Epstein-Barr Virus Load in Children Infected With Human Immunodeficiency Virus Type 1 in Uganda

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Background. Epstein-Barr Virus (EBV) is involved in a wide range of malignancies, particularly in immunocompromised subjects. In Africa, EBV primary infection occurs during early childhood, but little is known about the EBV load in Human Immunodeficiency Virus type 1 (HIV-1)–infected children.

Methods. Blood samples from 213 HIV-1–infected children, 140 of whom were receiving antiretroviral therapy (ART), were collected at the Nsambya Hospital in Kampala, Uganda, and obtained for dried blood spot analysis. Nucleic acids were extracted and analyzed for quantification of EBV types 1 and 2; 16S ribosomal DNA (rDNA), a marker of microbial translocation; and HIV-1 RNA.

Results. Ninety-two of 140 children (66%) receiving ART and 57 of 73 ART-naive children (78%) had detectable EBV DNA levels. Coinfection with both EBV types was less frequent in ART-treated children than in ART-naive children (odds ratio, 0.54 [95% confidence interval {CI}, .30–.98]; P = .042). Mean EBV DNA levels (±standard deviation) were lower in the former (3.99 ± 0.59 vs 4.22 ± 0.54 log10 copies/mL; P = .006) and tended to be inversely associated with ART duration. EBV DNA levels were higher in children with an HIV-1 RNA load of > 3 log10 copies/mL of blood (regression coefficient, 0.32 [95% CI, .05–.59]; P = .020) and correlated with circulating 16S rDNA levels (rs = 0.25 [95% CI, .02–.46]; P = .031).

Conclusions. These findings suggest that ART, by limiting HIV-1 replication, microbial translocation, and related immune activation, prevents superinfection with both EBV types and keeps EBV viremia down, thus potentially reducing the risk of EBV-associated lymphomas.

Keywords. EBV; HIV-1; African children; immune activation; antiretroviral therapy.

Epstein-Barr Virus (EBV) is associated with a wide range of malignancies, particularly in immunocompromised subjects, ranging from lymphoproliferative disorders to B-cell non-Hodgkin lymphomas (NHLs) [1, 2]. Besides immunodepression, chronic immune activation, a hallmark of Human Immunodeficiency Virus type 1 (HIV-1) pathogenesis [3], may play a critical role in the genesis of B-cell lymphomas [4, 5]. Cell activation driven by HIV-1 antigens, together with impaired immunosurveillance against EBV, may result in chronic B-cell stimulation and expansion of EBV-infected B cells [6–9], thus increasing the risk of EBV-related malignancies. The factors contributing to HIV-1–induced B-cell activation and expansion of EBV-infected cells are largely unknown. Massive HIV-1–induced T-cell depletion causes damage to intestinal mucosa, promoting...
translocation of microbial products into circulation. Pathogen-associated molecular patterns, such as 16S ribosomal DNA (rDNA), trigger a potent innate immune response through the engagement of several Toll-like receptors (TLRs), which also involve B cells, causing polyclonal B-cell activation [10].

Antiretroviral therapy (ART) has greatly modified the natural course of HIV-1 infection, resulting in a decreased HIV-1 load, an increased CD4+ T-cell count, and a decreased incidence of HIV-1–associated opportunistic infections, indicating the restoration of immune function [11]. Although the incidence of HIV-1–related malignancies, such as Kaposi sarcoma, has declined markedly following expanded access to ART, the incidence of NHL still remains elevated [12]. Two EBV types, type 1 and type 2, are recognized. EBV type 1 is more common in Africa and New Guinea [13]. HIV-1–infected subjects may have either type 1 or type 2, and coinfection with both types is also possible [13].

In Africa, EBV primary infection occurs during infancy and early childhood, and EBV-associated lymphomas represent a substantial cause of morbidity and mortality in children. NHL commonly occurs in African children, endemic Burkitt lymphoma being the most common type of cancer [14]. Endemic Burkitt lymphoma is closely associated with EBV and accounts for up to 75% of all childhood malignancies [15], reaching an annual incidence of 5–10 cases per 100 000 in Central Africa [16]. Other tumors, such as EBV-associated immunoblastic lymphomas, may occur in HIV-1–infected children [17, 18].

