Genetic Analyses of HIV-1 env Sequences Demonstrate Limited Compartmentalization in Breast Milk and Suggest Viral Replication within the Breast That Increases with Mastitis

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The concentration of human immunodeficiency virus type 1 (HIV-1) is generally lower in breast milk than in blood. Mastitis, or inflammation of the breast, is associated with increased levels of milk HIV-1 and risk of mother-to-child transmission through breastfeeding. We hypothesized that mastitis facilitates the passage of HIV-1 from blood into milk or stimulates virus production within the breast. HIV-1 env sequences were generated from single amplicons obtained from breast milk and blood samples in a cross-sectional study. Viral compartmentalization was evaluated using several statistical methods, including the Slatkin and Maddison (SM) test. Mastitis was defined as an elevated milk sodium (Na+) concentration. The association between milk Na+ and the pairwise genetic distance between milk and blood viral sequences was modeled using linear regression. HIV-1 was compartmentalized within milk by SM testing in 6/17 (35%) specimens obtained from 9 women, but all phylogenetic clades included viral sequences from milk and blood samples. Monotypic sequences were more prevalent in milk samples than in blood samples (22% versus 13%; P = 0.012), which accounted for half of the compartmentalization observed. Mastitis was not associated with compartmentalization by SM testing (P = 0.621), but Na+ was correlated with greater genetic distance between milk and blood HIV-1 populations (P = 0.041). In conclusion, local production of HIV-1 within the breast is suggested by compartmentalization of virus and a higher prevalence of monotypic viruses in milk specimens. However, phylogenetic trees demonstrate extensive mixing of viruses between milk and blood specimens. HIV-1 replication in breast milk appears to increase with inflammation, contributing to higher milk viral loads during mastitis.

The risk of MTCT is strongly associated with the concentration of HIV-1 in breast milk (28, 46, 47). Although breast milk HIV-1 RNA concentrations correlate with those in plasma, levels in milk are typically 2 log10 lower (15, 24, 43). This suggests that HIV-1 in blood and milk may not mix freely, likely because of the closure of tight junctions between mammary alveolar cells that occurs once milk production is established and before weaning (16). Thus, HIV-1 may evolve in the breast without substantial mixing with blood, i.e., evolving viral variants would become compartmentalized—a phenomenon that has been observed in the central nervous system (50) and in some studies of the genital tract (10, 44, 57). Compartmentalization of HIV-1 variants has been detected in the breast milk of a small number of women (3, 4), but other data suggest that compartmentalization in breast milk may be uncommon (22).}

Breast inflammation (mastitis) occurs frequently during lactation, most commonly without symptoms. Mastitis is associated with elevations in HIV-1 RNA levels in milk (15, 31, 47, 55), an increase in the number of inflammatory cells in milk, and opening of tight junctions in the mammary epithelium that

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allows passage of subcellular blood components, of which sodium (Na\(^+\)) serves as a marker (15, 16, 36, 47, 55). Greater permeability of mammary epithelia may allow the passage of free virus from the blood into breast milk, which would result in the mixing of HIV-1 subpopulations from blood and milk. Alternatively, inflammation in the breast may induce replication of virus by HIV-1-infected cells within the breast, which would result in divergence between milk and blood HIV-1 subpopulations. Here we describe detailed genetic analyses of HIV-1 subpopulations in the blood and breast milk to determine whether mastitis affects the structure of these populations and to gain understanding of the processes that may lead to increased concentrations of HIV-1 in milk.

**MATERIALS AND METHODS**

**Study design.** Specimens were selected from participants in a previously described (15) cross-sectional study of Zimbabwean women who were between 6 and 16 weeks postpartum, HIV-1 infected, and breastfeeding. All participants provided written informed consent, and all procedures were approved by the Institutional Review Board of Seattle Children's Hospital and the Medical Research Council of Zimbabwe. Specimens were selected based on a high HIV-1 concentration in milk and adequate specimen volume for generation of multiple sequences by single-template PCR, and a subset were selected to have mastitis, defined by a milk Na\(^+\) concentration of >12 mM.

**Specimen collection and processing.** Briefly, breast milk was collected by manual expression, and skim milk was separated from cells using centrifugation (15). The Na\(^+\) concentration in skim milk was determined using a Roche/Hitachi 902 clinical chemistry analyzer (15). HIV-1 RNA was quantified using the Amplipcr Monitor kit (version 1.5; Roche Diagnostics) in plasma using the standard method (detection limit, 400 copies/ml) and in skim milk using the ultrasensitive method (detection limit, 50 copies/ml) (15).

