HIV-1 Tropism and Survival in Vertically Infected Ugandan Infants

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Background. Human immunodeficiency virus type 1 (HIV-1) may utilize the CXCR4 coreceptor (X4 virus), the CCR5 coreceptor (R5 virus), or both (dual/mixed [DM] virus). We analyzed HIV-1 coreceptor tropism in Ugandan infants enrolled in the HIVNET (HIV Network for Prevention Trials) 012 trial.

Methods. Plasma or serum was analyzed using a commercial coreceptor tropism assay. HIV env subtype was determined by phylogenetic methods.

Results. Tropism results were obtained for 57 samples from infants collected 6–14 weeks after birth. Fifty-two infants had only R5 virus, and 5 had either X4 or DM virus. The mothers of those 5 infants also had X4 or DM virus. In infants, subtype D infection was associated with high-level infectivity in CCR5-bearing cells and also with the detection of X4 or DM strains. High-level infectivity in CCR5-bearing cells was associated with decreased infant survival, but infection with X4 or DM virus was not. HIV clones from infants with DM viral populations showed different patterns of coreceptor use. V3 loop sequence–based algorithms predicted the tropism of some, but not all, env clones.

Conclusions. Complex patterns of HIV tropism were found in HIV-infected newborn infants. Subtype D infection was associated with X4 virus and with high-level replication in CCR5-bearing cells. High-level replication of R5 virus was associated with decreased infant survival.

HIV interacts with CD4 and a coreceptor for cell entry. Early studies of HIV tropism found that the virus could be either syncitia inducing (SI) or non-syncitia inducing (NSI), depending on the type of target cells infected and the tropism of the virus. The majority of variants classified as SI were subsequently found to use the CXCR4 coreceptor for cell entry (X4 virus). In contrast, most NSI variants were found to use the CCR5 coreceptor (R5 virus; reviewed in [1]).

Most cases of pediatric HIV infection result from mother-to-child transmission. Most infants are infected with R5 virus or with virus presumed to be R5 on the basis of the results of older assays (e.g., NSI or macrophage tropic) [2–5]. Small numbers of infants harboring HIV strains that are X4 virus (or virus presumed to be X4 on the basis of the results of older assays) have been described in a few reports [6–8]. In one study, X4 virus was detected in only 1 of 14 infants, who was also noted to have a defect in the CCR5 coreceptor (homozygous for the Δ32 CCR5 allele [8]). It is unclear whether the

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presence of X4 virus in infants is associated with more rapid disease progression and death [9, 10].

Recent studies have reported differences in coreceptor use among HIV subtypes [11–13]. A higher portion of X4 viruses have been found in adults with subtype D infection compared with infection with other subtypes [13–15]; this trend has also been observed in pregnant women [13]. Detection of subtype D HIV is associated with faster disease progression, possibly because of the presence of X4 viruses [14–16]. It is not known whether infants with subtype D HIV infection are more likely to harbor X4 strains or to progress more rapidly to AIDS or death.

To date, many studies of coreceptor use in HIV-infected infants have focused on the analysis of tropism in viral populations. Using this approach, it is not possible to determine whether viral populations that are capable of infecting CXCR4-bearing and CCR5-bearing cells (dual/mixed [DM] viruses) contain mixed populations of pure X4-using and R5-using strains or contain more complex mixtures of viruses, including those that can use both coreceptors (R5X4 viruses). A recent study found that some pregnant Ugandan women with subtype D HIV infection harbor heterogeneous populations composed of X4, R5, and R5X4 variants [13].

In the present report, we extend previous studies of HIV tropism in infants by analyzing HIV in 57 Ugandan infants, including those with subtype D infection. We examined the relationship between HIV tropism and other clinical and laboratory variables, including infant survival. In addition, we analyzed the relationship between HIV tropism and the sequence of the third variable (V3) region of env in individual HIV clones from selected infant samples.

**METHODS**

**Study cohort.** Samples were obtained from Ugandan women and infants enrolled in the HIVNET (HIV Network for Prevention Trials) 012 trial, which compared 2 antiretroviral regimens for prevention of HIV mother-to-child transmission [17, 18]. In the nevirapine (NVP) arm of the trial, women received a single dose of NVP during labor, and their infants received a single dose of NVP within 72 h of birth. In the zidovudine (ZDV) arm, women received ZDV during labor, and their infants received ZDV twice daily for 7 days. Fifty-nine infants in the ZDV arm and 36 infants in the NVP arm were found to be HIV infected by 6–8 weeks of age (total, 95 infants). The present report includes analysis of 57 infants (37 from the ZDV arm and 20 from the NVP arm). Samples from 5 women whose infants had X4 or R5X4 virus were also analyzed.