To date, there are very few data concerning EBV infection in HIV-1–infected African children. The recent finding that primary EBV infection occurs earlier and with a higher peak of viremia in HIV-infected versus HIV-uninfected Kenyan infants born to HIV-seropositive mothers [19] stresses the importance of investigating EBV types and viremia in the context of HIV-1 infection, particularly in HIV-1–infected children who did or did not receive ART. The use of dried blood spot sampling may represent a feasible method of collecting and storing blood samples and may be instrumental in expanding studies in resource-limited settings.

**MATERIALS AND METHODS**

**Patients and Sample Collection**

All children enrolled in this cross-sectional study belonged to an observational cohort set up among HIV-1–infected children at the Home Care Department of the St. Raphael of St. Francis Hospital at Nsambya in Kampala, Uganda. Children were evaluated monthly for clinical follow-up and self-reported adherence. Laboratory investigations, including a full blood count, liver function tests, and measurement of creatinine level and CD4+ T-cell count, were performed every 6 months or, if clinically required, more frequently. World Health Organization (WHO) guidelines from 2006 [20] were followed for initiation of trimethoprim/sulfamethoxazole prophylaxis, ART, and management of opportunistic infections. In the absence of contraindications, ART was prescribed after provision of counseling to and receipt of written consent from caregivers.

Blood samples from 213 HIV-1–infected children (age, 0–18 years) who attended the Home Care Department from March 2010 to July 2010 were collected for this study. At the time of sampling for this study, 140 children (66%) were receiving ART. Ninety-seven children received nevirapine (NVP)–based regimens, 34 received efavirenz (EFV)–based therapy, and 8 received lopinavir/ritonavir (LPV/r)–based treatment (Table 1). Blood from each child was collected in ethylenediaminetetraacetic acid (EDTA) tubes, centrifuged at 2000 g for 10 minutes, and stored at −70°C until analysis. Blood samples from 140 children (66%) were receiving ART. Children not receiving ART included those who did not meet WHO guidelines for the initiation of ART and parents who refused ART. All children were tested for HIV-1 RNA, EBV-DNA, and EBV-DNAemia levels.

**Table 1. Baseline Characteristics of Human Immunodeficiency Virus Type 1–Infected Children**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y, median (range)</td>
<td>8 (0–18)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>105/213 (49)</td>
</tr>
<tr>
<td>Female</td>
<td>108/213 (51)</td>
</tr>
<tr>
<td>Children receiving ART</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>73/213 (34)</td>
</tr>
<tr>
<td>Yes</td>
<td>140/213 (66)</td>
</tr>
<tr>
<td>WHO disease stage</td>
<td></td>
</tr>
<tr>
<td>All children</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>60/209 (29)</td>
</tr>
<tr>
<td>II</td>
<td>120/209 (57)</td>
</tr>
<tr>
<td>III</td>
<td>27/209 (13)</td>
</tr>
<tr>
<td>IV</td>
<td>2/209 (1)</td>
</tr>
<tr>
<td>Children receiving ART</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>23/140 (16)</td>
</tr>
<tr>
<td>II</td>
<td>97/140 (69)</td>
</tr>
<tr>
<td>III</td>
<td>18/140 (13)</td>
</tr>
<tr>
<td>IV</td>
<td>2/140 (2)</td>
</tr>
<tr>
<td>ART type</td>
<td></td>
</tr>
<tr>
<td>NVP based</td>
<td>97/140 (69)</td>
</tr>
<tr>
<td>EFV based</td>
<td>34/140 (24)</td>
</tr>
<tr>
<td>LPV/r based</td>
<td>8/140 (6)</td>
</tr>
<tr>
<td>Triple NRTI</td>
<td>1/140 (1)</td>
</tr>
<tr>
<td>CD4+ T-cell count, cells/μL</td>
<td></td>
</tr>
<tr>
<td>&lt;350</td>
<td>18/177 (10)</td>
</tr>
<tr>
<td>350–750</td>
<td>68/177 (38)</td>
</tr>
<tr>
<td>750–1000</td>
<td>41/177 (23)</td>
</tr>
<tr>
<td>&gt;1000</td>
<td>50/177 (29)</td>
</tr>
</tbody>
</table>

Data are no. of children with the characteristic/total for whom data were available (%), unless otherwise indicated.

Abbreviations: ART, antiretroviral therapy; EFV, efavirenz; LPV/r, lopinavir/ritonavir; NRTI, nucleoside reverse transcriptase inhibitor; NVP, nevirapine; WHO, World Health Organization.

* All characteristics refer to time of sample collection.

