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Short Communication
HIV Type 1 Subtype C Variants Transmitted Through the Bottleneck of Breastfeeding Are Sensitive to New Generation Broadly Neutralizing Antibodies Directed Against Quaternary and CD4-Binding Site Epitopes

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Abstract

Mother-to-child transmission of HIV-1 subtype C can occur *in utero*, intrapartum, or via breast milk exposure. While not well understood, there are putative differences in the mechanisms involved with the distinct routes of vertical HIV transmission. Here, we address the question of whether specific viral characteristics are common to variants transmitted through breastfeeding that may facilitate evasion of innate or adaptive immune responses. We amplified the *envelope* gene (*env*) from the plasma of six infants during acute infection who were infected with HIV-1 subtype C through breastfeeding, and from three available matched maternal samples. We sequenced the full-length *env* genes in these subjects revealing heterogeneous viral populations in the mothers and homogeneous populations in the infants. In five infants, the viral population arose from a single variant, while two variants were detected in the remaining infant. Infant *env* sequences had fewer N-linked glycosylation sites and shorter sequences than those of the available matched maternal samples. Though the small size of the study precluded our ability to test statistical significance, these results are consistent with selection for virus with shorter variable loops and fewer glycosylation sites during transmission of HIV-1 subtype C in other settings. Transmitted *envs* were resistant to neutralization by antibodies 2G12 and 2F5, but were generally sensitive to the more broadly neutralizing PG9, PG16, and VRC01, indicating that this new generation of broadly neutralizing monoclonal antibodies could be efficacious in passive immunization strategies.

Transmission of human immunodeficiency virus-1 (HIV-1) through breastfeeding (BF) makes up one-third to one-half of all mother-to-child transmission events.[1] The mechanism(s) of transmission, however, are poorly understood. The oral cavity and gastrointestinal tract of breastfed infants are exposed daily to both cell-free and cell-associated HIV-1,[2–4] yet the majority of infants remain uninfected even if neither mother nor baby receive antiretroviral prophylaxis.[5] This inefficiency of transmission indicates that anatomical, innate, and/or adaptive mechanisms of protection are able to prevent transmission to a great extent.[6–11] Maternal antibodies could prevent infection either through direct binding of virus in the breast milk, or by their systemic and mucosal presence in the infant. This passive maternal immunity in the infant increases in concentration during the last trimester of gestation, and continues to pass into the infant through breastfeeding.

Studies of *in utero* and intrapartum transmission have shown a universal bottleneck in genetic diversity from mother to child, as well as differences in the characteristics of transmitted virus for *in utero* versus intrapartum transmission.[12,13] Data are very limited for breastfeeding pairs, but one study of three breast milk transmission events found a similar bottleneck for HIV-1 subtype A.[14] We previously demonstrated that the viral population found in infants infected intrapartum tended to be more heterogeneous than populations from

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infants infected *in utero*, and that viruses from infants infected intrapartum tended to have shorter variable loops and fewer glycosylation sites than the parent viral population.[13,15] Others had similar findings for infants mostly infected intrapartum, though they only found differences in glycosylation sites and not variable loop lengths.[16]

In this article, we present data on viral sequences from three mother–child pairs plus three additional infants where the infant became HIV DNA positive between 6 and 12 weeks postpartum through exposure to breast milk, with the goal of exploring whether known selective pressures influence this route of mother-to-child transmission. Participant plasma samples were collected from the Malaria and HIV-1 in Pregnancy (MHP) prospective cohort.[17–20] The MHP study was approved by the Malawi College of Medicine Research Committee and the Institutional Review Board at the University of North Carolina at Chapel Hill. Informed consent was obtained from all mothers. Women and their newborn infants received single-dose nevirapine according to the HIVNET 012 protocol[20] and breastfeeding was initiated. Plasma and cell pellets were isolated from blood collected from the mothers at labor-ward admission, and blood was collected via heel-sticks from the infants at three time points: within 48 h of birth, at 6 weeks, and at 12 weeks of age. Infants who were HIV-1 DNA negative by real-time polymerase chain reaction (PCR)[21] at 0 and 6 weeks, then positive at 12 weeks were classified as infected postpartum through breastfeeding (BF). Viral RNA and cell-associated DNA were isolated and amplified using single-genome *env* gene amplification, as has been described previously,[15,22] to ensure that most amplifications were initiated with a single template without artifactual recombination during PCR between multiple template sequences. The HIV-1 *env* DNA single genome amplification protocol was the same as the RNA protocol following reverse transcription (GenBank accession numbers JN983803–JN983805).