**Analysis of HIV tropism.** Coreceptor tropism was analyzed using the Trofile assay (Monogram Biosciences). In the Trofile assay, env region cDNA from HIV in the test sample is cloned into a vector. The resulting env vector and a recombinant HIV vector containing the luciferase gene (∆env) are cotransfected into HEK293 cells to produce pseudovirus. The pseudovirus is cultured in U87 cells expressing either CXCR4 or CCR5, and the level of luciferase production (expressed as relative light units [RLU]) is used to determine viral tropism. Coreceptor tropism determination is based on the detection of luciferase above the background level, and a significant reduction in RLU in the presence of a high concentration of a CXCR4 or CCR5 antagonist [19]. env clones were isolated from 5 infants with X4 or DM HIV coreceptor tropism, and V3 amino acid sequences of these clones were analyzed.

**Sequencing of env and subtype determination.** Methods used for HIV env sequencing from viral populations have been described elsewhere [13]. HIV subtypes were determined by phylogenetic analysis of sequences based on the constant region 5 (C5) of gp120 and the entire gp41 region of env, using reference sequences recommended for subtyping by the Los Alamos National Laboratory. Phylogenies were constructed using a 1000-replicate bootstrap-consensus neighbor-joining method, as implemented in MEGA [20].

**Analysis of V3 sequences.** HIV tropism was predicted using 2 sequence-based algorithms, the 11/25 rule and the position-specific scoring matrix (PSSM). The 11/25 rule predicts HIV tropism based on the presence of positively charged amino acids at positions 11 and 25 in the V3 loop [21, 22]. In the PSSM, V3 sequences of viral variants with different tropism are compared. A particular sequence is assigned a score that indicates the probability that the virus is X4 or R5 virus [23, 24].

**Statistical methods.** We used the χ² and Fisher’s exact tests to compare categorical variables. For comparing the distribution of continuous variables, we calculated 2-sample Wilcoxon rank tests and exact Wilcoxon rank tests. Exact tests were used when the sample size was sufficiently small for asymptomatic approximations to be questionable. Cox proportional hazards regression was used to compare infant survival rates.

**GenBank accession numbers.** The GenBank accession numbers for the env sequences from the 5 infants with X4 or R5X4 virus are EU281995-EU281999.

**RESULTS**

**Analysis of HIV tropism.** Plasma or serum samples collected at 6–14 weeks of age were available for 75 (78.9%) of the 95 HIV-infected infants in HIVNET 012 (46 infants in the ZDV arm and 29 infants in the NVP arm; see Methods). Analysis of HIV tropism by use of a commercial assay (Trofile) was successful for 57 (76.0%) of 75 infant samples. Results for the remaining samples were most likely not obtained because of the low volume of plasma available for testing (100–200 μL). The median baseline maternal CD4 cell count was higher for the 57 infants with results than for the 38 infants without results (374 vs. 246 cells/μL; P = .0071). However, baseline maternal viral load was similar between these 2 groups (4.7 vs. 4.8 log₁₀ HIV RNA copies/
Infants who did not have Trofile assay results (18/36 [50.0%] vs. 10/20 [50.0%]; \(P = 1.00\), \(\chi^2\) test).

Median maternal baseline CD4 cell count and median baseline maternal HIV load were similar among infants with high and those with low R5-RLU (for CD4 cell count, 348 vs. 382 cells/µL [\(P = .46\]); for HIV load, 4.8 vs. 4.9 log10 copies/mL [\(P = .61\)]. We also compared R5-RLU among the 32 infants who tested positive for HIV infection at birth versus the 24 infants who tested negative for HIV infection at birth but were diagnosed with HIV infection at 6 weeks of age. The proportion of infants with high R5-RLU was the same for these 2 groups (16/32 [50.0%] vs. 12/24 [50.0%]; \(P = 1.00\)). Data on HIV load from 6–8 weeks of age were available for only 27 of the 57 infants. In this small sample set, we did not detect a significant difference in HIV load between infants with high and those with low R5-RLU (median, 5.8 vs. 5.5 log10 copies/mL; \(P = .82\)). We did find a statistically significant association between HIV env subtype (C5-gp41 region) and R5-RLU in infants. A high R5-RLU was detected in 15 (78.9%) of 19 infants with subtype D infection, compared with only 13 (35.1%) of 37 infants infected with other subtypes (including 31 with A, 2 with C, and 4 intersubtype recombinant strains; \(P = .002\)).