**Generation of single-template sequences.** The nucleic acids in 0.2 to 1.0 ml of plasma samples and 0.7 to 2.0 ml of skim milk samples were extracted using Boom silica methods, with slight modifications (6, 8). Purified RNA was reverse transcribed into cDNA using primer BH2JC (TAATGTTGGTCTCTCCTACGCAAAC; HXB2 coordinates 7701 to 7723) or ED12 (AGTGCCTCTCTGCTCTTCACCAAG; HXB2 coordinates 7822 to 7792) for env (1) and primer RT1-C (GTGTGACTCACCTGGATGAC; HXB2 coordinates 2519 to 2539) for pol. DNA from 1.2 ml of whole-blood or frozen milk cell pellets derived from 4 to 14 ml of whole breast milk (15) were extracted using the QIAamp minikit (Qiagen, Hilden, Germany) into 50 \(\mu\)l of elution buffer, according to the manufacturer's instructions. The concentration of amplifiable viral templates in each specimen was determined using limiting dilution nested PCR (45), with a multiplexed first round containing the above-mentioned sequences (RT1-C) and a second-round forward primer and ED5JC (TGCATGAGGATATAATCAGTTTATGGGA; HXB2 coordinates 6535 to 6562) to amplify env and RT2-C (GTATATCATTGGACAGTCCACGC; HXB2 coordinates 3321 to 3360) to amplify pol. In a second-round PCR, the C2-V5 region of env was amplified with primers DR7JC (GTGCATCAACTCACAATGCTTTGAAATGG; HXB2 coordinates 6984 to 7012) and DR8 (CAGCTCCCAATTGCTCATAATCCCTCC; HXB2 coordinates 7638 to 7668). Viral gene sequences were then derived by one of the two following methods. First, up to 100 amplification reactions were performed on the nucleic acid from each specimen after dilution to ~0.3 HIV-1 amplifiable copies/reaction to amplify a single viral template (45, 53). Positive reactions were identified by gel electrophoresis, processed with ExoSAP-IT (USB Corporation, Cleveland, OH), and sequenced directly using fluorescent dye terminators (BigDye Terminator version 3.1, cycle sequencing kit; Applied Biosystems, Foster City, CA) and a Prism 3730XL DNA analyzer (Applied Biosciences). Second, when the average viral input exceeded 0.3 copies/reaction, amplicons were cloned as previously described (8), and one clone per PCR was sequenced to ensure derivation from separate viral templates (32). Each type of specimen (plasma RNA, whole-blood DNA, skim milk RNA, and milk cell pellet DNA) obtained from a participant was evaluated on a different day to minimize the risk of cross-contaminating specimens.

**Sequence analysis and phylogenetic tree construction.** Sequences were assembled and checked for read errors in Sequencher version 3.4 (Gene Codes Corporation, Ann Arbor, MI). Alignments were generated in CLUSTALW (52), and hypermutated sequences were excluded and manually adjusted using MacClade version 4.08 (34). An all-inclusive phylogenetic tree was used to verify that each sequence segregated only with others from the identified participant. Sequences from all participants were carefully examined for contamination by comparisons to known reference sequences and to all other sequences generated in the laboratories, and no matches were found. We also screened each alignment for recombination, since this could confound compartmentalization (56). For all subjects, phylogenetic trees were constructed using the DIVEIN interface (11) (http://indra.mullins.microbiol.washington.edu/DIVEIN/index.html), with a general time-reversible model plus invariant plus gamma distribution (GTR+I+G) model evolution within PhyML (version 3.0) (18). Four representative subtype C HIV-1 sequences from GenBank accession numbers AY265932, AY265936, AF268277, and AY265952 were used to root the phylogenies of each participant's sequences. In order to better evaluate the possibility of dual HIV-1 infection in the phylogenies of those individuals (participants A, D, E, G, and H) with pronounced separation of discrete clades, eight additional subtype C sequences (GenBank accession numbers AF434091, AF772691, AY162224, AY1772699, AF067155, AF286224, AF443112, and AF443106) as well as one each from subtypes B, A, and D (GenBank accession numbers AF286241, U63632, and AY253311) were included. HIV-1 coreceptor usage was predicted for the V3 region amino acid sequences using the subtype C position-specific scoring X4/R5 and syncytium-inducing and non-syncytium-inducing matrices (23) (http://indra.mullins.microbiol.washington.edu/pssm/).