Sequences were aligned using the L-INS-I method in MAFFT version 5.8.[23] A maximum likelihood phylogenetic tree was constructed with PHYML[24] using the general time reversible plus gamma ($\alpha \sim 0.25$ for each tree) evolutionary model chosen by FindModel (hiv.lanl.gov) with four rate substitution categories. Trees were resampled 100 times and bootstrap values greater than 70 were considered significant. In a tree including all sequences, each infant or mother–infant pair clustered together as a distinct clade (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/aid). Cell-free (viral RNA) and cell-associated (viral DNA) viral populations were highly similar in the infant, as expected in acute infection, and allowed for analysis of viral sequences from blood plasma or cell pellets from BF-infected infants as available (Fig. 1 and Supplementary Table S1). Five of six infants appeared to be infected with a single variant, while in the remaining infant (942) a second minor variant was amplified from two distinct reactions. In the three mother–infant paired samples, maternal populations were more heterogeneous than the infant populations, demonstrating a bottleneck in viral diversity during HIV-1 subtype C BF transmission (Fig. 1 and Supplementary Table S1). Within-participant sequence diversity was conducted using the Kimura two-parameter method in MEGA4.[25,26] Infant viral *env* populations were more homogeneous than maternal populations, and the infant populations were highly similar in their low diversity, with $\leq 0.2\%$ diversity in infants with single variants. In addition, using the Poisson-Fitter tool,[27] sequence populations from all infants had a Poisson distribution of mutations and a phylogeny that coalesced to an inferred consensus sequence representing a virus present at or near the time of HIV-1 transmission (data not shown) and predicted time since most recent common ancestor (MRCA) was also less than 12 weeks for all (84 days), supporting transmission during breastfeeding. For infant 942 the minor variant and recombinant sequences were excluded in this analysis. Thus, we infer that in five of six BF mother–infant pairs a single variant was transmitted or established the infant infection, while in the sixth infant a second minor variant was identified.

In maternal–infant pair 942 there is evidence for the transmission/replication of two maternal variants in the infant (Fig. 1). Using a Highlighter plot (hiv.lanl.gov) (data not shown), we found 41/49 infant sequences were nearly identical with mutations following a model of random evolution. Two infant sequences were distinct, indicating a second transmitted variant; the final viral RNA infant sequence was a recombinant of the two sequences. Because we used single-genome amplification, a method with a very low probability of recombination during amplification, we propose recombination within the infant as the likely source. Analysis with Poisson-Fitter found all 942 infant sequences had a mean hamming distance (HD) of 3.4 and MRCA of 64 days. Yet upon removal of the two minor variant sequences the mean HD decreased to 1.6 and the MRCA to 31 days, demonstrating their influence in the analysis. Both sequences could have been transmitted to the infant during the same transmission event, or through two separate events. Our data are similar to those reported in cohorts of heterosexual transmission, where the likelihood of transmission of multiple variants is around 20%, and overall suggest that the low probability of transmission for any given exposure generally results in the transmission of a single variant when an infection does occur.[12,28–34]

We next compared the number of putative N-linked glycosylation sites (N-glycosite program, hiv.lanl.gov) and sequence length between mother and infant viral populations. Fewer glycosylation sites and shorter full-length *env* sequences were seen in infants compared to their mothers over *env* in all three transmission pairs (Supplementary Table S1). These differences were not common to a particular variable or constant region (data not shown). The small sample size of this study does not allow testing for statistical significance of these differences between matched pairs, but the differences are of a magnitude similar to the significant results from our previous work with intrapartum transmission pairs and other studies of undetermined intrapartum (IP)/BF transmission.[16,35] A larger sample size would be needed to confirm an association.