Analysis of HIV infection of CCR5-bearing cells. HIV from all but 1 of the 57 infants was able to infect CCR5-bearing cells. The level of infectivity for HIV from these 56 infants ranged from 967 to 1,556,740 RLU (median, 159,291 RLU). We examined the relationship between the efficiency of infection in CCR5-bearing cells (R5-RLU) and other factors. For this analysis, infants were divided into 2 groups: high R5-RLU (R5-RLU greater than or equal to the median of 159,291) and low R5-RLU (R5-RLU less than the median of 159,291). The one infant who had R5-RLU below the limit of detection of the assay was excluded from the analysis of HIV infectivity in CCR5-bearing cells. The proportion of infants with high R5-RLU was similar among infants in the NVP and ZDV arms of the HIVNET 012 trial (18/36 [50.0%] vs. 10/20 [50.0%]; \(P = 1.00\), \(\chi^2\) test).

Table 1. Association of infectivity in CCR5-bearing cells and other factors with infant survival (Cox proportional hazards model).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate model</th>
<th>Multivariate model</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;br&gt;Infected in CCR5-bearing cells, high vs. low (n = 56)</td>
<td>3.28 (1.55–6.90)</td>
<td>.0018</td>
</tr>
<tr>
<td>Maternal baseline HIV RNA level, per increase of 1 log10 copies/mL (n = 56)</td>
<td>1.97 (1.07–3.65)</td>
<td>.03</td>
</tr>
<tr>
<td>Maternal baseline CD4 cell count, per decrease of 100 cells/µL (n = 56)</td>
<td>1.17 (0.99–1.38)</td>
<td>.06</td>
</tr>
<tr>
<td>HIV positive at birth vs. not (n = 56)</td>
<td>1.07 (0.54–2.11)</td>
<td>.86</td>
</tr>
<tr>
<td>Subtype D vs. non-D (n = 56)</td>
<td>1.55 (0.78–3.07)</td>
<td>.21</td>
</tr>
<tr>
<td>Subtype D vs. A (n = 50)</td>
<td>1.28 (0.89–1.83)</td>
<td>.18</td>
</tr>
</tbody>
</table>

**NOTE.** CI, confidence interval; HR, hazard ratio. Boldface indicates statistical significance.

[58x426]n[58x426]38 infants who did not have Trofile assay results (P arm of the HIVNET 012 trial, compared with 14 (36.8%) of the infants with Trofile assay results were randomized to the NVP

Figure 1. Analysis of infant survival. Shown is a comparison of survival among infants with high vs. low HIV infectivity in CCR5-bearing cells.

Table 1. Association of infectivity in CCR5-bearing cells and other factors with infant survival (Cox proportional hazards model).
Analysis of the relationship between infectivity in CCR5-bearing cells and infant survival. Infants in HIVNET 012 were followed up until 5 years of age. We examined the relationship between the level of infectivity in CCR5-bearing cells (R5-RLU) and infant survival. High R5-RLU was associated with decreased infant survival (figure 1). Twenty-four (85.7%) of 28 of infants with high R5-RLU died during the study, compared with only 10 (35.7%) of 28 of infants with low R5-RLU (P = .001). In univariate analyses, both high R5-RLU and high baseline maternal HIV load were associated with decreased infant survival, but maternal baseline CD4 cell count, infant HIV status at birth, and HIV subtype were not (table 1). High R5-RLU was still associated with decreased infant survival in a Cox proportional hazards model adjusting for maternal HIV load (table 1).

Analysis of infants with X4 and DM HIV. As described above, we identified 5 infants who had evidence of CXC4-using virus at 6–14 weeks of age (4 with DM virus and 1 with X4 virus) (table 2). We performed a more detailed analysis of HIV tropism for those infants and their mothers. Four of the 5 infants had evidence of HIV infection at the time of birth. For all 4 of these infants, DM virus was present in the birth sample (table 2). At the time of delivery, 3 of the mothers had DM HIV, 1 had X4 HIV, and 1 had predominantly R5 virus, with a minor population of CXC4-using variants (RLU in CXC4-bearing cells was below the level of detection of the Trofile assay for this woman; X4 variants were detected in env clones from her sample). Samples collected at 12–18 months of age were available for 2 of the 5 infants with DM HIV (table 2). In one of the infants, DM HIV was present in the follow-up sample. In the other infant, only R5 virus was detected.

