Published in final edited form as:

J Acquir Immune Defic Syndr. 2010 October ; 55(2): 143–147. doi:10.1097/QAI.0b013e3181eb301e.

The role of transplacental micro-transfusions of maternal lymphocytes in *in utero* HIV transmission

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Abstract

Background—The mechanisms of HIV transmission from mothers to infants are poorly understood. A possible mechanism of *in utero* transmission is transplacental transfer of HIV-infected maternal leukocytes into the fetal circulation during pregnancy.

Objective—To determine if the frequency of *in utero* HIV infection correlates with presence or levels of maternal cells (MC) in placenta-derived cord blood.

Methods—DNA was extracted from dried cord blood spots (DBS) from newborns born to HIV+ mothers and corresponding maternal DBS specimens. Paired mother-infant samples were probed to identify unique maternal sequences targeted by 24 allele-specific real-time PCR assays. Infant DBS-derived DNA was then probed in replicate analyses for non-inherited maternal allelic-sequences. Rates of detection and levels of maternal cells in DBS samples of HIV(+) and HIV(–) newborns were compared.

Results—Of 114 mother-infant pairs with informative alleles, 38 newborns were HIV(+) and 76 HIV(–), based on detection of HIV DNA/RNA at birth. MC were detected in 23 of 38 HIV(+) newborns (60.5%) and in 47 of 76 HIV(–) newborns (61.8%). The mean and median concentrations of nucleated maternal cells in DBS for the HIV(+)/MC(+) newborns (N=23) were 0.33% and 0.27%, respectively, compared with 0.09% and 0.10% for the HIV(–)/MC(+) newborns (N=47) (Two-Sample T-test for means: p=0.78).

Conclusions—There was no significant difference in rates of detection or concentrations of MC in DBS between HIV(+) and HIV(–) newborns. Therefore, we could not demonstrate a correlation between MC in DBS, assumed to reflect levels of *in utero* maternal-fetal cell trafficking, and the risk of *in utero* HIV transmission.

Keywords

HIV; maternal-fetal transmission; polymerase chain reaction; PCR; microchimerism; placenta

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Introduction

In a study conducted in Malawi before antiretroviral treatment was available, about 6–8% of infants born to HIV+ women were found to become infected *in utero*. [1] At birth, many infants with HIV DNA (+) cord bloods have quantitative viral levels that are as high as or higher than those in their mothers, indicating sustained *in utero* replication well preceding parturition. [2] Furthermore, while antiretroviral therapy is partially effective when given only at parturition [3], the efficacy is greater when it given throughout the pregnancy. [4] Thus, the mechanisms by which the virus is transmitted *in utero* are of interest, even though they remain poorly understood. One possible mechanism of HIV *in utero* transmission is by “transfusion” of HIV-infected maternal blood cells into the infants’ circulation during pregnancy. The continuous blood exchange between maternal circulation and fetal circulation during pregnancy has been established, [5–7] with blood exchange peaking at the perinatal stage, [5] a finding consistent with the highest transmission risk being at the time of delivery. [1]

We have previously hypothesized that perinatal transmission might occur because of transplacental passage of infected maternal cells during delivery. However, using polymerase chain reaction (PCR) testing, we failed to detect statistically significant differences in the likelihood that maternal cells would be found in umbilical cord blood of perinatally-infected infants (cord blood PCR-negative but first sample at 6 weeks found to be PCR positive). [8] In contrast, another group did find maternal cells more commonly in perinatally-infected infants. [9] Furthermore, they reported a marginally significant increase in risk of *in utero* infection [i.e., cord blood PCR HIV (+)], with increasing numbers of maternal cells in umbilical cord blood at delivery. We therefore examined new samples from our study to assess if *in utero* infection risk might be correlated with the presence of maternal cells in cord blood.

Materials & Methods

Patients and Materials

The dried blood spots (DBS) analyzed in this study were collected at a clinical trial in Blantyre, Malawi in 1994, which enrolled women giving birth to determine rates of HIV transmission with and without vaginal cleansing with chlorhexidine. Details of the study have been published elsewhere. [1] HIV infection in the mother was determined by testing umbilical cord blood for passively transmitted antibodies against HIV. HIV infection in the infant was determined by PCR testing of DBS made from the umbilical cord blood or heel sticks at various times after birth. DBS samples were stored in individual sealed plastic envelopes at 4°C. Laboratory methods for sample collection and HIV testing have been reported in previous publications. [1, 10, 11] Infants found to be uninfected postnatally were assumed to be HIV uninfected at all earlier ages, including *in utero*. Cord blood samples from infants found to be infected on their first postnatal visit were further tested by PCR to determine their HIV status at delivery.