**Evaluation of HIV-1 population structure and statistical testing.** The topology of each phylogenetic tree was examined, with particular attention to the identification of breast milk- or peripheral blood-specific clades. The compartmental structure of viral sequences in blood and milk specimens obtained from each subject was evaluated by the Slatkin and Maddison (SM) test, which evaluates whether the distribution of discrete clades, eight additional subtype C sequences (GenBank accession numbers AF434091, AF772691, AY162224, AY1772699, AF067155, AF286224, AF443112, and AF443106) as well as one each from subtypes B, A, and D (GenBank accession numbers AF286241, U63632, and AY253311) were included. HIV-1 coreceptor usage was predicted for the V3 region amino acid sequences using the subtype C position-specific scoring X4/R5 and syncytium-inducing and non-syncytium-inducing matrices (23) (http://indra.mullins.microbiol.washington.edu/pssm/).

**RESULTS**

**Participant and specimen characteristics.** Blood and breast milk specimens obtained from both breasts of nine women...
collected 6 to 16 weeks postpartum were evaluated in a cross-sectional study (15). All participants had received a single dose of nevirapine for the prevention of MTCT (17) but were not otherwise exposed to antiretroviral drugs. The median HIV-1 RNA viral load in plasma samples was 146,224 copies/ml (range, 25,831 to 986,896 copies/ml) and in milk samples was 4,105 copies/ml (range, 241 to 124,254 copies/ml) (Table 1). A total of 515 individual HIV-1 env sequences were generated, with a median of 54 sequences from each participant (range, 42 to 74) (Table 1 and Fig. 1). Of these sequences, 186 were generated from HIV-1 RNA, and 329 were generated from HIV-1 DNA. A total of 171 of these sequences were derived from blood samples, and 344 were derived from milk samples (Table 1 and Fig. 1).

Analysis of HIV-1 population structure. Pronounced separation of discrete clades was apparent in phylogenetic trees obtained from five of the nine participants (A, D, E, G, and H). The trees obtained from two of these individuals, participants A and E, displayed separation of viral clades by reference sequences without evidence of recombination, indicating infection with two unrelated strains of subtype C HIV-1 (Fig. 1). A lack of genetic diversity in cell-associated HIV-1, suggestive of recent infection, was notable in each infecting virus population of participant E. Phylogenies obtained from each of the other participants displayed a monophyletic cluster of sequences distinct from reference sequences, without evidence of dual infection. The sequences obtained from participant H were monophyletic with respect to reference sequences yet displayed two discrete clades that were found to differ by genotypic coreceptor usage pattern (Fig. 1) (phenotypic analysis for definitive determination of coreceptor usage was not performed [23]). Sequences obtained from participants D and G formed distinct clades that were not explained by dual infection or coreceptor use.

Across all the participants’ phylogenies, HIV-1 sequences from breast milk specimens were interspersed with those from blood specimens, without clades comprised exclusively of blood or milk viruses. RNA and DNA sequences were largely interspersed across the individual’s phylogenies. Because cell-free and cell-associated HIV-1 sequences in untreated individuals largely reflect actively replicating virus (33, 35) and because of the small numbers of sequences of each type available for analysis, RNA and DNA sequences were combined in the evaluations of compartmentalization.

The frequency of finding two or more monotypic sequences (those with zero nucleotide differences) was similar across breast milk and blood specimens (15/18 versus 5/9, respectively; P = 0.175). However, monotypic variants comprised a greater proportion of all sequences in milk specimens than in blood specimens (22.4% versus 12.9%; P = 0.012) (Table 2).

Despite the interspersion of sequences on phylogenetic trees noted above, statistical evaluation of the population structure of viral sequences using SM testing based on maximum likelihood phylogenies detected compartmentalization of virus between breast milk and blood in 6 of 17 (35.3%) specimens obtained from 4 of 9 individuals (participants B, C, D, and E) (Table 3). Results were similar when statistical estimation of compartmentalization was performed using parsimony scores, association indices, and maximum monophyletic clade sizes based on Bayesian phylogenies (12, 41) (Table 3). In addition, performing all analyses using balanced numbers of sequences from each participant’s samples did not substantially change estimates of compartmentalization (data not shown). No evidence for compartmentalization between right and left breast milk specimens was observed (data not shown). After monotypic sequences, which can inflate statistical estimates of population structure (7, 8, 21), were collapsed into a single sequence, 3 of 6 (50%) specimens no longer showed evidence of compartmentalization based on Bayesian phylogenies (12, 41) (Table 3). In addition, performing all analyses using balanced numbers of sequences from each participant’s samples did not substantially change estimates of compartmentalization (data not shown). No evidence for compartmentalization between right and left breast milk specimens was observed (data not shown).

