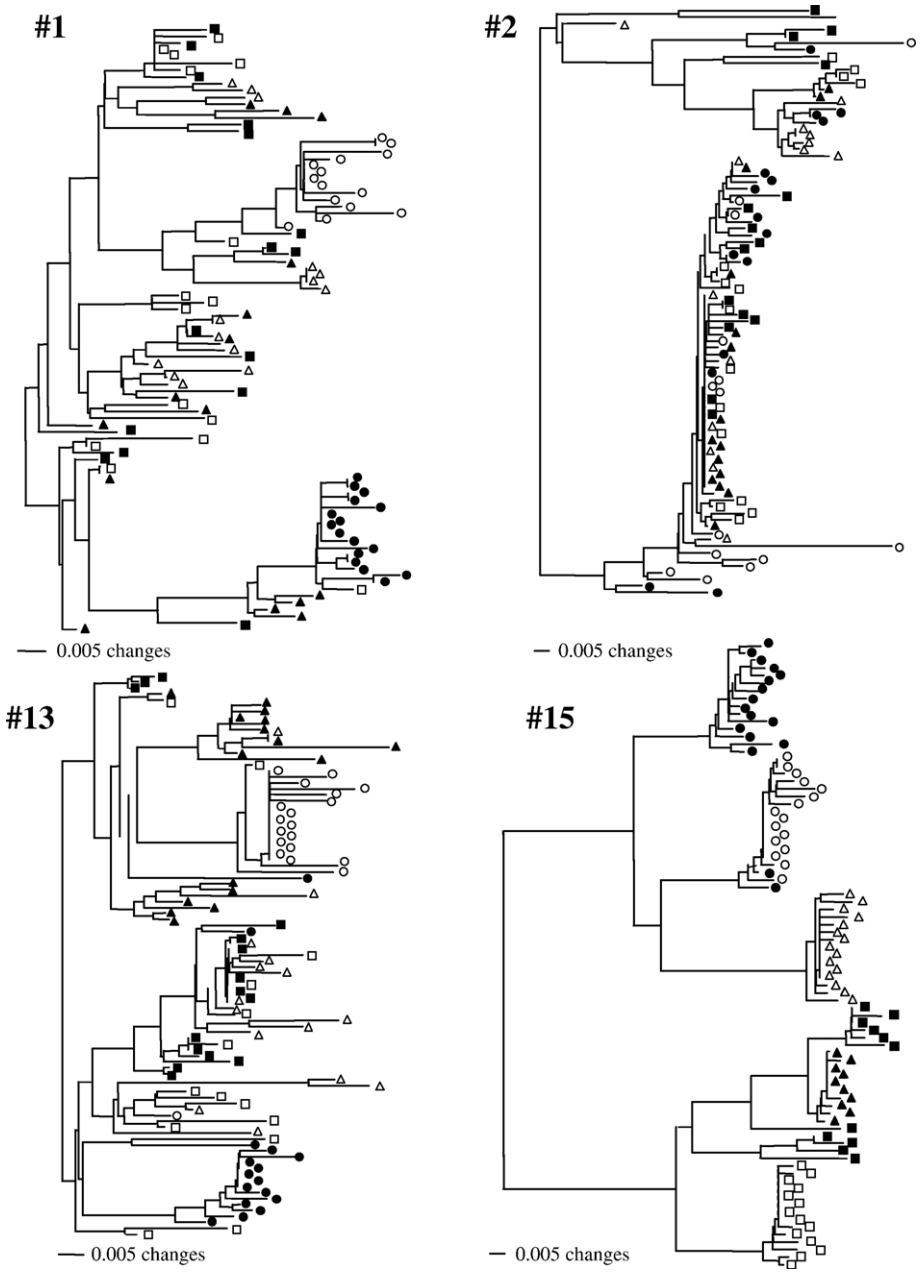

Fig. 1. Phylogenetic relationships of *env* C2-V5 sequences from the breast milk of 4 HIV-1-infected mothers. Proviral sequences derived from cell pellet are shown with circles. Viral sequences derived from lipid and lactoserum fractions are shown as triangles and squares, respectively. Filled and open symbols represent sequences from the right and the left breast, respectively. An asterisk along a branch indicates a percentage of bootstrap replicates (out of 100) >70. Horizontal branch lengths are drawn to scale.

exhibited particular pattern of chemokine coreceptor usage. Thus, the X4− and R5− tropism of viruses in breast milk were predicted, based on the analysis of the deduced amino acid sequences of gp120-V3 hypervariable regions. In all mothers, milk HIV-1 DNA and RNA variants were R5-tropic.