The presence of multiple variants in infant 942 could represent separate transmission events, or multiple variants simultaneously transmitted in a single event. It is worth noting that the two variants clustered on the same tree branch compared to the more diverse maternal viral population (Fig. 1). This phenomenon was also seen in an IP transmission pair for HIV-1 subtype B.[36] This similarity between variants could be stochastic or it could suggest selection for certain characteristics (in breast milk, during transmission, or through selective amplification in the infant).[37–39] Other studies have presented conflicting results testing the hypothesis that selection is influenced by neutralizing antibodies, and

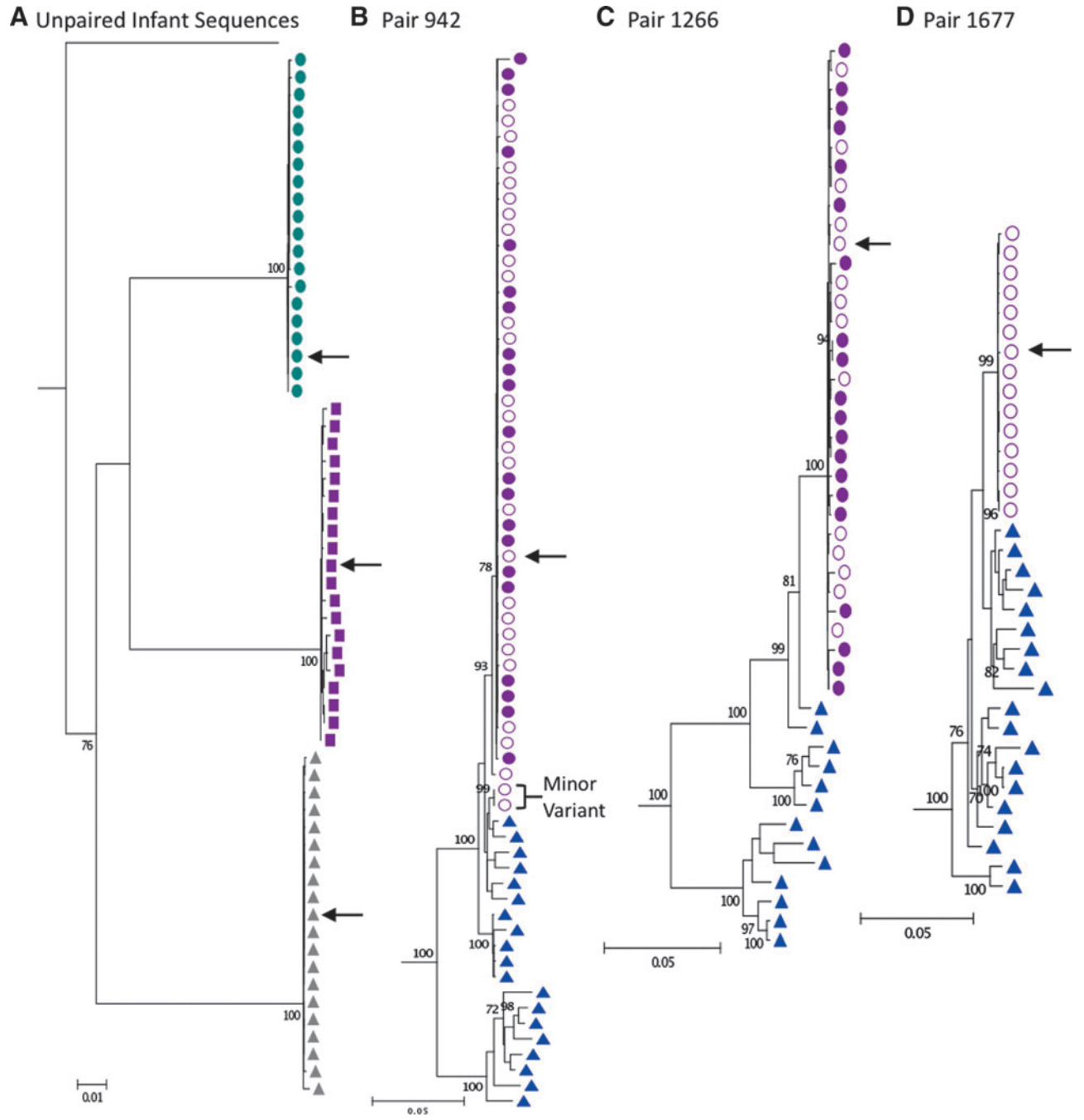

FIG. 1. Maximum likelihood phylogenetic trees for **(A)** unmatched infant cell-associated HIV DNA *env* sequences and (**B–D**) matched mother–infant pairs. **(B–D)** Filled triangles indicate maternal *env* RNA HIV sequences, open circles infant HIV *env* RNA sequences, and filled circles infant HIV *env* DNA sequences. Arrows point to cloned *env* genes. Outgroups have been cropped for space.

transmitted variants are generally resistant to neutralization.[14,36,39] Thus, we investigated the neutralizing antibody resistance profile of these subtype C infant viral variants transmitted through breast milk for comparison.

