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Analysis of HIV tropism in Ugandan infants

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Abstract

HIV-infected infants may have CXCR4-using (X4-tropic) HIV, CCR5-using (R5-tropic) HIV, or a mixture of R5-tropic and X4-tropic HIV (dual/mixed, DM HIV). The level of infectivity for R5 virus (R5-RLU) varies among HIV-infected infants. HIV tropism and R5-RLU were measured in samples from HIV-infected Ugandan infants using a commercial assay. DM HIV was detected in 7/72 (9.7%) infants at the time of HIV diagnosis (birth or 6–8 weeks of age, 4/15 (26.7%) with subtype D, 3/57 (5.3 %) with other subtypes, P=0.013). A transition from R5-tropic to DM HIV was observed in only two (6.7%) of 30 infants over 6–12 months. Six (85.7%) of seven infants with DM HIV died, compared to 21/67 (31.3%) infants with R5-tropic HIV (p=0.09). Higher R5-RLU at 6–8 weeks was not associated with decreased survival. Infants with *in utero* infection had a higher median R5-RLU than infants who were HIV-uninfected at birth (p=0.025).

Keywords

CCR5; CXCR4; HIV-1; infant; survival; transmission; tropism

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CONFLICT OF INTEREST

Wei Huang is anemployee of Monogram Biosciences. Susan Eshleman is a member of the Clinical Advisory Board of Monogram Biosciences.

DISCLAIMER

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Centers for Disease Control and Prevention. Use of trade names is for identification purposes only and does not constitute endorsement by the U.S. Centers for Disease Control and Prevention or the Department of Health and Human Services.

INTRODUCTION

HIV entry into cells involves interactions between viral particles, CD4, and a co-receptor, usually CCR5 or CXCR4 [1]. In an HIV-infected individual, viruses may use CCR5 (R5-tropic), CXCR4 (X4-tropic), or both CCR5 and CXCR4 (dual-tropic) [2]. Previous studies examined the association of co-receptor tropism and mother-to-child transmission of HIV using cell culture-based methods to assess HIV tropism. Several studies found that infants are more likely to be infected with non-synctia inducing virus or macrophage-tropic virus (later classified as R5-tropic virus) [3–7]. However, non-synctia inducing and T cell-tropic virus (later classified as X4-tropic virus) was also identified in some infants [3,8,9]. In adults, a switch from R5-tropic to X4-tropic HIV has been associated with disease progression. In other studies, 20% of recently infected adults had X4-tropic HIV, compared to 50% of adults with chronic HIV infection [10–12].

Disease progression in untreated HIV-infected infants and young children is often rapid, particularly in resource-limited settings [13–15]; mortality rates of 50% by 2 years of age have been observed [16]. There are relatively few studies examining HIV tropism in HIV-infected infants. In Uganda, most infants we studied previously had R5-tropic HIV at 6–14 weeks of age; X4- or dual-tropic HIV was only detected in 5 (8.8%) of 57 infants [17]. In that study, the presence of X4-tropic or DM HIV was not associated with an increased risk of death [17]. Switches in HIV tropism (both from R5-tropic to X4-tropic or DM HIV [5,18], and from X4-tropic or DM HIV to R5-tropic HIV [17,18]) have been observed in HIV-infected children in the first 1–2 years of life; however, children can progress to AIDS without detectable X4-tropic HIV [19,20]. In one study, HIV-infected children with X4-tropic HIV had lower CD4 cell counts than those who had R5-tropic HIV [21]. However, it is not known whether emergence of X4-tropic HIV is a cause or consequence of disease progression in HIV-infected children. In one study, the presence of X4-tropic HIV was observed in children only after immunological deterioration [5].

