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Xiaohong Kong
University of Nebraska-Lincoln, xkong2@unl.edu

John T. West
University of Nebraska-Lincoln, jwest2@unl.edu

Hong Zhang
Univesity of Nebraska - Lincoln, hzhang2@unl.edu

Danielle Shea
University of Nebraska-Lincoln, dshea2@unl.edu

Tendai J. M'Soka
University Teaching Hospital, Lusaka 10101, Zambia

See next page for additional authors

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Authors

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The Human Immunodeficiency Virus Type 1 Envelope Confers Higher Rates of Replicative Fitness to Perinatally Transmitted Viruses than to Nontransmitted Viruses[∇][†]

Xiaohong Kong,[1][‡] John T. West,[1][§] Hong Zhang,[1] Danielle M. Shea,[1] Tendai J. M'soka,[2] and Charles Wood[1]*

Nebraska Center for Virology, and The School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68583-0900,[1] and Department of Pediatrics, University Teaching Hospital, Lusaka 10101, Zambia[2]

Selection of a minor viral genotype during perinatal transmission of human Immunodeficiency virus type 1 (HIV-1) has been observed, but there is a lack of information on the correlation of the restrictive transmission with biological properties of the virus, such as replicative fitness. Recombinant viruses expressing the enhanced green fluorescent protein or the *Discosoma* sp. red fluorescent (DsRed2) protein carrying the V1 to V5 regions of *env* from seven mother-infant pairs (MIPs) infected by subtype C HIV-1 were constructed, and competition assays were carried out to compare the fitness between the transmitted and nontransmitted viruses. Flow cytometry was used to quantify the frequency of infected cells, and the replicative fitness was determined based on a calculation that takes into account replication of competing viruses in a single infection versus dual infections. Transmitted viruses from five MIPs with the mothers chronically infected showed a restrictive *env* genotype, and all the recombinant viruses carrying the infants' Env had higher replicative fitness than those carrying the Env from the mothers. This growth fitness is lineage specific and can be observed only within the same MIP. In contrast, in two MIPs where the mothers had undergone recent acute infection, the viral Env sequences were similar between the mothers and infants and showed no further restriction in quasispecies during perinatal transmission. The recombinant viruses carrying the Env from the infants' viruses also showed replication fitness similar to those carrying the mothers' Env proteins. Our results suggest that newly transmitted viruses from chronically infected mothers have been selected to have higher replicative fitness to favor transmission, and this advantage is conferred by the V1 to V5 region of Env of the transmitted viruses. This finding has important implications for vaccine design or development of strategies to prevent HIV-1 transmission.

Perinatal transmission of human immunodeficiency virus type 1 (HIV-1) accounts for 90% of infections in infants worldwide, and it occurs in 30 to 45% of children born to untreated HIV-1-infected mothers. This is especially significant in sub-Saharan Africa where HIV-1 infection is epidemic, and antiretroviral treatment is still not widely available (1). The HIV-1 subtypes that are transmitted in the region are also different from those found in developed countries, with subtype C being the most prevalent. Understanding the viral factors that influence perinatal transmission of subtype C virus is thus critical for development of strategies to prevent transmission. However, much of the accumulated knowledge on transmission comes from studies of cases of subtype B infection which have focused on adult donors who were chronically infected. Very little is known about the transmission in the context of mother-to-child transmission, and the applicability of such findings to subtype C remains to be determined. Several studies in adult donors have shown that only a limited number of viruses within the viral quasispecies population are transmitted from the donor, suggesting that minor viral species are transmitted (8, 11). Similar observations were made with perinatal transmission (34, 37, 40).

A number of factors have been suggested to affect the transmission of specific viral species. Genetic analyses of the *env* gene have suggested selective transmission of R5-utilizing HIV-1 during perinatal transmission from chronically infected donors (37, 40). However, other analyses have shown a stochastic pattern with transmission of multiple and/or major maternal HIV-1 variants (10, 19, 24). It has also been suggested that the virus variants more resistant to neutralization are preferentially transmitted perinatally (33). Neutralizing antibody response in the host may be associated with a replicative cost to the virus, and a compromise for the virus between immune escape and replication adaptability to the host could occur (5, 9, 28). It is likely that other biological properties of the virus may play roles to favor perinatal transmission; such properties may include enhanced viral replicative fitness of the transmitted viruses.

HIV-1 replicative fitness is related to pathogenicity, virulence, drug-resistant mutations, and disease progression (4, 13, 30, 35). The fitness of different HIV-1 subtypes was also found

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* Corresponding author. Mailing address: School of Biological Sciences, University of Nebraska-Lincoln, Rm 102C, Morrison Center, 4240 Fair St., Lincoln, NE 68583-0900. Phone: (402) 472-3323. Fax: (402) 472-8722. E-mail: cwood1@unl.edu.

‡ Present address: School of Medicine, Nankai University, Tianjin, China, 300072.

§ Present address: University of Oklahoma Health Sciences Center, Department of Microbiology and Immunology, Oklahoma City, OK 73104.

to correlate with their prevalence in the human population (17, 26, 31). Despite these observations, the association between HIV-1 replicative fitness and transmission is poorly defined, and information addressing an association with perinatal transmission is essentially nonexistent. Since, in the absence of drug treatment, HIV-1 replicative fitness is largely determined by the functions of Env (4, 22, 31), it is anticipated that replicative fitness might also impact virus transmission. This concept has heretofore not been tested, and therapy-naive mother-infant pairs (MIPs) in our study provide a unique opportunity to examine the properties of transmitted viruses from mother to child. As the perinatally transmitted virus should be the target of vaccine strategies, information on its biological phenotype could be directly relevant to the development of a vaccine. Such information also provides insight into mechanisms to prevent such transmission.