For the current study, we analyzed all available maternal-infant pairs from the 1994 intervention study. [1] All infants found to be PCR HIV[+] in the cord blood were assumed to have been infected *in utero*, an assumption justified by earlier studies of viral levels in umbilical cord blood which showed that HIV levels in infected infants are typically as high or higher as those in the mother. [2] For each *in utero*-infected infant, two uninfected controls were obtained. We confirmed that cord blood samples from infants previously found to be HIV-negative were indeed negative by retesting cord blood DBS for HIV with a highly sensitive reverse transcription-PCR method that identified sequences specific to HIV. [12] Both case and control infants were vaginally-delivered singleton infants born at term to

untreated HIV-infected women. [1] The original study was approved by the University of Malawi Medical School, the Government of Malawi, and the National Institute of Health (USA). The current study was approved by the National Cancer Institute as exempt from further ethical review because it used only anonymous and unlinkable materials collected from a previously approved study.

Testing strategy

Previously, we have developed PCR assays to detect the presence of microchimeric cells in the context of a high background of allogeneic cells, [13] including the detection of maternal cells in fetal tissue. [7] These assays are based on two panels: an insertion/deletion (INDEL) panel consisting of 12 different insertion/deletion polymorphisms first described by Alizadeh et al., [14] and an HLA-DR panel consisting of 12 HLA-DR alleles (DR1, DR3, DR4, DR7, DR8, DR9, DR10, DR11, DR12, DR13, DR15, and DR16). The mother and infant were both typed using these panels. Alleles for which the mother was positive but the infant was negative were termed “informative alleles.” Maternal cells were detected in the cord blood by amplifying for these informative alleles.

Extraction of DNA from dried blood spots

Each blood spot representing 50 uL of whole blood was cut out using sterile scissors, immersed in 400 uL of PBS, and incubated in PBS overnight at room temperature. A total of 200 uL of Corbett Robotics digest buffer (Sigma, St Louis, Missouri, USA) was then added and incubated at 60°C for 2 hours. Both the liquid and paper were transferred to a QIAamp spin column (Qiagen, Valencia, CA, USA) and DNA was extracted, as per the manufacturer’s instructions. After elution in water, the samples were dried and reconstituted in 300 uL of Solution A+B. [15]

HLA-DR and INDEL typing of maternal and cord blood samples

Plates for PCR typing were prepared and frozen at –80°C. The 24×16 typing plate formats consisted of 12 columns of INDEL PCR assays and 12 columns of HLA-DR PCR assays. Five microliters of DNA were added to 10 uL of PCR mixture consisting of 15 mM KCl, 5 mM Mg, 20 mM Tris-HCl pH 8.0, BSA (0.1 mg/mL), Triton X-100 (1%), 1 mM of dNTPs (Roche Diagnostics GMBH, Mannheim, Germany), 1 uM of each primer (Integrated DNA Technologies, Coralville, IA), SYBR Green (18.75 units/reaction; BioWhittaker Molecular Applications, Rockland, ME), and FastStart Taq (1.5 units/reaction; Roche Diagnostics GMBH, Mannheim, Germany). Real-time PCR was performed with a sequence detection system (Roche LightCycler 480, Roche Diagnostics, Basel, Switzerland) with the following cycle conditions: 1 minute at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 64°C, and 45 seconds at 72°C.

Amplification for microchimerism detection and quantification

After the mother and child were both typed with the INDEL and HLA-DR panels, an informative allele was determined for each pair. We used the INDEL allele preferentially. If an informative INDEL allele was not found, we used the HLA-DR allele.