Discussion

In the present study, we selected milk samples with HIV-1 load higher than 2500 copies/ml in order to minimize bias in the selection of variants out of a small viral particles quantity (Liu et al., 1996). In order to obtain an adequate sampling representative of the viral quasispecies content, we used primers allowing to detect a wide range of HIV-1 subtypes (Delwart et al., 1993). Thus, we were able to amplify viral quasispecies in a heterogeneous template and, by means of phylogenetic analyses of at least fifteen *env* clones in each breast milk fraction, to accurately estimate the distribution of HIV-1 variants.

Comparison of viral RNA and DNA in breast milk confirmed the results obtained from a previous study (Becquart et al., 2002). Viral DNA (reflecting the proviral reservoir) was clearly different from viral RNA (reflecting virions) in 3 out of the 4

studied mothers (mothers #01, #13 and #15). In 3 mothers (mothers #01, #13 and #15), proviral quasispecies detected in milk differed according to right or left breast, as did cell-free viral quasispecies in lactoserum from two of them. This suggests that HIV-1 present in breast milk both as proviruses or virions is submitted to a specific microenvironment (involving cytokines, antibodies, CTLs) driving quasispecies viral selection.

Cellular composition of breast milk and blood differs markedly. In breast milk, epithelial cells represent the predominant cell population (>90%) (Becquart, unpublished), but these cells are not highly permissive to HIV-1 infection and *in vitro* produce only small quantities of virus (Toniolo et al., 1995). Consequently, viral DNA detected in breast milk most probably originated from the other two major cell populations, macrophages and lymphocytes (Southern, 1998). T cells and macrophages from breast milk could be infected into, and migrate from the sub-mucosae (Becquart et al., 2002). In the prototype lentivirinae model causing slow infection in goats and sheep, Caprine Arthritis Encephalitis Virus/visna maedi virus, the virus is directly excreted in milk due to the presence of highly productive germinal centers into the mammary gland in the direct vicinity of lactiferous ducts (Narayan and Cork, 1985). Similarly, HIV-1 replication could occur locally in the sub-mucosal cells of the mammary gland. Alternatively, HIV-1 virions could transsudate from blood. In case of mastitis (mother #13 and #15), factors such as inflammatory chemokine could both trigger viral replication in the sub-mucosal cells of the mammary gland and impair the blood–mammary permeability.

Cell-free HIV-1 quasispecies could therefore accumulate due to replication errors produced by the RT. Consequently, new cells could become infected and archive additional viral DNA quasispecies. As the composition of breast milk is changing overtime, archived DNA with different characteristics due to evolving selection conditions is also likely to accumulate.

Phylogenetic analyses show that the HIV-1 variants detected in the lipid fraction and the lactoserum were mixed in mothers #01, #02 and #13, while the viral populations in the 2 fractions were clearly distinct (although phylogenetically very close) in breast milk from mother #15. These differences may result from a bias in the variants sampling in cases where nucleic acids were extracted from a small quantity of lipids and/or a limited repertoire of quasispecies.

The predicted tropism of the virus was determined from the V3 loop amino acid sequence (Jensen and van 't Wout, 2003) variants. Confirming a previous study (Becquart et al., 2002), all breast milk HIV-1 strains were predicted to be R5-tropic viruses, consistent with the preferential mucosal transmission of R5-tropic HIV-1 (Zhu et al., 1996).