We have been studying a Zambian mother-infant cohort for perinatal transmission of subtype C HIV-1 and have analyzed the viral *env* quasispecies of several MIPs during perinatal transmission. We found that there was a reduction in the viral quasispecies during perinatal transmission from mothers who were chronically infected (41). In contrast, such a reduction was not observed in mothers who were acutely infected (14). In this study, we investigated the fitness of the viral Env, which may affect perinatal transmission, from seven MIPs infected by subtype C HIV-1. Five pairs were from a chronically infected source, and the other two pairs were from an acutely infected source. We found that recombinant viruses carrying the V1 to V5 region of Env of the infant viruses from all chronically infected MIPs have higher replicative fitness levels than the viruses infecting the mothers. In contrast, as expected, the Env from the acutely infected MIPs did not show such properties since both the mother and infant viruses had undergone recent transmission. Our results suggest that the Env protein affects viral fitness, which plays a role in perinatal transmission. Here, we show that transmitted viruses have biological properties, such as enhanced replicative fitness, that favor perinatal transmission and may provide new targets for intervention.

MATERIALS AND METHODS

Patient information and sample collection. HIV-1 from seven HIV-1-infected MIPs was studied. The mothers of five MIPs (pairs 2617, 1449, 1084, 2669, and 2873) were found to be infected at delivery, possibly by sexual transmission, and their infants were determined by PCR to be infected at either 2 months (pairs 2617, 1449, 2669, and 2873) or 4 months (1084) after birth. The infants were either infected in utero/intrapartum or through breast-feeding. These five MIPs were defined as the chronically transmitted MIPs. For the other two MIPs (pairs 834 and 2660), mothers and infants were found to have seroconverted at the same follow-up time point after birth. MIPs 834 and 2660 were found to be infected at 4 and 18 months after birth, respectively. They were defined as acutely infected MIPs, where the mothers were infected via sexual contact and the infants were infected through breast-feeding. The baseline HIV-1 serological status of the mothers was determined at delivery by two rapid assays, Capillus (Cambridge Biotech, Ireland) and Determine (Abbott laboratories). Positive serological results were confirmed by immunofluorescence assay as previously described (20). Informed consent and human subject protocols were approved by the University of Nebraska-Lincoln Institutional Review Board and the University of Zambia, School of Medicine Research Ethics Committee.

Cell and virus cultures. 293T and TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin-streptomycin. U87-CD4-CCR5 cells (kindly provided by Lee Ratner, Washington University) were propagated in DMEM supplemented with 10% FBS, 4 mM L-glutamine, 1 mM sodium pyruvate, 200 µg/ml G418, and 1

µg/ml puromycin (27). The 50% tissue culture infective dose of each recombinant virus stock was determined by infecting TZM-bl cells (39) with fourfold serial dilutions of virus as described previously (21).
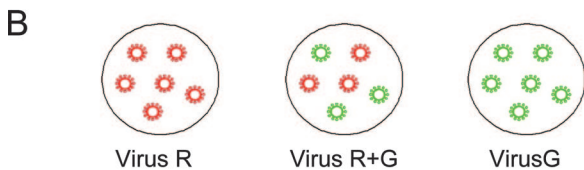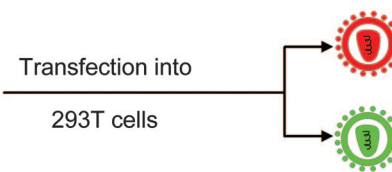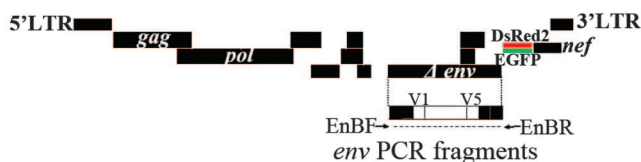
Cloning and expression of Env glycoprotein. The cloning of the V1 to V5 (V1-V5) regions of *env* derived from patients and the sequences of the Env clones used for this study were described previously (41). For the chronically infected MIPs (pairs 2617, 1449, 1084, 2669, and 2873), maternal samples collected at delivery and infant samples collected at the first postpartum HIV-1 PCR-positive time point were defined as baseline specimens. For acutely infected MIPs (pairs 834 and 2660), the baseline specimens were obtained at the time of seroconversion. For this study, the *env* V1-V5 regions were subcloned from the selected clones by amplifying the insert from the pGEM-T Env clones using the sense primer C-DraIII (5′-TGACCCCACTCTGTGTCACTTTA-3′) or C-PshAI (5′-TGGGACCAAAGTCTAAAGCCATGTGT-3′) and antisense primer C-AvrII (5′-CTATTCCTAGGGGCTTAATTTCTACCACTT-3′) containing restriction enzyme DraIII/PshAI and AvrII sites for further subcloning. PCR was carried out at 95°C for 2 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 68°C for 1 min, followed by 1 cycle at 68°C for 7 min. The amplified products were subcloned into pSP72 NLA/S/Av (envelope gene of NL4.3 modified with AgeI/SbfI/AvrII for cloning purposes) shuttle vector and pSRHS NLA/S/Av expression vector to determine if the Env sequences were functional by fusion analysis. Env was expressed as a chimeric protein with the patient's Env V1-V5 region and HIV-1 strain NL4.3. Fusion assays were carried out as described previously (8). Between four and seven representative functional and fusion-positive Env clones from each MIP baseline were available for the growth competition analysis.