In this small sample set, we did not find a significant difference between either baseline maternal HIV load or maternal baseline CD4 cell count among the 5 infants with X4 or DM virus versus the 52 infants who had only R5 virus (for viral load, P = .35; for CD4 cell count, P = .67). Two (10.0%) of 20 infants in the NVP arm of the HIVNET 012 trial had X4 or DM virus, compared with 3 (8.1%) of 37 infants in the ZDV arm (P = 1.00, Fisher’s exact test). However, our data suggest an association between HIV subtype (C5-gp41 region) and X4 virus. Four (20.0%) of the 20 infants with subtype D infection had X4 or DM HIV, compared with only 1 (2.7%) of 37 infants infected with other subtypes (subtype A, C, or recombinant HIV) (P = .047). X4 or DM virus was also more common among infants with subtype D infection (4/20 [20.0%]) than among infants with subtype A infection (1/31 [3.2%]) (P = .07). Among the 50 women in this study with subtype A and D who had CD4 cell count results (30 with A and 20 with D), the median value was 354 cells/μL for subtype A and 283 cells/μL for subtype D (P = .57, Wilcoxon 2-sample test).

The median survival of the 52 infants who had R5 HIV at 6–14 weeks of age was 23.6 months (table 2). This was not significantly different from the median survival of the 5 infants with CXCR4-using virus (24.3 months) (P = .293). However, the 2 infants with a higher level of infectivity in CXCR4-bearing cells (65,411 and 481,819 RLU) died earlier (at 10.5 and 14.5 months of age) than the 3 infants with low-level infectivity in CXCR4-bearing cells (200, 225, and 358 RLU; survival to 4.7, 2.0, and 2.5 years, respectively) (table 2).

Table 3. Tropism of env clones isolated from infants with CXCR4-using viruses.

<table>
<thead>
<tr>
<th>Infant</th>
<th>Subtype</th>
<th>Age, weeks</th>
<th>Tropism</th>
<th>Clones, no.</th>
<th>Clones, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>185</td>
<td>A</td>
<td>14</td>
<td>DM</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>197</td>
<td>D</td>
<td>6–8</td>
<td>X4</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>223</td>
<td>D</td>
<td>6–8</td>
<td>DM</td>
<td>&gt;99</td>
<td>&lt;1</td>
</tr>
<tr>
<td>632</td>
<td>D</td>
<td>6–8</td>
<td>DM</td>
<td>62</td>
<td>47</td>
</tr>
<tr>
<td>827</td>
<td>D</td>
<td>6–8</td>
<td>DM</td>
<td>70</td>
<td>50</td>
</tr>
</tbody>
</table>

a Coreceptor tropism of viral populations and clones is as follows: X4, clone or viral population able to replicate in CCR5-bearing cells only; R5, clone or viral population able to replicate in CXCR4-bearing cells only; dual/mixed (DM), clone or viral population able to replicate in both CXCR4-bearing and CCR5-bearing cells; R5X4, clone able to replicate in both CXCR4-bearing and CCR5-bearing cells; dual/mixed (DM), viral population able to replicate in CXCR4-bearing and CCR5-bearing cells.
Analysis of env clones from infants with DM HIV. HIV populations from the 5 infants with X4 or DM HIV were further characterized by evaluating the HIV tropism of individual HIV env clones (24–140 clones per sample) (table 3). In one sample, all clones were R5X4 (clones from infant 185); in another sample, all clones were X4 (clones from infant 197). Analysis of clones from the other 3 infants revealed mixed viral populations. One infant had a mixture of R5 and X4 clones (clones from infant 223), and the other 2 infants had mixtures of R5 and R5X4 clones (clones from infants 632 and 827) (table 3).

Analysis of V3 loop sequences of clones from infants with DM HIV. Important determinants of HIV tropism reside within the V3 region of the HIV env gene. We analyzed the V3 loop sequences of selected clones isolated from the infants with X4 or DM HIV (12–15 clones per infant) (table 4). Each infant had between 1 and 4 distinct V3 loop sequences among the clones analyzed. Tropism predictions for both the 11/25 rule and the PSSM algorithms were the same except for 2 V3 sequences from infant 827 (table 4). Both algorithms predicted X4-using viruses for 4 sequences; X4-using viruses were confirmed by the Trofile assay (detection of X4 or R5X4 HIV). The 11/25 rule predicted R5 usage for the remaining 9 sequences; 6 of these predictions were consistent with results from the Trofile assay. However, clones with the other 3 sequences were R5X4 by the Trofile assay. Two of these 3 discordant sequences were correctly predicted by the PSSM algorithm.
predicted to be X4 by the PSSM. The remaining sequence, which the PSSM predicted to have R5 virus, had R5X4 virus when the clone was analyzed by the Trofile assay (infant 632).