In Uganda, most HIV infections are caused by HIV subtypes A and D. Previous studies have found a higher frequency of X4-tropic HIV in individuals infected with subtype D strains. This association has been observed in Ugandan adults [22], pregnant Ugandan women [2], and Ugandan infants [17]. Furthermore, the level of HIV infectivity on CCR5-bearing cells (measured as relative light units in a commercial tropism assay, R5-RLU) was higher in Ugandan infants with subtype D infection than in infants with subtype A infection, and associated with decreased infant survival in a previous study [17]. In this report, we combined data from three clinical trials in Uganda with data from our previous study to examine the relationship between HIV tropism, R5-RLU, HIV subtype, and infant survival in a larger cohort of HIV-infected Ugandan infants. We also extended our previous study by analyzing of changes in HIV tropism and R5-RLU over time in longitudinal samples from the HIV-infected infants.

METHODS

Study Cohort

Plasma samples were obtained from infants enrolled in four clinical studies (HIVNET 012, Pathophysiology of Breast Milk, Repeat Pregnancy, and SWEN) conducted in Kampala, Uganda at the Mulago Hospital and the Makerere University–Johns Hopkins University Clinic (see Table 1) [23–27]; for infants in the HIVNET 012 trial, if a plasma sample prepared from peripheral blood was not available, plasma from a cord blood sample was used for analysis. Plasma samples (100–200 ul) were available from 125 infants at the time of HIV diagnosis (birth or 6 weeks of age). All of the infants analyzed received single dose nevirapine (sdNVP) for prevention of mother-to-child transmission of HIV (pMTCT) prior

to HIV diagnosis, with the following exception: 21 infants in the HIVNET 012 trial received a short course of zidovudine for pMTCT [25,26]. Viral load and CD4 cell count data were obtained in each clinical trial.

Analysis of HIV tropism, R5 infectivity, and *env* subtype

The clinically validated Trofile™ assay (Monogram Biosciences, South San Francisco, CA) was used for analysis, as previously described [28]. In the Trofile assay, DNA encoding the HIV envelope protein is amplified from a test sample, and is used to generate envelope test vectors. These test vectors are co-transfected with a replication-defective retroviral vector containing a luciferase reporter gene into HEK 293 cells, and pseudoviruses are generated to infect U87 cells expressing CD4, alone with either the CXCR4 or CCR5 co-receptor. Infection in each cell type is monitored by production of light (relative light units, RLU) from the luciferase reporter gene in the test vector. The HIV tropism of the viral population in a test sample is characterized as R5 tropic, X4 tropic, or DM (dual/mixed) tropic, based on infectivity in each cell type, and inhibition of infectivity by CCR5 and CXCR4 inhibitors [28]. The Trofile assay also provides a quantitative measure of infectivity in each cell type (e.g. R5-RLU in CCR5-bearing cells) [28]. Methods for determination of *env* subtype and for phylogenetic analysis of *env* sequences are described in previous reports [2,17].

Statistical analysis

Associations between R5-RLU, HIV subtype and timing of infection (birth vs. 6–8 weeks) were assessed by Wilcoxon rank sum tests. Student T-tests were used to compare baseline maternal CD4 cell counts and viral load between infants with vs. without Trofile results. Univariate Cox proportional hazards models were used to estimate the hazard ratios for infant death for predictors of interest including tropism status at HIV diagnosis, HIV *env* subtype, HIV *pol* subtype and maternal viral load. In this analysis, infants who initiated antiretroviral treatment for HIV were censored at the age when treatment began. Similar Cox models were used for the joint outcome of infant death or infant antiretroviral treatment. Statistical analyses were performed using SAS version 9.1.3 on the SunOS 5.9 platform.

Informed Consent

Guidelines of the U.S. Dept. of Health and Human Services and the authors' institutions were followed in the conduct of this research. Informed consent was obtained from all subjects for participation in the studies. Each study was approved by Institutional Review Boards (IRBs) in Uganda and at Johns Hopkins University School of Medicine. The Repeat Pregnancy and Breast Feeding studies were also approved by a U.S. Centers for Disease Control and Prevention IRB.