Another cord blood spot was then processed into 300 uL of DNA lysate and amplified for its informative allele. A total of 25 uL of DNA lysate was added to 50 uL of PCR reaction mix and amplified with the above conditions for 4 replicates. To quantify the recovery of genomic DNA, the cord blood samples were amplified with primers specific for HLA DQ-alpha to measure levels of genomic DNA. To quantify the maternal cells, 10-fold serial dilutions of quantified standards (i.e., peripheral blood mononuclear cells at known concentrations based on replicate determination using a Coulter counter) were amplified

along with the clinical samples. Fluorescence was recorded at each extension step and computer software (Roche LightCycler 480, Roche Diagnostics, Basel, Switzerland) was used to plot the fluorescence values against cycle number.

Results

We tested dried blood spot samples from 145 mothers and their infants. A total of 31 mother-infant pairs had no informative allele in either INDEL or HLA-DR tests. Of 114 mother-infant pairs with informative alleles, 38 pairs were HIV positive in cord blood [HIV(+)] and 76 pairs were HIV negative in cord blood [HIV(-)].

The mean genomic DNA concentration (copies/50 uL cord blood, i.e., the number of nucleated cord blood cells in one dried blood spot, assuming a volume of 50 uL in each spot) of cord blood samples for the HIV(+) group was 20,759 human cell equivalents, with a median of 17,200 and a range of 3,876 to 62,220. Similarly, the mean genomic DNA concentration of cord blood samples for the HIV(-) group was 15,055 human cell equivalents, with a median of 13,236 and a range of 768 to 49,140. (Fig. 1) The genomic DNA concentrations arising from nucleated cord blood cells in the HIV(+) group were significantly higher than those of the HIV(-) group (Two-Sample T-test: $p=0.01$).

Maternal cells (MC) were detected in the cord blood in 23 of 38 HIV(+) pairs (60.5%) and in 47 of 76 HIV(-) pairs (61.8%). The mean number of maternal cells in 50 uL of cord blood for the HIV(+) group was 39, with a median of 18 and a range of 6 – 439. The mean number of maternal cells for the HIV(-) group was 30, with a median of 12 and a range of 6 – 289. The difference in the number of maternal cells in cord blood samples between the HIV(+) group and the HIV(-) group was not statistically significant (Two-Sample T-test: $p=0.65$). (Fig. 2)

The proportion of maternal cells in the cord blood sample was obtained by dividing the quantity of maternal cells by the quantity of the total cord blood nucleated cells (maternal and fetal). The mean proportion of maternal cells for the HIV(+)/MC(+) group was 0.33%, with a median of 0.27%, and a range of 0.03% – 5.12%. The mean proportion of maternal cells for the HIV(-)/MC(+) group was 0.09%, with a median of 0.10%, and a range of 0.02% – 3.08%. (Fig. 3) There was no significant difference in the concentration of maternal cells in cord blood samples between the HIV(+) group and the HIV(-) group (Two-Sample T-test: $p=0.78$).

Discussion

The current study was designed to test the hypothesis that a high concentration of maternal cells in cord blood could reflect more *in utero* exposure to maternal transplacental microtransfusions and, hence, a greater likelihood of HIV transmission from HIV+ mothers to their infants. To examine this hypothesis, we used a highly sensitive and specific quantitative real-time PCR assay to identify specific maternal DNA sequences, which were then used to quantify maternal cells in cord blood. This quantitative real-time PCR assay has been applied in many studies which required a very sensitive assay to detect very low-level chimeric cell populations. [13, 15, 16]

We found that about half of the cord blood samples contained a significant number of maternal cells, which is similar to the same frequency of detection of maternal cells in cord blood DBS in our previous publication which focused on perinatal HIV transmission. [8] When we compared the genomic DNA (amplification of HLA DQ-alpha) recovery from DBS, we found that the genomic DNA concentrations of cord blood samples for the HIV(+) group to be significantly higher than those of the HIV(-) group (Two-Sample T-test:

$p=0.01$), which implies that the HIV(+) cord blood contained a higher number of WBCs than the HIV(−) cord blood. This finding suggests that *in utero* HIV infection is associated with leukocytosis, possibly reflecting inflammation or cell activation that results from infection of the fetus.