The luciferase activity of the clones described above is shown in figure 2. X4 and R5X4 clones with different levels of luciferase activity in infected CXCR4 target cells were observed in these infants. Note that in some cases clones had different tropism patterns, even though their V3 loop sequences were identical. For example, all 12 clones from infant 632 had the same V3 loop sequence; 3 of those clones were R5 using, and 9 were R5X4.

**DISCUSSION**

In the present study, 5 of 57 Ugandan infants had either X4 or DM HIV at 6–14 weeks of age, 4 of whom were diagnosed with HIV infection at birth. We cannot determine whether HIV infection was initiated in those infants with X4 or DM HIV or whether an R5-using strain evolved in utero or shortly after birth to produce a DM viral population.

Survival of infants with X4 or DM HIV was not significantly different from that of infants who had only R5 HIV in this study. However, because of the small number of infants with CXCR4-using virus in this study, this finding is not conclusive. Among the 56 infants with R5 virus, high-level infectivity in CCR5-bearing cells (R5-RLU) and high baseline maternal viral load each independently predicted reduced infant survival. In the Trofile assay, the envelope protein is the only portion of the pseudovirus that is derived from the patient (test sample). Therefore, these results indicate that env gene determinants influence the survival of HIV-infected infants. Other studies have shown that mortality in HIV-infected infants is associated with high baseline maternal viral load, high infant viral load (birth and peak), time of infection, and CD4 cell percentage [26–29]. We did not see an association between infant survival and either infant viral load or time of infection in the univariate models. This may reflect the use of ZDV or NVP for prevention of mother-to-child transmission of HIV in this cohort and/or the small number of infants available for analysis.

HIV-infected children can progress to AIDS without evidence of X4 virus, and some young children with SI isolates can remain clinically stable for a year or more [9]. In one study, enhanced replication of NSI HIV in monocyte-derived macrophages was associated with more advanced HIV disease in a cohort that included infants and children [30]. Follow-up data from 12–18 months was available for only 2 of the 4 infants in this study who survived beyond 1 year. Both infants had DM virus at 6 weeks, and one still had DM virus at 12–18 months; the other had R5 virus only at 12–18 months. Evolution from SI strains to NSI strains has been observed previously in young children, and the reverse switch (evolution from NSI strains to SI strains) has also been observed [9]. Evolution from R5 to X4 HIV has been observed in children months or years after a decline in CD4 cell count, suggesting that the switch to X4 virus may have been a consequence, rather than a cause, of immunodeficiency [10]. Further studies are needed to evaluate the relationship of HIV tropism and disease progression in infants and children.

In adults, individuals infected with HIV subtype D are more likely to have CXCR4-using virus and are more likely to progress to AIDS and death than individuals with other subtypes [13–16]. In a previous study of infants in the HIVNET 012 cohort, we did not see a statistically significant association between maternal HIV pol subtype and infant survival, although there was a trend toward decreased survival in infants with pol subtype A infection [31]. That analysis included a larger number of infants than the present study, since some infants did not have samples available for tropism analysis or did not have tropism results. In the present study, infection with env subtype D was associated with both high-level infectivity in CCR5-bearing cells and the presence of X4 or DM virus. However, HIV env subtype was not a predictor of infant survival. Further studies are needed to examine the impact of HIV env-mediated entry activity and subtype in clinical outcome in infants. It is important to note that the analysis of raw RLU data from the Trofile assay (as opposed to the validated designation of tropism as R5, X4, or DM) has not been standardized and that the methods of analysis and the arbitrary threshold used in this study may not be applicable to other settings.

Our analysis of env clones revealed that some infants harbor complex viral populations, even at 6–14 weeks of age. Important determinants of HIV tropism have been shown to reside within the V3 loop of the HIV envelope protein [32]. This is consistent with our finding that clones with identical V3 regions can have different patterns of coreceptor use. Further studies are needed to assess whether subtype-specific differences in V3 loop sequences affect HIV tropism, thereby influencing the predictive value of V3 sequence-based algorithms derived from analysis of subtype B sequences. Other studies demonstrate some determinants of HIV tropism reside in regions outside of the V3 loop [13, 33–36]; these determinants may be particularly relevant in defining HIV tropism in R5X4 strains [13]. Further studies are needed to define the determinants of HIV tropism in subtype B and non–subtype B HIV infection.

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**References**