RESULTS

Analysis of HIV tropism

In four clinical studies conducted in Uganda, 168 infants were HIV-infected by 6–8 weeks of age. Plasma samples collected at the time of HIV diagnosis (birth or 6–8 weeks of age) were available for 125 (74.4%) of the 168 infants. HIV tropism data at the visit when HIV was diagnosed was obtained using the Trofile assay for 74 (59.2%) of the 125 samples (Table 1). The median baseline maternal HIV viral load and CD4 cell count at the time of delivery were similar for the 94 infants without Trofile results (either no sample available, or no Trofile result obtained) and the 74 infants with Trofile results (for viral load: 4.9 vs. 4.7 copies/ml, p=0.10; for CD4 cell count: 382.9 vs. 374.5 cells/ul, p=0.82). We think it is unlikely that low HIV viral loads were a cause of assay failure in some samples. We

provided 100 ul for each sample for Trofile testing, and the median $\log_{10}$ HIV viral load was similar among infants with Trofile results (5.8, 37/74 samples had viral load data) and those without Trofile results due to assay failure (5.7, 23/51 samples had viral load data, P=0.41, Wilcoxon rank test). Failure to obtain Trofile results for some infants may have reflected the small volume of plasma available for testing, or other factors related to testing of non-subtype B samples.

Among the 74 infants with Trofile data from the time of HIV diagnosis, 67 had R5-tropic HIV, and seven had DM-tropic HIV (dual/mixed, Table 1); the seven infants with DM HIV included 4 (26.7%) of 15 infants with subtype D infection and 3 (5.1%) of 57 infants who were infected with other HIV subtypes (P=0.013); subtype results were unavailable for two infants with R5-tropic HIV. Five (11.1%) of 45 infants with *in utero* infection had DM HIV compared to 2 (6.9%) of 29 infants who were HIV-uninfected at birth (P=0.55). CD4 cell count and CD4 cell % were measured in infants at the time of birth in the HIVNET 012 trial; these data were not obtained in the other studies. Among infants in the HIVNET 012 trial, there was no significant difference in CD4 cell count or CD4 cell % at the time of diagnosis between infants who had DM vs. R5 HIV at the time of diagnosis (P=0.223 for CD4 cell count; P=0.491 for CD4 cell %, Exact Wilcoxon test). HIV viral load data was only available for 37 of the 74 infants who had Trofile results (four infants with DM HIV, 33 infants with R5 HIV). The median HIV viral load at 6–8 weeks of age was similar among the infants with DM and R5 virus (5.5 vs. 5.9, P=0.15, Wilcoxon rank test).

Fifty-four (73.0%) of the 74 infants with Trofile results also had a sample available for testing that was collected at 6 or 12 months of age; this included two of seven infants who had DM HIV at the time of HIV diagnosis. Trofile results were obtained for 31 (57.4%) of the 54 infants with follow-up samples. Two infants who had DM HIV at the time of HIV diagnosis also had DM HIV at 6–12 months of age (Table 2). Among the 29 infants who had R5-tropic HIV at the time of HIV diagnosis and also had a follow-up sample available, 28 infants had R5-tropic HIV at follow-up; the remaining infant had DM HIV at 6 months and R5-tropic HIV at 12 months of age (Table 2). *Env* sequences from infants who had DM virus at one or more visits grouped in phylogenetic analysis.

Analysis of HIV infectivity on CCR5-bearing cells

We next measured the level of infectivity of HIV on CCR5-bearing cells, expressed as R5-RLU (see Methods). Among the 74 infants with Trofile results at the time of HIV diagnosis, the median $\log_{10}$ R5-RLU was 5.45 (range 2.71–6.34). There was no significant difference in the median R5-RLU at the time of HIV diagnosis among infants with subtype A vs. D HIV (5.18 vs. 5.43, p=0.09). The median R5-RLU was higher among the 45 infants with *in utero* HIV infection (those diagnosed with HIV infection at birth) than among the 29 infants who were HIV-uninfected at birth and were diagnosed with HIV infection at 6–8 weeks of age (5.62 vs. 5.24, respectively, p=0.025). Among the 31 infants who had paired R5-RLU results from the time of HIV diagnosis and from 6–12 months of age, median $\log_{10}$ R5-RLU was similar at two time points (p=0.1496, paired samples sign test).