However, when we compared the detection rates of maternal cells among cord blood samples from infected and non-infected infants, we found no significant difference between the HIV(+) and the HIV(−) groups (60.5% and 61.8%, respectively, Two-Sample T-test: $p=0.89$). Moreover, the difference in the concentrations of maternal cells in cord blood and in the proportion of total cord blood leukocytes that were of maternal origin between the HIV(+) and HIV(−) groups was also not statistically significant (Two-Sample T-test: $p=0.65$ and 0.78 , respectively). These results do not support the hypothesis that the frequency or magnitude of maternal fetal “transfusion” is positively associated with the frequency of *in utero* HIV transmission. Possibly, infection of the fetus *in utero* is not necessarily accompanied (and possibly caused) by transplacental movement of maternal cells carrying HIV and/or by fetal regulatory T cells responses induced by such cells. Alternatively, some fetuses exposed to infected HIV+ maternal cells may be able to mount protective immune response against HIV while others can not. For instance, given recent observations that maternal microchimerism is associated with the development of fetal regulatory T cells that suppress fetal responses against non-inherited maternal alleles, [16] it is possible that concomitant exposure of the fetus to HIV might also result in the generation of fetal regulatory T cells specific for HIV. [17] If so, the fetus might be more susceptible to HIV infection (because protective adaptive antiviral immunity is suppressed) or, alternatively, protected (because pro-inflammatory responses that drive viral replication and spread might be suppressed).

There are several limitations to this study. One is that we do not know when the infants were infected *in utero*. We presume that the infection must have occurred much earlier than near delivery, because HIV levels in cord blood were already as high as in the mother and such levels would likely take several weeks to establish following active viral replication in the fetus. [2] Yet, infection may have occurred at varying times and the concentration of maternal cells at delivery might not be representative of the levels of transplacentally-passed maternal cells at those times. Hypothetically, some break in the placental barrier might have sent a transient burst (acute “transfusion”) of maternal cells (and HIV) across the placenta much earlier in the pregnancy, the evidence for which would have been gone by the time of delivery.

Another limitation is that samples used in the current study were stored for a long time, which could affect the quantity and quality of the recovered DNA. Although we were able to recover a reasonable amount of human DNA from the majority of samples, some samples had very low DNA recovery. With optimal and high volume samples, we would have had higher total DNA inputs in the PCR amplifications, which would have enabled detection of maternal cells in a larger proportion of cord blood samples. However, this limitation should not have undermined our key findings of relatively higher levels of total WBC but similar levels of chimeric maternal WBC in cord blood DBS eluates from infected and non-infected infants, respectively, since the DBS from both groups were stored and processed under identical conditions and testing was performed under code. We believe that the observation of significantly higher levels of cell genomes in DBS from HIV(+) than HIV(−) infants is valid, albeit unexplained, since the HIV status was unknown at birth and could not have confounded the collection or processing of the blood samples. Additionally, confounding factors that were not available, such as the viral load of maternal HIV and maternal-fetal HLA concordance, should also be taken into consideration, [18–21] although we doubt they explain our findings.

In summary, we did not find support for a correlation between maternal cells found in umbilical cord blood at birth and the risk of HIV infection *in utero*. Nevertheless, we cannot refute the possibility that *in utero* infection occurred via transplacental transmission of infected cells earlier in pregnancy that did not result in persistently elevated levels of microchimeric maternal cells or of HIV in cord blood at delivery.

Acknowledgments

These studies were supported in part by R01-HL-083388, NHLBI (D.M.C., T.H.L., M.B.), R37 AI40312, and DPI OD00329 (J.M.M.). J.M.M. is a recipient of the NIH Director's Pioneer Award Program, part of the NIH Roadmap for Medical Research. The authors would like to acknowledge the contributions of Taha E. Taha for the establishment of the cohort and assistance with access to the DBS specimens.

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Figure 1.

Comparison of genomic DNA recovery of dry blood samples between the HIV(+) group and the HIV(-) group. The Y-axis represents the concentration of genomic DNA (copies/50 uL of whole blood) and the X-axis represents the HIV infection status of dry blood samples.

Figure 2.

Comparison of the number of maternal cells in cord blood between HIV(+) and HIV(-) cord blood. The Y-axis represents the number of maternal cells in 50 uL of cord blood and the X-axis represents the HIV infection status of dry blood samples.

Figure 3. Comparison of the concentration of maternal cells in cord blood between HIV(+) and HIV(-) cord blood. The Y-axis represents the percentage of maternal cells in cord blood and the X-axis represents the HIV infection status of dry blood samples.