The consensus (or transmitted/founder) infant *env* gene (or a close match with 1 nt change for 942 and 1266) from each infant was cloned as previously described[15] (Fig. 1). Viruses were assayed as Env pseudotyped viruses for sensitivity to soluble CD4 (sCD4) and to a panel of monoclonal antibodies [2G12, 2F5, 4E10, IgG1b12, PG9, PG16 (gifts of Dennis Burton), VRC01 (gift of John Mascola), CH31 (gift of Bart Haynes)], and subtype C HIVIG. Titers are reported as the antibody concentration or reciprocal serum dilution with a 50% reduction in relative luminescence units, as previously described.[40] Values were interpolated using 5 parameter curve-fitting. As expected for subtype C virus, no pseudotyped infant virus was sensitive to monoclonal antibodies 2G12 or 2F5 up to a concentration of 25 μg/ml.[40] Five of the six

Table 1. 50% Neutralization Sensitivity of Infant Envelope Clones

Infant MHP ID	*1B12 μg/ml*	*2G12 μg/ml*	*2F5 μg/ml*	*4E1O μg/ml*	*sCD4 μg/ml*	*PG-9 μg/ml*	*PG-16 μg/ml*	*VRC01 μg/ml*	*CH31 μg/ml*	*HIVIG-C dilution*
329	>25	>25	>25	17.11	13.54	<0.01	<0.01	<0.01	7.62	11.23
591	8.88	>25	>25	5.78	15.11	0.17	0.03	0.08	0.16	71.69
703	>25	>25	>25	7.52	7.14	0.43	0.03	0.15	0.06	59.35
942	>25	>25	>25	0.75	21.15	0.06	0.03	>25	>25	65.05
1266	>25	>25	>25	13.36	>25	0.02	<0.01	0.05	0.13	93.65
1677	>25	>25	>25	>25	>25	1.06	>25	1.5	0.43	103.89

MHP, malaria and HIV-1 in pregnancy.

pseudotyped infant viruses were sensitive to 4E10 and PG16 within this range, while 1677 was resistant to both. All infant viruses were sensitive to PG9, and all but 942 were sensitive to VRC01 and CH31.

The panel of neutralizing antibodies used in this study represents both the previous generation of monoclonal antibodies (mAbs) with potent neutralization of subtype B HIV-1, yet importantly also includes more recently identified mAbs with extensive breadth even against the previously "difficult to neutralize" HIV-1 subtype C. The resistance profiles found for the infant clones described here are similar to other studies of newly infected infant viral variants of several HIV-1 subtypes.[14,16,36,41] While we saw little neutralization to 1b12 (Table 1) compared to some other studies where 50% or more of viruses were sensitive,[41,42] the results were similar to our previous study of uncultured Envelope pseudotypes from virus newly transmitted from mother to child.[15] Only one study of three infants infected through breast milk[39] also analyzed sensitivity to the recently identified broadly neutralizing PG9 and PG16 antibodies.[43,44] Similarly, all six infant clones tested herein were sensitive to PG9, and all but one were sensitive to PG16, which have a conformational epitope in the V1/V2 region. In addition, the sensitivity of these variants to the CD4 binding-site antibodies VRC01 and CH31 further demonstrates that transmitted infant subtype C viruses are not inherently resistant to neutralization and that recently transmitted viruses may have neutralizing epitopes in common among all prevalent subtypes. These antibodies clearly have enhanced breadth that includes neutralization of subtype C, and for this reason may represent a useful tool for passive immunization in regions where clade C HIV-1 is prevalent.[41] These data in particular support the CD4 binding site as a promising target for infant vaccine design.

In summary, we analyzed *env* sequence data from six infants infected with HIV-1 subtype C through breastfeeding, including three transmission pairs. There was a strong genetic bottleneck during transmission, and more than one variant was transmitted in only one of six infants. Viral envelopes were generally resistant to the monoclonal neutralizing antibodies 2G12 and 2F5, yet were generally sensitive to 4E10 and the more recently identified antibodies PG9, PG16, VRC01, and CH31. The neutralizing sensitivity of these postnatally transmitted virus variants supports the use of the new generation of broadly neutralizing antibodies directed against quaternary V1/V2 epitopes or the CD4 binding site for infant passive immunization strategies, and also implicates these epitopes in infant HIV vaccine design.

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Author Disclosure Statement

No competing financial interests exist.

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