Analysis of infant survival

Among the 74 infants who had tropism results from the time of HIV diagnosis, 14 (19%) started antiretroviral (ARV) therapy and 27 (36%) died during study follow-up, which ranged from 6 months to 5-years in the four trials (Table 1). Note that ARV treatment was initiated based on the standard of care in Uganda at the time each trial was performed, and that access to ARV treatment for infants and children increased in Uganda over time; ARV treatment was only initiated in infants in the more recent Repeat Pregnancy and SWEN trials (Table 1).

The 27 infants who died included six of the seven infants with DM HIV at the time of diagnosis (median survival time for those 6 infants was 15 months, 95% CI 10–24 months). We analyzed the association of infant survival with HIV tropism, R5-RLU (above vs. below the median R5-RLU value for the cohort), and other factors (maternal HIV viral load and CD4 cell count at delivery, *in utero* HIV infection (yes/no) and HIV envelope subtype, Table 3). In this analysis, infants who started ARV therapy were censored at the time of treatment initiation. In a second analysis, we included both death and ARV treatment initiation as outcomes (Table 3). In both models, baseline maternal HIV viral load was the only significant predictor for infant death and for the joint outcome of death or initiation of ARV treatment. DM HIV at diagnosis was not a statistically significant predictor of death in either model. Six (85.7%) of seven infants with DM HIV died by 5 years, compared to only 21 (31.3%) of 67 infants without X4-tropic strains (p=0.09). Our inability to detect a significant association between DM tropism and survival may have reflected the limited power in our sample, or the limited length of follow-up in three of the four clinical trials (Table 1). While the association we observed was not statistically significant, it does suggest that infants who have X4-tropic HIV may have reduced survival. We considered the possibility that treatment initiation was a confounder in this analysis. ARV treatment was initiated in 14 of the 67 infants without X4-tropic strains, while none of the seven infants with DM HIV started treatment during the follow-up period. However, in the subset of 60 infants who did not start ARV treatment, a higher proportion of infants with DM HIV died compared to infants who did not have X4-tropic strains (6/7=86% with DM HIV died vs. 21/53=40% with R5-tropic HIV); however, this association was not statistically significant (p=0.15).

DISCUSSION

Previous studies have demonstrated that most HIV-infected infants are infected with HIV that is likely to be R5-tropic based on results from cell culture-based methods (i.e., non-syntcytia inducing or macrophage tropic) [3–7]. In this study, the majority of infants (67/74=90.5%) had R5-tropic HIV at the time of HIV diagnosis (birth or 6–8 weeks of age). We identified seven infants (9.5%) who had DM HIV at the time of HIV diagnosis; four (57.1%) of these infants had subtype D infection. While we did not observe a statistically significantly higher rate of DM HIV among infants with subtype D vs. other HIV subtypes, these data are consistent with previous studies that found a higher rate of X4-tropic HIV in individuals with subtype D HIV infection [2,17,22,29]. One potential limitation of this study is that infants in the HIVNET 012 study were born earlier (1997–1999) than those in the other three clinical trials (2003–2006); it is noteworthy that the proportion of infants with DM-tropic virus was higher in the HIVNET 012 trial (6/40=15%) that in the other three trials combined (1/34=3%, P=0.116, Fisher's exact test). While the infants in all four studies received the same regimen for pMTCT (sdNVP), there may have been other unidentified differences between the cohorts, possibly related to the differences in the enrollment dates of the trials that could have influenced the study results.