* Data are for 213 children.
acid (EDTA)–containing tubes by trained nurses. A 50-µL specimen of this blood was spotted onto each of the 5 circles of a Protein Saver 903 Card (Whatman, Hahnenstra, Germany). The blood samples were dried at room temperature overnight, stored in individual ziplock bags containing a desiccant, and shipped to the laboratory of the Viral Oncology Unit and AIDS Reference Center, Section of Oncology and Immunology, Padua, Italy. The study was approved by the Internal Review Board and Ethics Committee of Nsambya Hospital and registered by the Uganda National Council for Science and Technology (reference HS724).

DNA Elution
From each 50-µL dried blood spot, three 3-mm-diameter circles, equivalent to 5 µL of whole blood each (15 µL total), were used to extract DNA by means of the QiaPrep DNA Micro-Kit (Qiagen, Hilden, Germany). DNA was then resuspended in a final volume of 50 µL. To check whether the eluted DNA could be amplified, 5 µL of the final elution were amplified for detection of the human telomerase reverse transcriptase (TERT) gene (GenBank accession number AF128893), which was used as housekeeping gene. Amplification was performed as previously described [21].

EBV DNA Typing and Quantification
A quantitative method, based on multiplex real-time polymerase chain reaction (PCR) assay, was used to quantify EBV types 1 and 2, as described elsewhere [22]. Each PCR was performed in a 25-µL reaction mix containing 5 µL of eluted DNA (corresponding to 1.5 µL of blood). A standard reference curve was obtained by 5-fold serial dilution of 2 amplicons, one for EBV type 1 and one for EBV type 2, and amplification was performed as already described [22]. The multiplex assay showed a dynamic range from 5 to 2 × 10^5 copies. The lower limit of detection was up to 1 copy and was established by analyzing replicate dilution samples containing 1 EBV copy. According to Poisson distribution, repeated measurement of these samples gave positive results for EBV detection in 9 of 15 replicates. As amplification was performed in a final volume of 1.5 µL of blood, and a sample was considered positive when there were at least 660 EBV DNA copies/mL blood. Results are expressed as log_{10} EBV DNA copies/mL.

16S rDNA Quantification
A quantitative method based on real-time PCR assay was performed to quantify 16S rDNA, with the primer pair and probe described elsewhere [23]. A standard curve was generated from 5-fold serial dilutions of plasmid DNA containing known copy numbers of the template. The assay showed a dynamic range from 3 to 2.5 × 10^5 copies. Levels of 16S rDNA were quantified in 109 available samples. Results are expressed as log_{10} 16S rDNA copies/µL.

HIV-1 RNA Quantification
For each sample, the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) was used to extract RNA from 2 entire dried blood spots, corresponding to 100 µL of whole blood. RNA was eluted with 75 µL of the elution buffer heated to 70°C. HIV-1 RNA levels were determined with the Amplicor HIV-1 Monitor Test (Roche Diagnostic Systems, Branchburg, NJ). The lower limit of detection with the dried blood spot samples was 170 HIV-1 RNA copies/mL of blood. Results are expressed in log_{10} HIV-1 RNA copies/mL of blood. Because an HIV-1 RNA load of up to 3 log_{10} copies/mL plasma is currently considered the most appropriate cutoff to define treatment failure [24], and because ART was recommended for all women with HIV-1 RNA levels of ≥3 log_{10} copies/mL of plasma to prevent mother-to-child transmission of HIV [25], the relationship between EBV and HIV-1 was estimated according to this cutoff value.

Statistical Analyses
EBV DNA, HIV-1 RNA, and 16S rDNA data were log_{10} transformed to obtain more normal distributions. The χ^2 test for categorical variables and the Kruskal–Wallis test for continuous variables were used. Logistic regression analysis was used to assess the relationship between EBV detection status and whether ART was received, after adjustment for multiple confounders such as CD4^+ T-cell count z score, WHO disease stage, age, and sex. Logistic regression analysis and the Spearman correlation coefficient were used to evaluate relationships between status and levels of EBV DNA and ART duration. Linear regression analysis was used to assess the relationship between HIV-1 RNA load and EBV DNA levels in univariate analysis and after adjustment for multiple confounders. The association between EBV DNA and circulating bacterial 16S rDNA levels was evaluated by Spearman correlation and a linear regression model. All statistical analyses were performed with SPSS, version 18, and Stata, version 12, software.