Generation of recombinant HIV-1 expressing EGFP or DsRed2 fluorescent proteins. To generate enhanced green fluorescent protein (EGFP)-tagged or *Discosoma* sp. red fluorescent protein (DsRed2)-tagged recombinant HIV-1, the entire chimeric *env* was amplified from the pSP72 NLA/S/Av or pSRHS NLA/S/Av plasmid clones with sense primer EnvB-F (5′-AGAAAGAGCAGAAGA CAGTGGCAATGA-3′) and antisense primer EnvB-R (5′-TTGTACTACTTC TATAACCTTATCTGT-3′). The PCR parameters were 1 cycle at 95°C for 2 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, and 68°C for 2 min and 30 s; and 1 cycle at 68°C for 10 min. The NL4.3ΔEnvEGFP or NL4.3ΔEnvDsRed2 vectors (kindly provided by Miguel E. Quinones-Mateu, Case Western Reserve University) were linearized with XbaI (New England Biolabs) and used as the backbone for the generation of recombinant viruses with the Env fragments. The EGFP- or DsRed2-tagged recombinant viruses were generated by cotransfecting equal molar amounts of the PCR-amplified entire *env* fragment and linearized NL4.3ΔEnvEGFP or NL4.3ΔEnvDsRed2 vector into 293T cells (at a density of 2.5×10^6 cells/per 10-cm culture dish) with FuGENE 6, according to the manufacturer's protocol (Roche, Indianapolis, IN). Culture supernatants were harvested at 60 h posttransfection, filtered through 0.45-µm-pore-size sterile syringe filters (Millipore), and stored at −80°C for titration and growth competition analyses.
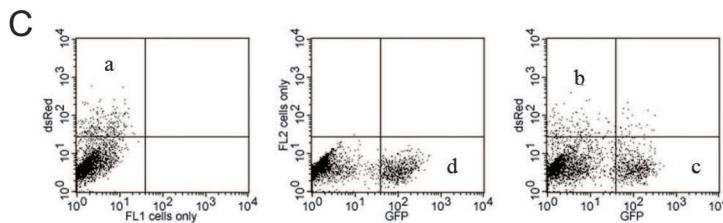
Growth competition assays for evaluation of viral fitness. Growth competition experiments involve coinfection of a cell culture by the different HIV isolates. In our assays, cells were exposed to a mixture of two viruses or to each of the two virus individually; the number of cells infected by each virus in the dual infection after several rounds of infection was compared to the number of infected cells during monoinfection by each of the two viruses (7, 25, 29, 30). Single infection and competition experiments were performed in triplicate with EGFP- or DsRed2-tagged recombinant viruses using TZM-bl or U87-CD4-CCR5 cells grown in 24-well cell culture plates. NL4.3 EGFP- or DsRed2-tagged recombinant viruses were used as positive controls. Cells were infected with two competing viruses at an equal multiplicity of infection (MOI): 0.01 for TZM-bl and 0.005 for U87-CD4-CCR5 cells. Infections were carried out with 5×10^4 cells/well in 24-well cell culture plates at 37°C and 5% CO_2 for 24 h. The cells were washed three times with phosphate-buffered saline and then cultured in complete DMEM until harvesting. For the infection of peripheral blood mononuclear cells (PBMC), the PBMC from HIV-seronegative blood donors were obtained by Ficoll-Hypaque density gradient centrifugation of heparin-treated venous blood. Prior to HIV-1 infection, the cells were stimulated with 5 µg of phytohemagglutinin (Gibco BRL) per ml for 3 days and maintained in RPMI 1640 medium supplemented with 10% FBS (HyClone) and 10 U/ml of interleukin-2 (Gibco-BRL). All mono- and dual-infection/competition experiments were performed at an MOI of 0.001 in PBMC in triplicate. Following a 6-h incubation at 37°C with 5% CO_2, cells were washed three times with 1× phosphate-buffered saline and then resuspended in complete medium (10^6/ml) on 24-well plates. At 14 days postinfection, viruses were harvested and analyzed.

Quantitation of cells infected with EGFP- or DsRed2-tagged HIV-1 by flow cytometry. Infection was monitored daily for the presence of fluorescent cells using a Eclipse TE300 inverted upright wide-field fluorescence microscope

FIG. 1. Construction of EGFP/DsRed2-tagged recombinant HIV-1 and fitness analysis. (A) Generation of HIV-1-EGFP/DsRed2-tagged recombinant viruses. PCR-amplified products of the entire *env* fragment were cotransfected with Xba1-linearized HIV-1 NL4.3Δenv EGFP/DsRed2 backbone vector into 293T cells. Viruses were harvested at 60 h after cotransfection. LTR, long terminal repeat. (B) Competition assay. Virus was added alone or in pairs to U87-CD4-CCR5 cells at an equal MOI of 0.005. Cells were harvested at day 5 postinfection and analyzed by fluorescence-activated cell sorter analysis. R, DsRed2-tagged virus; G, EGFP-tagged virus. (C) Relative fitness values calculation. Relative fitness values were calculated as shown. FL1 is the background intensity on the EGFP detector in a culture infected by a DsRed2-tagged virus (R), and FL2 is the background intensity on the DsRed2 detector in a culture infected by a EGFP-tagged virus (G). The number in the quadrant shows the percentage of green or red fluorescent cells in each single or dual infection. (D) EGFP and DsRed2 expression level in TZM-bl cells after single or dual infection with HIV-1 NL4.3 EGFP- or HIV-1 NL4.3 DsRed2-tagged recombinant viruses at an MOI of 0.01. The bars show the percentage of cells expressing EGFP or DsRed2 in each single or dual infection. (E) The relative fitness values of NL4.3 EGFP and DsRed2 viruses, respectively. (F) Analysis of dual infection/competition. HIV-1 NL4.3 DsRed2 competed against two randomly selected subtype C EGFP-tagged recombinant viruses in TZM-bl cells. The bars show the relative replicative fitness value of each virus. The scale of the *y* axis is 0.4, with higher relative fitness values above 1 and lower relative fitness values below 1.

(Nikon, Japan). Optimal signal of the cells infected with EGFP- or DsRed2-tagged HIV-1 from both single and dual infections was observed at 5 days postinfection. Cells were then harvested and analyzed by flow cytometry using a FACSCalibur instrument and Cell Quest Pro software (Becton Dickinson).

Evaluation of viral fitness. The fitness values were calculated based on the amounts of viruses produced during dual infections compared to amounts obtained from monoinfections. The amount of virus produced was determined by counting the number of cells that were infected by either the green- or red-tagged virus at 5 days postinfection using flow cytometry. In our HIV-1 competition experiments, the initial ratio of the competing viruses was 1:1. The fitness values were calculated with the formula shown in Fig. 1C. The amount of an individual HIV-1 isolate in a dual infection at the end of competition (f_0) was divided by the amount of the same virus resulting from a monoinfection assay (i_0), using the same initial inoculum as in the competition assay to derive a single-virus fitness value, W ($W = f_0/i_0$). The relative fitness value (W) for each virus was obtained from the average of the triplicate results in the competition

assay. The ratio of the relative fitness value of each HIV-1 variant in the competition is a measurement of the fitness differences (W_d) between the infant and maternal viruses ($W_d = W_i/W_m$), where W_i and W_m correspond to the relative fitness of the infant and maternal viruses, respectively (30). Any variation in quantitation efficiency of flow cytometry was compensated by dividing the level of fluorescence of the specific virus from competition by the level of fluorescence from a single infection (2).