Five of the seven infants who had DM HIV were HIV-infected *in utero* and two were HIV-uninfected at birth (diagnosed at 6 weeks of age). In a previous study, maternal *env* sequences from delivery and infant *env* sequences from the time of diagnosis collected from five of these mother-infant pairs were cloned and analyzed in the Trofile assay [30]. Phylogenetic analysis of the clones revealed that both X4- and dual-tropic variants were transmitted from the mother to the infant; there was no evidence that X4-tropic variants emerged from R5-tropic virus in these infants [30]. Only two of 30 infants in this study switched from R5-tropic HIV at the time of diagnosis to DM-tropic HIV at 6–12 months of age. Other studies have shown that infants can have rapid disease progression with R5-tropic

virus (M-tropic virus in older studies) [20]. We also identified one infant who switched from DM HIV to R5-tropic HIV during follow-up.

In this study, six (85.7%) of the seven infants with DM HIV died during the follow-up period, compared to only 21 (31.3%) of 67 infants without X4-tropic strains. This suggests an association between the presence of X4-using virus at or near the time of birth and shortened survival. While this association was not statistically significant in this study, we feel that this issue merits further analysis in a larger study cohort. Our failure to find a statistically significant association between the presence of DM HIV near the time of birth and survival may be due to small sample size (only seven infants with DM HIV), or the short follow-up (6–12 months) in three of the four clinical studies from which the samples were obtained.

In this study, we also measured the infectivity of the virus on CCR5-bearing cells (R5-RLU). Infants who were infected *in utero* had a higher R5-RLU compared to infants who were HIV-infected by 6–8 weeks of age. However, R5-RLU did not change significantly over time, and we did not see an association between R5-RLU at the time of HIV diagnosis and infant survival. In contrast, our previous study of infants in the HIVNET 012 cohort found an association between high R5-RLU and decreased survival [17]. Differences between the two studies, such as the source of the samples (HIVNET 012 only vs. the four trials in this report), the number of infants analyzed (57 vs. 74 in this report), the age of the infants at the time of sample collection (6–14 weeks vs. birth to 6–8 weeks in this report), or length of follow-up (5 years vs. 6 months to 5 years in this report) may account for the different results. Also, the difference in the two studies may also have been influenced by use of antiretroviral therapy for infant treatment. In the more recent studies (Breastfeeding, Redosing), ARV therapy was given to some of the infants at a young age. In the survival analyses we attempted to account for this with the joint endpoint of death or initiation of ARV treatment, as infants who may have been most likely to die without treatment were not included as endpoints in the analysis that censored infants at time of ART initiation.

Further studies are needed to fully define the relationship between HIV tropism and R5-RLU and clinical outcome in HIV-infected infants. Such studies will likely require analysis of a large cohort of HIV-infected infants from a country such as Uganda, where subtype D is prevalent, in a cohort with extended follow-up. Analysis of the natural history of HIV infection in infants infected with DM HIV may require analysis of cohorts of infants who did not initiate antiretroviral therapy during the follow-up period. However, analysis of clinical outcome of infants with DM HIV who are able to access antiretroviral treatment may also provide important information. Currently, the portion of HIV-infected children in sub-Saharan Africa who have access to antiretroviral therapy (estimated to be 35%) is lagging behind that of adults despite the significant survival benefit of early antiretroviral therapy [31,32].

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Table 1

Characteristics of infants (Four studies, Uganda).

Study name	Pathophysiology of Breast Milk [27]	HIVNET 012 [25,26]	Repeat Pregnancy [23]	SWEN[24]	Overall
Years of enrollment	2003–2004	1997–1999	2004–2006	2004–2006	1997–2006
Length of follow-up	6 months	5 years	1 year	12 months	6 months to 5 years
# infants with a Trofile result	10	40	10	14	74
# of infants with *in utero* HIV infection	6/10	25/40	6/10	8/14	45/74
# who started ARV therapy during follow-up	0/10	0/40	4/10	10/14	14/74
# who died during follow-up	0/10	23/40	2/10	2/14	27/74
HIV *env* subtype A/D/R	7/1/1 [c]	24/10/5 [c]	4/3/3	7/1/6	42/15/15
# infants with R5-tropic HIV at diagnosis/# infants with a Trofile result [a]	10/10	34/40	9/10	14/14	67/74
# infants with DM HIV at diagnosis/# infants with a Trofile result [a]	0/10	6/40	1/10	0/14	7/74
Median $\log_{10}$ R5-RLU (range) at diagnosis [b]	5.57 (3.50–6.33)	5.24 (2.71–6.25)	5.49 (3.97–6.03)	5.45 (4.14–6.34)	5.45 (2.71–6.34)