RESULTS
EBV DNA Levels in Children
Table 1 lists the baseline characteristics of the 213 HIV-1–infected children. A total of 149 children, 92 of 140 (66%) who were receiving ART and 57 of 73 (78%) who were ART naive, had detectable EBV in blood. EBV types 1 or 2 were detected in 31 (22%) and 21 (15%) children on ART, and in 15 (21%) and 11 (15%) ART-naive children, respectively. Coinfection with both EBV types was observed in 40 (29%) ART-treated and 31 (42%) ART-naive children. Children on ART had less probability of being coinfected with both EBV types than ART-naive children (odds ratio [OR], 0.54 [95% confidence interval [CI], 0.30–0.98]; P = 0.042), and overall antiretroviral-treated children were less likely than ART-naive children to...
Table 2. Epstein-Barr Virus (EBV) Detection Among Human Immunodeficiency Virus Type 1 (HIV-1)–Infected Antiretroviral-Treated and Antiretroviral-Naive Children

<table>
<thead>
<tr>
<th>EBV Detection</th>
<th>Antiretroviral Treated, No. (%)</th>
<th>Antiretroviral Naive, No. (%)</th>
<th>Univariate</th>
<th>Multivariatea</th>
<th>Univariate</th>
<th>Multivariatea</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV type 1</td>
<td>31 (22)</td>
<td>15 (21)</td>
<td>1.10 (.55–2.20)</td>
<td>0.85 (.35–2.06)</td>
<td>.788</td>
<td>.727</td>
</tr>
<tr>
<td>EBV type 2</td>
<td>21 (15)</td>
<td>11 (15)</td>
<td>0.99 (.45–2.02)</td>
<td>1.00 (.37–2.71)</td>
<td>.989</td>
<td>.997</td>
</tr>
<tr>
<td>EBV type 1 and 2</td>
<td>40 (29)</td>
<td>31 (42)</td>
<td>0.54 (.30–.98)</td>
<td>0.50 (.23–1.08)</td>
<td>.042</td>
<td>.069</td>
</tr>
<tr>
<td>Overall</td>
<td>92 (66)</td>
<td>57 (78)</td>
<td>0.54 (.28–1.04)</td>
<td>0.40 (.17–.97)</td>
<td>.064</td>
<td>.043</td>
</tr>
</tbody>
</table>

Abbreviation: CI, confidence interval; OR, odds ratio.

* Covariates considered for multivariate model: age, CD4+ T-cell count z score, WHO stage.

EBV DNA Levels in Relation to a Marker of Immune Activation

Mean levels (±SD) of 16S rDNA were significantly lower in antiretroviral-treated children, compared with ART-naive children (2.09 ± 0.23 vs 2.16 ± 0.25 log10 copies/µL; P = .007; Figure 2A). In 109 children, 72 had detectable EBV, 30 had EBV type 1, 13 had EBV type 2, and 29 were coinfected with both EBV types. Mean levels (±SD) of 16S rDNA were similar in children infected with EBV type 1 (2.09 ± 0.27 log10 copies/µL) or type 2 (2.14 ± 0.11 log10 copies/µL) but higher in children coinfected with both EBV types (2.22 ± 0.12 log10 copies/µL; Figure 2B). Levels of 16S rDNA were correlated with EBV DNA levels (r = 0.250 [95% CI, .02–.46]; P = .031; Figure 2C). A linear regression model confirmed the significant relationship between 16S rDNA and EBV (regression coefficient = 1.01 [95% CI, 1.00–1.02]; P = .020).

**DISCUSSION**

This is the first study describing the relationship between HIV-1 infection, a marker of microbial translocation, and EBV types and viremia in HIV-1–infected African children. First of all, we found that, although healthy individuals usually harbor a single EBV type, HIV-1–infected children were more likely to be coinfected with both EBV types than with type 1 or type 2 alone. The fact that EBV type 1 transforms B cells in vitro more efficiently than type 2 has suggested type-specific differences in oncogenic activity in vivo [13, 26]. However, both types have been found in HIV-1-related lymphomas [7, 13, 26, 27], indicating that both viruses may play an oncogenic role in the context of a weakened immune system. In addition, LMP1, the master oncprotein of EBV, differs among the EBV strains, according to the number of 33 base-pair repeat elements, but not specifically between the two types [28, 29].

We found that children co-infected with both EBV types had higher EBV levels than those infected with only type 1 or type
Co-infection and levels of EBV were also related to blood levels of HIV-1 RNA and 16S rDNA, a marker of microbial translocation [10]. These results suggest that HIV-1 replication and circulating microbial products may lead to EBV replication and expansion of EBV-infected B cells, thus increasing the EBV DNA load. Superinfection with both EBV types may represent an additional risk.