Nucleotide sequence analysis and divergence analyses. The pSP72 NLA/S/Av or pSRHS NLA/S/Av clones containing *env* V1-V5 were confirmed by sequence analysis using primers EnF1 (5′-GATGCATGAGGATATAATCAGTTTATG GGA-3′) and EnR1 (5′-ATTGATGCTGCGCCCATAGTGCT-3′). Sequence analyses were carried out using an ABI Prism BigDye 3 terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequence data were manually edited with Sequencher, version 4.6 (Gene Codes Corporation). Neighbor-joining trees based upon Kimura two-parameter distance matrices were constructed using the MEGA3 software package (18). The most recent common ancestor

(MRCA) of the *env* sequences from the previously available sample was reconstructed using maximum likelihood in GARL1 (41, 42). Divergence was defined as the pairwise distance between the individual sequence and the MRCA from the virus population.

Statistical analyses. A Shapiro-Wilk test was used to analyze the pattern of distribution of fitness values. A relative fitness value (W) and standard deviation of relative fitness values were calculated for each MIP; the statistical significance between two different groups of MIPs was determined by the Student paired t test. P values of ≤ 0.05 were considered to be significant.

RESULTS

Testing and validation of the recombination and competition assay system. Chimeric recombinant viruses containing the Env V1-V5 region derived from the MIPs in the subtype B clone NL4.3 backbone, expressing EGFP or DsRed2 protein, were constructed and utilized in a dual infection competition assay (Fig. 1A). The relative fitness of each Env chimeric virus was determined by infecting U87-CD4-CCR5 cells with viruses tagged with either EGFP or DsRed2 (Fig. 1B). Flow cytometry was used to quantify the frequency of infected cells, and the relative fitness value was calculated based on the amount of virus produced in the single- versus dual-infection assay (Fig. 1C). To validate the dual-infection assay, we first determined whether the recombinant viruses with the fluorescent tag were replication competent using standard HIV-1 subtype B NL4.3 Env recombinant viruses carrying the EGFP or DsRed2 tag. As expected, both were replication competent and generated similar growth kinetics in TZM-bl cells, with virus production increased exponentially and reaching a maximum at day 5 postinfection (data not shown). Harvesting the cells earlier gave a much weaker signal and was not optimal for fitness determination in the competition assay. Since both recombinant viruses are identical except for the fluorescent protein gene, we tested whether they possessed equivalent replicative fitness in competitions. Indeed, they have the same relative fitness value (W of 1.0) regardless of the marker they carry (Fig. 1E). The DsRed2 label consistently gave a weaker fluorescence signal than EGFP either in the single- or dual-infection assay (Fig. 1D), but this did not adversely affect the overall replicative fitness value (Fig. 1E). The weaker fluorescent signal for DsRed2 is due to suboptimal excitation of the protein by the 488-nm laser line of the cytometer.

To further validate the assay, dual infections were carried out between NL4.3 and subtype C Env chimeric viruses from our patients. Based on published studies (4), we anticipated that subtype B NL4.3 would outcompete recombinant subtype C viruses. As expected, NL4.3 outcompeted a representative recombinant subtype C virus (Fig. 1F), irrespective of whether DsRed2 or EGFP was used to identify the subtype C virus and the NL4.3 virus in the dual infection. Hence, the replicative fitness variation between the two competing viruses is attributed to differences in Env and not due to the viral backbone or the fluorescent tags.

The infant recombinant viruses are more fit than the maternal recombinant viruses from chronically infected MIPs. We had previously characterized the *env* genes from the five infants whose mothers were chronically infected (41). The phylogenetic analyses of Env V1-V5 sequences showed that the baseline infant viruses were more homogeneous than the mothers' in five MIPs where mothers were infected with HIV-1
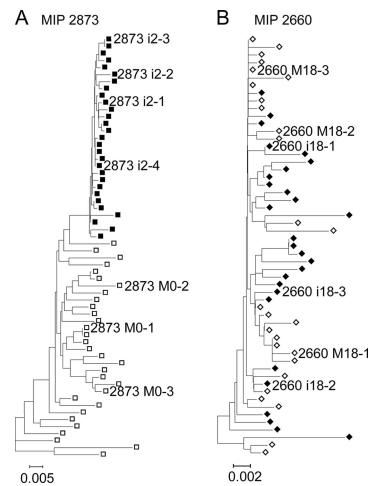

FIG. 2. Phylogenetic analysis of HIV-1 Env V1-V5 sequences obtained from the baseline time point of two representative MIPs. (A) MIP 2873 is a representative of a chronically infected MIP described previously (41). (B) MIP 2660 is a representative of an acutely infected MIP described previously (14). Samples from infants are shown in filled symbols, and samples from mothers are shown in open symbols.

chronically; a representative example of them is shown in Fig. 2A. This indicates either that selective transmission of a minor viral population occurred during perinatal transmission from each of these chronically infected mothers or that multiple HIV-1 variants may have entered the recipient but only minor variants were selectively amplified. To determine whether the replicative fitness of transmitted viruses in the infants was different from those in the maternal donor population, dual-infection competitions were performed between viruses carrying different maternal and infant viral *env* genes from the five MIPs that were previously analyzed (41). The *env* clones were selected in each MIP based on the following criteria: (i) the length of the Env V1-V5 fragment, (ii) the branch length of each Env in the phylogenetic tree, and (iii) the number of putative N-linked glycosylation sites. Four to seven *env* clones were selected from both the mother and the infant to generate recombinant viruses. To eliminate the possibility that the selected clones for the competition assay could be outliers, we then calculated the divergence for each selected clone of the MIP as the genetic distance between any sequence and the MRCA of the total previously analyzed archived virus sequences (41). The results showed that divergence from each selected *env* is within the range of the characterized population, and no outlier of divergence was used in our competition assays. As expected, the analyses of the representative MIP 2873 showed that divergence in pairwise comparison of the individual sequence and MRCA is somewhat higher in the *env* of the maternal viruses than the *env* of infant viruses, with the divergence distribution in the infant being more homogeneous (Fig. 3A and B). The divergence within each individual ranged from 0.001 to 0.043 (median, 0.007) and 0.001 to 0.008 (median, 0.002) in mother and infant, respectively. Similar results were obtained for the other four chronically infected MIPs (data not shown).