Effect of mastitis on HIV-1 population structure. The median concentration of breast milk Na+ across all specimens analyzed was 8 mM (range, 2 to 86 mM), and 6 participants had mastitis in one breast, as defined by a milk Na+ concentration of >12 mM (Table 1) (15, 47). Milk specimens with mastitis had a median HIV-1 viral load of 45,228 copies/ml (range, 1,553 to 124,254 copies/ml) compared with that of 2,660 copies/ml (range, 400 to 55,227 copies/ml) in specimens without mastitis (P = 0.043).

The proportion of monotypic viruses in milk specimens was not associated with mastitis (18.4% versus 24.4% in samples with and without mastitis, respectively; P = 0.218) (Table 2).

### Table 1. Plasma and breast milk HIV-1 viral loads, breast milk Na+ concentrations, and numbers of single-template env DNA and RNA sequences analyzed

<table>
<thead>
<tr>
<th>Specimen results</th>
<th>Blood</th>
<th>Left breast milk</th>
<th>Right breast milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participant</td>
<td>Plasma viral load (log copies/ml)</td>
<td>No. of HIV-1 env sequences</td>
<td>Skim milk viral load (log copies/ml)</td>
</tr>
<tr>
<td>A</td>
<td>4.4</td>
<td>11 7</td>
<td>3.6</td>
</tr>
<tr>
<td>B</td>
<td>4.8</td>
<td>11 10</td>
<td>3.6</td>
</tr>
<tr>
<td>C</td>
<td>5.0</td>
<td>12 3</td>
<td>3.5</td>
</tr>
<tr>
<td>D</td>
<td>5.1</td>
<td>11 8</td>
<td>4.7</td>
</tr>
<tr>
<td>E</td>
<td>5.2</td>
<td>11 13</td>
<td>2.4</td>
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<tr>
<td>F</td>
<td>5.5</td>
<td>12 13</td>
<td>4.2</td>
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<tr>
<td>G</td>
<td>5.7</td>
<td>11 0</td>
<td>3.2</td>
</tr>
<tr>
<td>H</td>
<td>5.7</td>
<td>25 0</td>
<td>4.1</td>
</tr>
<tr>
<td>I</td>
<td>6.0</td>
<td>13 0</td>
<td>3.4</td>
</tr>
</tbody>
</table>

a Na+ sample not available.
FIG. 1. HIV-1 env sequences from both breast milk and blood specimens are present in all phylogenetic clades, suggesting a lack of viral compartmentalization. Maximum likelihood phylogenetic analysis of the HIV-1 env region C2-V5 derived from single-amplicon-derived sequences from RNA (squares) and DNA (circles) from blood specimens (black filled symbols) and breast milk specimens (left breast, gray filled symbols; right breast, open symbols). Dual HIV-1 infection is evident in participants A and E, with each infecting strain delineated by a bracket. The phylogeny for participant H shows distinct clades, shown with brackets, which differ by predicted coreceptor usage (CCR5 or CXCR4). Mastitis, as defined by a breast milk Na\(^+\)/H\(^+\) concentration of \(>12\) mM, was present in samples from subject A (left breast), B (right breast), D to F (right breast), and G (left breast). Sequences obtained from all participants are rooted by four reference sequences of subtype C (labeled RC1, etc.). Eight additional subtype C sequences and one each from subtypes A, B, and D were used to root phylogenies for participants A and E in order to better characterize their dual infections. The number of substitutions per site is indicated by the horizontal line below each phylogeny.
Similarly, compartmentalization of virus was not associated with mastitis, with compartmentalization observed in 1 of 6 (16.7%) breast milk specimens with mastitis compared to 5 of 11 (45.5%) of those without mastitis (P = 0.334) (Table 3) by SM testing. Furthermore, the median Na\(^+\) concentration did not differ significantly between milk specimens with compartmentalization detected by SM testing and those without (P = 0.155).

The relationship between milk Na\(^+\) concentration as a marker of inflammation within the breast and the genetic distance between HIV-1 populations in breast milk and blood samples was evaluated using the GEE. Breast milk Na\(^+\) was positively correlated with the genetic distance between HIV-1 in milk and blood specimens, indicating that with increasing inflammation HIV-1 populations in milk specimens progressively diverged from those in blood specimens (β coefficient = 3.8 \times 10^{-5}; 95% confidence interval, 1.6 \times 10^{-6} to 7.4 \times 10^{-5}; P = 0.041).