Our results show that ART affects EBV infection and levels. In particular, antiretroviral-treated children have a lower probability of being coinfected with both EBV types and, overall, their EBV levels were significantly lower than those in ART-naive children. Of interest, EBV load tended to be inversely associated with ART duration, and children with a prolonged ART duration had lower EBV DNA levels. These findings suggest that ART constrains EBV superinfection and keeps EBV viremia down.

As expected, HIV-1 RNA load in total blood was significantly lower in antiretroviral-treated children, compared with ART-naive children. In this study, many children receiving ART had detectable HIV-1 RNA levels. It is worth noting that the

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**Figure 1.** Epstein-Barr Virus (EBV) DNA levels in Human Immunodeficiency Virus type 1 (HIV-1)–infected children, by EBV type (A) and antiretroviral therapy (ART) status (B). Each plot represents 1 child. Lines indicate mean values. Abbreviation: NS, not significant.
level of HIV-1 RNA extracted from a dried blood spot derives from plasma viremia plus cell-associated viral nucleic acids. Most studies agree that whole-blood samples yield higher levels of HIV-1 RNA than plasma samples, particularly when plasma viremia is low [30–33]. This aspect may be important, especially in children receiving ART, because the viral load from whole blood includes cell-associated HIV-1 RNA, which may persist even in patients with undetectable plasma viremia [34, 35], and may be considered as an indicator of residual virus replication in antiretroviral-treated patients [36]. Therefore, although we cannot rule out a few cases of poor adherence to therapy, which may lead to a weak virological response with persistent plasma viremia, most cases of detectable HIV-1 RNA among antiretroviral-treated children can be explained by residual cell-associated HIV-1 RNA.

The introduction of ART, which has resulted in a significant reduction in HIV-1 load, has also reduced circulating levels of microbial translocation products, including bacterial 16S rDNA [10, 23, 37–39]. As expected, we found that such levels were significantly lower in antiretroviral-treated children, compared with ART-naïve children. Overall, the relationships among EBV, HIV-1, and 16S rDNA suggest that ART, by limiting HIV-1 replication and reservoirs, microbial translocation, and related chronic immune activation, impedes superinfection by both EBV types, preventing stimulation of B cells and expansion of EBV-infected B cells, thus reducing the risk of the development of EBV-associated B-cell lymphomas. Further studies with more markers of immune activation on separate plasma and cell samples would be advisable to validate the relationship between immune activation and EBV load.

Analysis of dried blood spots is commonly used to diagnose and monitor different DNA/RNA viral loads [40]. However, to date only 1 study [41] has investigated EBV DNA levels from dried blood spots. EBV load assessment in peripheral blood may be an important instrument for diagnosing and monitoring EBV-associated lymphoproliferative diseases, particularly in some regions of Africa, such as Uganda, where EBV seroprevalence is nearly universal, with acquisition of infection in early childhood. Our results show that it is possible to quantify EBV DNA, HIV-1 RNA, and 16S rDNA by dried blood spot

**Figure 2.** 16S ribosomal DNA (rDNA) levels in Human Immunodeficiency Virus type 1 (HIV-1)–infected children. 16S rDNA levels in HIV-1–infected children, by antiretroviral therapy (ART) status (A), Epstein-Barr Virus (EBV) type (B), and EBV DNA load (C). Each plot represents 1 child. Lines indicate mean values. Abbreviation: NS, not significant.
analysis, thus improving diagnostic and monitoring capabilities in resource-limited settings.

Our findings should be interpreted in light of some potential limitations. First, blood specimens for dried blood spot analysis were collected in Uganda, stored at room temperature, and shipped to Italy, with a median time from collection to analysis of 49 days (range, 34–64 days). While amplification failure has been described when specimens are stored at room temperature for >30 days [42], the influence of storage conditions on the EBV amplification is unknown. Second, although multivariate analyses were performed, potential additional confounders, such as comorbidities or type of therapy, cannot be ruled out.

In conclusion, HIV-1, by inducing microbial translocation and a state of persistent immune activation, may lead to EBV replication and expansion of EBV-infected B cells, thus increasing the EBV DNA load. Superinfection by both types of EBV in HIV-1–infected subjects may represent an additional risk for the onset of EBV-related malignancies. ART, by limiting HIV–1 replication, microbial translocation, and related immune activation, may prevent superinfection by both EBV types and keep EBV viremia down, thus reducing the risk of EBV-associated lymphomas.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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