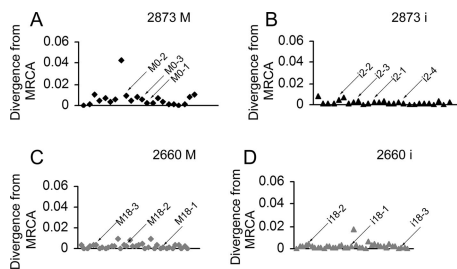The Env clones from infant 1084 were first used to generate

FIG. 3. Divergence of the selected clones from the MRCA *env* sequences from a representative chronically infected MIP (2873) and a representative acutely infected MIP (2660). Pairwise comparisons between each individual maternal (A) and infant (B) viral Env from MIP 2873 and MRCA are arbitrarily distributed along the *x* axis. Pairwise comparisons between each individual maternal (C) and infant (D) viruses from MIP 2660 and MRCA are arbitrarily distributed along the *x* axis. Samples selected for this study are indicated by the arrows.

DsRed2-tagged recombinant viruses and tested against EGFP-tagged recombinant viruses from mother 1084 (Fig. 4A). Statistical analysis showed that the fitness values were distributed normally (Shapiro-Wilk test, $P < 0.05$). Interestingly, a higher replicative fitness was consistently observed with all 1084 infant recombinant viruses than for the maternal variants (Fig. 4B and Table 1). Dual-infection assays were also carried out using viruses with the reciprocal Env and fluorescent protein combinations, and the same outcomes were observed (Fig. 4C and D). In general, viruses from infant 1084 outcompeted the maternal viruses, with fitness differences (W_d) ranging from 1.45- to 2.64-fold (median, 2.03-fold) in the dual-infection cultures (Table 1), and significant differences were evident ($P < 0.05$, Student paired *t* test) for all except one MIP (i6-3 versus M0-4; i indicates infant virus and M indicates maternal virus in all designations). Similar results were obtained with the other four chronically infected MIPs, including MIPs 1449 (W_d range, 1.31 to 2.23; median, 1.83), 2669 (W_d range, 1.27 to 2.64; median, 1.60), 2617 (W_d range, 1.13 to 3.35; median, 1.99), and 2873 (W_d range, 1.56 to 5; median, 2.52) (Fig. 5A to D). The relative fitness values (W) of infants' HIV-1 viruses were significantly higher than those of the mothers' (see Tables S1 to S4 in the supplemental material). Recombinant viruses from infants significantly outcompeted their corresponding maternal viruses, regardless of the target cell line (TZM-bl or U87-CD4-CCR5) used in 39 of the 42 pairwise competitions performed ($P < 0.05$, Student paired *t* test). To further confirm our results, pairwise HIV-1 competitions between MIP 1084 maternal and infant Env chimeric viruses were repeated in PBMC derived from an HIV-1-negative donor. Similar to the results from cell lines, higher fitness values from infant viruses were observed in the primary cells (Fig. 4E and F and Table 2). The fitness differences (W_d) ranged from 1.13 to 2.7 (median, 2.17). This result further suggests that the superior fitness observed for infant viruses is not a function of receptor overexpression on cell lines and is independent of the cell types utilized.
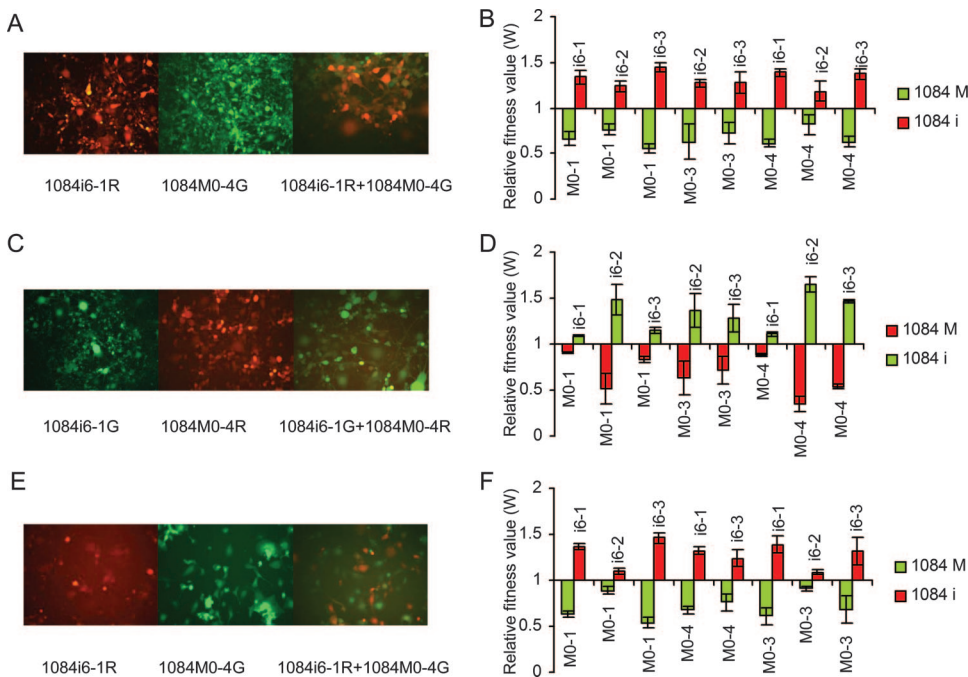
FIG. 4. The competition assays of mother and infant viruses from representative chronically infected MIP 1084 with different fluorescence tags in U87-CD4-CCR5 cells and in PBMC. (A) A representative of pairwise competition of MIP 1084 at the baseline time point in U87-CD4-CCR5 cells at an MOI of 0.005 (mother, EGFP-tagged viruses; infant, DsRed2-tagged viruses). (B) The relative fitness values of recombinant viruses obtained from chronically infected MIP 1084 in U87 cells. (C) A representative of pairwise competition of MIP 1084 viruses using reciprocal fluorescence tags at a baseline time point in U87-CD4-CCR5 cells at an MOI of 0.005 (mother, DsRed2-tagged viruses; infant, EGFP-tagged viruses). (D) The relative fitness values of recombinant viruses obtained from chronically infected MIP 1084 in reciprocal fluorescence tags in U87 cells. (E) A representative of pairwise competition of MIP 1084 at the baseline time point in PBMC at an MOI of 0.001. (F) The relative fitness values of recombinant viruses obtained from chronically infected MIP 1084 in PBMC.