[a] R5-tropic: HIV capable of replicating in CCR5-bearing cells; DM HIV: HIV capable of replicating in both CCR5- and CXCR4-bearing cells (dual or mixed tropic HIV)

[b] R5-RLU: Relative light units produced in the Trofile assay using CCR5-bearing cells.

[c] One sample did not have a subtype result.

Table 2

Tropism results for infants with one or more sample with dual-tropic HIV*

Study	*Env* ST	Maternal log_{10} HIV RNA (copies/ml)	Maternal CD4 cell count (cells/ul)	Age at diagnosis	Tropism at diagnosis	Tropism at 6 months	Tropism at 12 months	End of follow-up status	Total follow-up (days)
RP	D		229	Birth	R5	DM	R5	Alive	361
RP	A	4.71	459	6–8 weeks	DM	DM	DM	Alive	372
HIVNET 012	A	5.35	17	Birth	DM			Died	309
HIVNET 012	D	4.01	657	Birth	DM			Died	377
HIVNET 012	D	5.02	204	6–8 weeks	DM			Died	548
HIVNET 012	A	5.29	77	Birth	DM			Died	133
HIVNET 012	D	5.41	376	Birth	DM			Died	1655
HIVNET 012	D	4.27	299	Birth	DM		DM	Died	739

**Env* ST: subtype of the HIV envelope region; RP: Repeat Pregnancy study; maternal viral load and CD4 cell count were measured at the time of delivery. R5: CCR5-tropic HIV; DM: HIV capable of replicating in both CCR5- and CXCR4-bearing cells (dual or mixed tropic HIV)

Table 3

Factors associated with infant survival in univariate models including either death or death or antiretroviral treatment initiation as an outcome*.

		Outcome is death[c]		Outcome is death or initiation of antiretroviral treatment[d]	
	N	**HR (95% CI)**	**P value**	**HR (95% CI)**	**P value**
Baseline maternal $\log_{10}$ HIV RNA [a]	65	2.3 (1.2–4.4)	**0.012**	1.8 (1.1–3.1)	**0.02**
Maternal delivery CD4 cell count [b]	70	1.2 (1.0–1.4)	0.12	1.1 (1.0–1.3)	0.18
In utero HIV infection vs, HIV- uninfected at birth	74	1.3 (0.6–2.9)	0.50	1.3 (0.7–2.5)	0.41
DM HIV at diagnosis	74	2.2 (0.9–5.4)	0.09	1.3 (0.6–3.2)	0.52
High R5-RLU (> median value)	74	1.0 (0.5–2.1)	0.097	1.0 (0.6–1.9)	0.93
Env subtype (D vs. A)	57	1.6 (0.7–3.8)	0.28	1.4 (0.7–3.0)	0.38
Env subtype (D vs. non-D)	72	1.7 (0.7–3.9)	0.22	1.2 (0.6–2.5)	0.57

*N: number of children included in the univariate models; HR: hazard ratio: CI: confidence intervals.

[a]The hazard ratio is calculated for a 1 log increase in HIV RNA.

[b]The hazard ratio is calculated for a decline of 100 cells in CD4 counts.

[c]In this model, death is the outcome variable and infants who started antiretroviral treatment were censored at the time of treatment initiation; infants who survived were censored at the end of follow-up.

[d]In this model, both death and antiretroviral treatment initiation are outcome variables; infants who survived were censored at the end of follow-up.