### DISCUSSION

Three observations are noteworthy from our study. First, we found only limited compartmentalization of HIV-1 in breast milk specimens, suggesting substantial interchange of viruses between the milk and blood specimens obtained from the participants we studied. Second, monotypic sequences were overrepresented in the HIV-1 populations from milk specimens compared to those from blood specimens, suggesting local HIV-1 production within the breast, possibly from the proliferation of infected cells. Third, the mean genetic distance between milk and blood viruses correlated positively with breast milk Na\(^+\) concentrations, suggesting full cycles of viral replication within the breast and that the increased breast milk HIV-1 concentrations observed with mastitis are not simply due to the passage of virus from blood into breast milk.

Viral compartmentalization was detected in a minority of breast milk specimens using a panel of statistical methods commonly used to evaluate population structure. However, among the participants evaluated, no tissue-specific clades were observed in phylogenetic trees. Rather, viral sequences from milk and blood specimens were intermingled, suggesting that viruses mix readily between these fluids. Similarly, studies of HIV-1 populations in the genital tract and lung have found compartmentalization by statistical testing when segregation by fluid/tissue is not apparent in phylogenies (7, 21). The importance of such compartmentalization is unclear but is consistent with replication or proliferation within a small virus population (i.e., limited effective population size) (7, 21).

Detection and quantification of minority sequence variants are dependent on the method of sampling and the number of sequences obtained. We sequenced virus derived from single PCR amplicons, which prevents bias due to resampling of individual variants (9, 32). Resampling of viral sequences may explain the apparent compartmentalization of HIV-1 in breast milk specimens previously reported for some women (3, 4). In support of our findings, a study using a heteroduplex tracking assay was unable to distinguish differences between HIV-1 populations from breast milk and blood specimens obtained from 8 women (22), as did a recent study that cloned viruses but employed methods to avoid resampling of viral templates (20). The time and expense required for the generation of single-amplicon sequences limits the sample size feasible for phylogenetic analyses and therefore affects the precision of estimates of population differences. We analyzed a large number of sequences, but these sequences were derived from a small number of nonrandomly selected participants, potentially limiting the generalizability of our findings. In addition, it was not possible to perform analyses on cell-free virus populations separately, as few sequences were generated from breast milk specimens with low concentrations of HIV-1 RNA. Finally, because the sequences were derived at a single time point early in lactation, we cannot rule out the possibility that HIV-1 compartmentalization in an individual’s breast milk changes significantly over time. Relevant to this last point, a comparison of the compartmentalization of blood and milk when these specimens were collected on the same or different dates gave discrepant results (20). Viruses in specimens collected on the same dates were rarely compartmentalized,
We observed a statistically significant positive correlation between breast milk Na+/H+ exchange activity and the presence of mastitis. This correlation was consistent across different statistical analyses. However, when inflammation renders mammary epithelial tight junctions permeable, HIV-1 from the blood may more easily enter breast milk, allowing for increased production of HIV-1 in milk. We speculated that this increased production might contribute to the increase in milk HIV-1 concentration observed in the present study.

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the sole mechanism, because an influx of blood viruses would decrease the genetic differences between these subpopulations. While the absolute divergence of breast milk HIV-1 we detected in mastitic milk was small, it is important to note that the duration of increased viral replication would at most be 6 to 16 weeks in duration due to the timing of sample collection after the onset of lactation. In addition, the magnitude of the genetic differences between milk and blood HIV-1 may have been diminished by the countervailing contribution of blood virus leaking into milk. Of note, although there was a significant association between milk Na⁺ concentrations and the genetic distance between HIV populations in blood and milk specimens, we did not detect an association between mastitis and the prevalence of monotypic sequences in milk specimens. This discrepancy suggests that mastitis may stimulate viral replication across a larger population of virus in the infected breast compared to that in the healthy breast, perhaps due to an influx of infected inflammatory cells. Furthermore, compartmentalization of virus in breast milk specimens was not associated with mastitis, suggesting either that a relatively diverse group of viruses replicated in milk specimens or that the net effect of inflammation on replication within the breast was not sufficient to affect HIV-1 population structure by these measures.

In summary, phylogenetic analyses of HIV-1 genomes from breast milk and blood specimens obtained from lactating women found limited viral compartmentalization, indicating relatively free mixing of viruses between milk and blood specimens. The greater prevalence of monotypic HIV-1 in breast milk specimens suggests viral replication and/or proliferation of cells containing proviruses within the breast. Statistical modeling of the effect of inflammation on genetic distance between HIV-1 subpopulations in milk and blood specimens found evidence for increased HIV-1 replication in breasts with mastitis.

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We have no conflicts of interest to declare.

REFERENCES