TABLE 1. Relative fitness value from pairwise competition of viruses from MIP 1084 in U87-CD4-CCR5 cells[a]

Pair no. and virus variant	W	SD	W_d	P
Pair 1				
M0-1	0.7	0.1	2.03	0.01
i6-1	1.3	0.1		
Pair 2				
M0-1	0.8	0.1	1.62	0.002
i6-2	1.2	0.1		
Pair 3				
M0-1	0.6	0.1	2.64	0.002
i6-3	1.5	0.1		
Pair 4				
M0-3	0.6	0	2.02	0.03
i6-2	1.3	0.2		
Pair 5				
M0-3	0.7	0.1	1.78	0.002
i6-3	1.3	0.1		
Pair 6				
M0-4	0.6	0	2.28	0.05
i6-1	1.4	0		
Pair 7				
M0-4	0.8	0.1	1.45	0.004
i6-2	1.2	0.1		
Pair 8				
M0-4	0.6	0.1	2.17	0.06
i6-3	1.4	0.1		

[a] The W values and standard deviations (SD) were derived from triplicate competition assays as shown in Fig. 4B. W_d indicates the fitness difference between infant and maternal viruses as calculated by the relative fitness value of infant divided by the relative fitness value of mother. A W_d of >1 indicates that infant virus is more fit than maternal virus; a W_d of <1 indicates infant virus is less fit than maternal virus. A two-tailed Student paired t test was used to determine the statistical difference between mother and infant values. A P value of ≤0.05 was considered to be statistically significant.

Higher fitness is an intrinsic property of all newly acquired infant Env glycoproteins. Since all the infant Env variant viruses outcompeted the representatives derived from the corresponding maternal quasispecies, it was of interest to determine if higher fitness is an intrinsic property of all newly

TABLE 2. Relative fitness value (W) from pairwise competition of viruses from MIP1084 in PBMC

Pair no. and virus variant	W	SD	W_d	P
Pair 1				
M0-1	0.6	0	2.17	0.002
i6-1	1.4	0		
Pair 2				
M0-1	0.9	0	1.24	0.04
i6-2	1.1	0		
Pair 3				
M0-1	0.5	0.1	2.7	0.005
i6-3	1.5	0.1		
Pair 4				
M0-3	0.9	0	1.13	0.03
i6-2	1.1	0		
Pair 5				
M0-3	0.7	0.2	1.94	0.07
i6-3	1.3	0.2		
Pair 6				
M0-4	0.6	0.1	2.3	0.02
i6-1	1.4	0.1		
Pair 7				
M0-4	0.7	0	1.94	0.006
i6-2	1.3	0		
Pair 8				
M0-4	0.8	0.1	2.7	0.04
i6-3	1.2	0.1		

[a] The W values and standard deviations (SD) were derived from triplicate competition assays as shown in Fig. 4F. For an explanation of the other values, see the footnote to Table 1.

acquired infant Env glycoproteins. To investigate this possibility, representative viruses from each infant were tested against representative viruses from mothers with whom they shared no linkage via transmission. We found that not all infant viruses outcompeted all maternal viruses (Table 3). The virus 1084i6-2 from infant 1084 outcompeted the maternal virus 1084M0-4, with the highest fitness difference (W_d) of 4.26; lower fitness differences were obtained with 2669M0-2 (W_d of 2) and
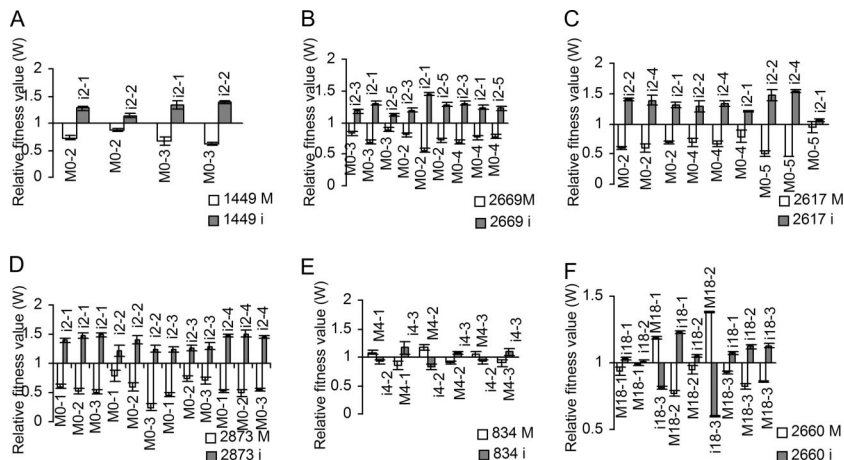
FIG. 5. The competition assays of mother and infant viruses from chronically and acutely infected MIPs. (A to D) The relative fitness values of recombinant viruses obtained from chronically infected MIPs 1449, 2669, 2617, and 2873 at the baseline time point in U87-CD4-CCR5 cells at an MOI of 0.005. (E and F) The relative fitness values of recombinant viruses obtained from acutely infected MIPs 834 and 2660. The gray bars represent infant viruses; the white bars represent maternal viruses from each pair. All competition experiments were performed in triplicate, and the error bars indicate standard deviation (SD) from triplicate results.