In summary, each mammary gland may be submitted to microenvironmental constraints differing from the contralateral breast. Breast milk HIV RNA levels and HIV genotypes are both predictors of breast milk transmission (Koulinska et al., 2006a, 2006b; Manigart et al., 2004). In addition, *in situ* immune response, ARV drug concentrations or cytokine secretion (such as IL7, IL8, TNFα or others) may act as driving force towards selection of viral populations and may be critically involved in breast-feeding transmission of HIV-1.

Materials and methods

Breast milk

Breast milk samples from HIV-infected mothers were collected from a study in Kwa Zulu-Natal, South Africa aimed at assessing determinants of breast milk RNA viral load (Willumsen et al., 2001). Presence of subclinical mastitis, defined as a raised sodium/potassium (Na+/K+) ratio in breast milk (Filteau et al., 1999a, 1999b; Semba et al., 1999), has been associated with increased breast milk viral loads (Willumsen et al., 2003). Among breast milk samples with HIV-1 RNA >2500 copies/ml, 15 were randomly selected for this study (#1 to #15).

DNA and RNA preparation

Cell pellets were washed twice with 1 ml of PBS. DNA extraction was carried out using the QIAmp blood and tissue kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. Whole milk was centrifuged at 1000×*g* for 10 min at 4 °C to separate cells, lactoserum and lipid fraction. Lactoserum was centrifuged at 23,500×*g* for 1.5 h at 4 °C to concentrate the virus. RNA was isolated from lipid fraction and virus pellet using RNeasy Lipid Tissue (Qiagen) according to the manufacturer's instructions. On-column DNase digestion was performed with the RNase-free DNase set to eliminate DNA contamination during RNA purification.

DNA and RNA amplification

The extracted RNA was immediately reverse transcribed into cDNA using Qiagen One-Step RT-PCR kit (Qiagen) following the manufacturer's instructions using 20 pmol of primers ED5 and ED12 (Delwart et al., 1993) and 10 U of protector RNase inhibitor (Roche Molecular Biochemicals, France). Then, 5 μl of the product was amplified with the inner primers ES7 and ES8 to generate a 650 bp fragment corresponding to the C2-V5 region of the envelope gene (Delwart et al., 1993). DNA samples were amplified by a two-step amplification procedure using Qiagen Taq DNA polymerase kit (Qiagen). The first PCR was performed with outer primer ED5 and ED12 (20 pM) and 2.5 U of *Taq* polymerase xxx (Qiagen). Following the first PCR round, 5 μl of the product was amplified as described above.

Molecular cloning and DNA sequencing

To increase the likelihood of detecting different viral variants, two nested PCRs were performed and PCR products were cloned separately. PCR products were gel purified and cloned into PGEM-TEasy vector (Promega, Charbonnières, France) according to the manufacturer's recommendations. Clones bearing inserts were identified by PCR. Double stranded recombinant plasmid DNA was sequenced using cycle sequencing and dye terminator methodologies (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 3.1) with AmpliTaq FS DNA polymerase (PE Biosystems, Warrington, England) on an automated capillary sequencer (ABI 3700

model, Applied Biosystems) using M13 primer. At least fifteen clones were sequenced for each breast milk fraction. Sequence editing was performed using Seqman II version 5.0 from the DNAstar package (http://www.dnastar.com).

Sequence analysis and phylogenetic analysis

Nucleotide and amino acid sequences were aligned by using ClustalW 1.8 program (Thompson et al., 1994) with references sequences of major HIV-1 subtypes available from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov/) with minor manual adjustments. Gaps and sequence corresponding to PCR primers in the alignment were omitted from further analyses.

Phylogenetic analyses were performed with PAUP* 4.0b10 software (Swofford, 1998) or using the ClustalW 1.8 package. Phylogenetic trees were constructed using the neighbor joining method with the Kimura's two-parameter method. The reliability of branching orders was tested using the bootstrap approach (100 replicates). Evolutionary distances were calculated with the Kimura's two-parameter method. HIV subtyping was done using Blast (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi).

Prediction of X4 and R5 viral tropism

The predicted tropism of the virus was determined from the V3 loop amino acid sequence (Jensen and van 't Wout, 2003). The R5 and X4 variants were confirmed by using a position-specific scoring matrix program: (http://ubik.microslu.washington.edu/computing/pssm/).

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