TABLE 3. Fitness value differences from cross-competitions between nonidentical MIPs

Maternal virus	W_d of the indicated infant virus relative to the corresponding maternal virus[a]				
	1084 i6-2	2617 i2-1	2669 i2-1	1449 i2-2	2873 i2-4
2873 M0-1	0.92	1.17	0.57	0.96	2.8
1084 M0-4	4.26	0.4	1.06	0.73	1.24
2617 M0-2	0.43	1.9	0.75	0.67	0.65
1449 M0-3	1	0.71	1.14	2.23	0.9
2669 M0-2	2	0.9	3	0.87	1.17

[a] See Materials and Methods for the calculation of W_d. A W_d of >1 indicates that the virus from infant was more fit than the virus from mother; a W_d of <1 indicates that the virus from the infant was less fit than the virus from the mother.

1449M0-3 (W_d of 1). Interestingly, 1084 i6-2 was outcompeted by nonidentical maternal viruses 2617M0-2 and 2873M0-1. Similar results were obtained with other infant viruses. These results suggest that the fitness advantage is lineage specific and can be observed only within the same MIP; the transmitted virus in the infants is derived from the maternal virus population but has been selected for consistently higher fitness. This trait does not imply that the transmitted viruses are universally more fit but likely reflects the overall fitness of the viral lineage in the transmission chain.

The replicative fitness values of the infant recombinant viruses are similar to their mothers' viruses in each acutely infected MIP. We have recently analyzed the *env* diversity of three acutely infected MIPs whose mothers have transmitted HIV-1 to the infants shortly after being infected themselves. We found that the viral Env protein sequences from the mothers' and infants' viruses did not phylogenetically segregate, and the infants did not possess a less diverse quasispecies (Fig. 2B) (14). These analyses suggest that no further restriction of the viral quasispecies occurred during transmission. This is possibly due to the absence of preexisting immunity and high viral loads present during acute infection of the mother. Alternatively, the virus recently transmitted to the mother had already undergone selection and was therefore more readily transmissible to a child undergoing exposure. A corollary to this premise is that one would not expect to detect differences in viral fitness, on average, between maternal and infant Env variants derived from such an acutely infected pair. To test this supposition, several previously characterized *env* clones were selected from the acutely infected MIPs to study their fitness. These selected clones were first analyzed for divergence as described earlier, and the results for MIP 2660 are shown in Fig. 3C and D. For the acutely infected MIP 2660, the divergences between maternal and infant *env* are similar (median, 0.002) (Fig. 3C and D). Although there is a slight difference in the two subsets, no above-average divergence value was observed. This confirmed that the selected clones were representative of the virus population. Similar results were obtained for MIP 834 (data not shown). Pairwise competitions of recombinant viruses obtained from selected clones from two acutely infected MIPs, 834 and 2660, were then performed. The fitness of the viruses conveying the acutely infected infant V1-V5 sequences demonstrated variable fitness in competitions with maternal variants; in some cases the infant was the winner, and in others it was the loser. For example with MIP 834, infant

TABLE 4. Relative fitness value from pairwise competition of viruses from acutely infected MIP 834 in U87-CD4-CCR5 cells[a]

Pair no. and virus variant	W	SD	W_d	P
Pair 1				
M4-1	1.08	0.04	0.85	0.12
i4-2	0.92	0.04		
Pair 2				
M4-1	0.85	0.07	1.38	0.13
i4-3	1.17	0.11		
Pair 3				
M4-2	1.17	0.06	0.71	0.06
i4-2	0.83	0.06		
Pair 4				
M4-2	0.91	0.02	1.18	0.04
i4-3	1.08	0.02		
Pair 5				
M4-3	1.05	0.04	0.87	0.33
i4-2	0.92	0.04		
Pair 6				
M4-3	0.9	0.06	1.2	0.17
i4-3	1.09	0.06		

[a] The W values and standard deviations (SD) were derived from triplicate competition assays as shown in Fig. 5E. For an explanation of the other values, see the footnote to Table 1.

virus i4-3 was more fit than any maternal variant, whereas i4-2 virus was less fit (Fig. 5E and Table 4). There was no significant difference between infant and maternal viruses ($P > 0.05$, Student paired t test) for all except one MIP (i4-3 versus M4-2). Similar results were obtained with MIP 2660 (Fig. 5F; see also Table S5 in the supplemental material). The fitness differences (W_d) between the viruses from infant and mother were not always >1; some W_d values were <1. Moreover, the results were not cell type dependent since using PBMC as target cells for infection yielded similar results (Table 5). These

TABLE 5. Relative fitness values from pairwise competition of viruses from acutely infected MIP 834 in PBMC[a]

Pair no. and virus variant	W	SD	W_d	P
Pair 1				
M4-1	1.08	0.06	0.85	0.14
i4-2	0.92	0.06		
Pair 2				
M4-1	0.75	0.07	1.67	0.02
i4-3	1.25	0.07		
Pair 3				
M4-2	1.07	0.02	0.87	0.02
i4-2	0.93	0.02		
Pair 4				
M4-2	0.93	0.06	1.15	0.16
i4-3	1.07	0.06		
Pair 5				
M4-3	1.29	0.11	0.54	0.05
i4-2	0.7	0.11		
Pair 6				
M4-3	0.9	0.06	1.22	0.1
i4-3	1.1	0.06		

[a] The W values and standard deviations (SD) were derived from triplicate competition assays. For an explanation of the other values, see the footnote to Table 1.

findings suggest that multiple Env variants are transmitted to the child perinatally if the mother is acutely infected.

DISCUSSION

In this study we have characterized the replicative fitness of perinatally transmitted viruses from chronically infected mothers and compared this with MIPs where perinatal transmission occurred when the mothers were acutely infected. Our results demonstrated that recombinant viruses containing Env V1-V5 regions derived from the infant viruses possessed a growth advantage over those derived from their chronically infected mothers. This advantage was seen in all five MIPs where chronically infected mothers transmitted their viruses to their infants but not from two MIPs evincing acute infection. Interestingly, these differences in the transmission phenotypes directly correlate with the viral genetic diversity near the time of transmission. The transmitted viruses from the chronically infected MIPs are more homogeneous due to a bottleneck transmission of a subset of the maternal quasispecies, similar to what other studies observed during perinatal and adult transmissions (12, 37). In contrast, the transmitted viruses from the acutely infected mothers are similar to the maternal quasispecies and did not result in a reduction in viral diversity during transmission.

There are a number of possible explanations to account for our observation that a minor viral population within the chronically infected mothers is transmitted. One is that only a minor population of the transmitted virus is infectious or replication competent in the new host (23, 34, 36, 40). A second is that the initial target cells restrict the replication of most transmitted viral genotypes. It has been postulated that macrophages, present in the mucosa and placental tissues, may be the first target cells that HIV-1 encounters and may select for a viral population that can preferentially infect these cells (6). The selective transmission could also be an outcome of immune selection by maternal antibodies. These antibodies could include neutralizing antibodies, which may play a role in limiting the transmission of neutralization-sensitive variants. Other studies using a macaque perinatal transmission model found that the infant viruses represent minor variants in their mothers and are more neutralization resistant (15, 16). Therefore, it is difficult to determine whether the transmission of more fit viruses to the infants is due to selective transmission or selective amplification in the recipient. In the five MIPs where mothers were chronically infected with HIV-1, most of the perinatally transmitted infants' specimens were obtained before 2 months of age except 1084i, which was obtained at 6 months. It is unlikely that the infants' immune systems mediate the selection since they are not yet fully developed. However, the maternal passive antibody that has neutralizing activity might play a role in selective amplification, in which minor variants were selectively amplified even though multiple HIV-1 variants might be perinatally transmitted, but not all studies are in agreement on the role of neutralizing antibodies in HIV-1 perinatal transmission. Maternal antibodies are known to cross the placenta beginning at 18 weeks of gestation to peak at delivery; antibodies persist in the infant for up to 18 month of age and may thus act to prevent HIV-1 infection in the infant both in utero and postpartum (9). In addition, during intrapartum exposure, major variants would be neutralized by passively transferred autologous neutralizing antibodies. However, minor variants which are resistant would be transmitted in the absence of broadly neutralizing antibodies, as suggested by Barin et al. (5). This possibility supports our data that only a subset of viruses with higher fitness, more adapted for transmission, was selected during perinatal transmission from the chronically infected mothers. This higher fitness may enable the transmitted virus to establish initial sites of replication (a beachhead) or to produce more progeny rapidly to adapt to growth in vivo and withstand the selection pressures imposed by perinatal transmission. Whether this subset of the viruses that we analyzed here is more resistant to neutralization needs to be determined. In contrast, in acutely infected mothers whose viruses had recently been selected during transmission, the viruses required no further selection during perinatal transmission, and viruses from both the mothers and infants had equal fitness, as we have observed. However, for the infants born to the chronically infected mothers analyzed, we do not know whether they were infected in utero, intrapartum, or postpartum via breast milk; further studies will be needed to determine whether selective transmission from chronically infected mothers is dependent on the route of transmission.

Although the growth competition assay used in our study uses a well-established methodology (38), recombination between the two competing viruses remained a concern. To address this concern, viral stocks were serially diluted to the minimal MOI to reduce the possibility of recombination between the two competing viruses. If recombination had been a problem, we would not have observed the consistently higher fitness of infant viruses. Another constraint is the small number of clones that were analyzed from each patient. This is due to the limited number of archived clones analyzed previously that were available for this study (41), and the clones were derived from PBMC rather than from plasma. Unfortunately, patient specimens such as plasma and PBMC DNA are no longer available to generate additional clones for further analyses, but quantitative analysis of Env V1-V5 divergence demonstrated that our available clones were representative of the virus population in our study. An additional constraint is that the clones were generated previously from the pooling of several bulk PCRs rather than from the single-genome PCR that has been used recently (32), and there is a possibility that the clones were generated from template resampling. However, our previous studies showed that the number of unique sequences is large and is similar at each time point (14, 41), suggesting that pooling individual PCR products is adequate to prevent virus template resampling in our study. If resampling were a problem, we would not have observed differences between the chronic and acute MIPs.

Selection of a minor viral genotype during perinatal transmission has been reported previously (3, 40); however, no report has correlated the restricted transmission of viral *env* genotypes to their biological properties. Our competition studies, which employed maternal and infant recombinant viruses that are identical except for the *env* V1-V5 region, suggest that this region of the HIV-1 *env* conferred the differences in fitness between the mother and infant viruses. However, we could not exclude the possibility that other viral genes such as *gag* and *pol* may also play a role in viral fitness. We also could not exclude

the possibility that the chimeric Env in the NL4.3 backbone may confer differences in fitness compared to the native non-chimeric structure. However, this effect should be similar for all recombinant viruses generated from both chronic and acute MIPs. The notion that differences were observed between the chronic and acute MIPs suggests that higher replicative fitness was determined by the Env V1-V5. Since Env is involved in the viral entry process, differences in the fitness profiles of mother and infant viruses indicate that a majority of the maternal viruses may be less efficient in entry, either in binding or fusion kinetics or both, and confer a fitness disadvantage compared to a minor population that was transmitted perinatally. At this point it is not clear whether superior fitness is due to more efficient viral entry by the transmitted viruses or whether postentry events may also have an effect on fitness.

Our study provides the first clear evidence of the higher fitness of viruses from infants conveyed by *env* during perinatal transmission. High fitness of the virus upon transmission to a new host, in combination with a bottleneck during perinatal transmission, suggests that during the course of disease progression in the chronically infected mothers, the majority of the maternal viruses may have evolved or been selected for growth and persistence in the host but that only a minor population has retained its fitness for perinatal transmission. It is possible that this selection occurs in the female reproductive tract or breast. Whether such a selection occurs during sexual or blood-borne transmission needs to be determined. This finding may provide important information for the development of a vaccine to prevent perinatal transmission.

ACKNOWLEDGMENTS

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