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CONCISE COMMUNICATION

Absolute Level of Epstein-Barr Virus DNA in Human Immunodeficiency Virus Type 1 Infection Is Not Predictive of AIDS-Related Non-Hodgkin Lymphoma

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To study whether Epstein-Barr virus (EBV) load can be used to predict the occurrence of acquired immunodeficiency syndrome-related non-Hodgkin lymphoma (AIDS-NHL), we determined EBV load longitudinally for individuals infected with human immunodeficiency virus type 1. EBV load in peripheral blood mononuclear cells (PBMC) was high and displayed considerable fluctuations over time, indicating that absolute EBV load in PBMC is not predictive of the development of AIDS-NHL. EBV DNA was also detectable in serum at some time points but at a lower level.

Epstein-Barr virus (EBV) is a widespread human gamma herpesvirus, which persists in a latent form in B lymphocytes as episomes in the B cell nucleus that can range from 5 to 500 episomes per infected B cell [1]. The latently infected B cells are controlled by specific cytotoxic T lymphocyte responses [2]. In human immunodeficiency virus (HIV) type 1-infected individuals, reactivation of EBV infection can lead to uncontrolled lymphoproliferation [3], resulting in AIDS-related non-Hodgkin lymphoma (AIDS-NHL). The majority of these AIDS-NHLs are EBV-positive diffuse large B cell lymphomas (DLCLs) [4].

EBV load is a possible predictor of the occurrence of lymphoproliferative disorders [5]. Cross-sectional studies that used semiquantitative and quantitative polymerase chain reaction (PCR) assays to measure EBV load in peripheral blood mononuclear cells (PBMC) or whole blood have shown that EBV load at diagnosis was much higher in transplant recipients and AIDS-NHL patients (4×10^4 to $>1.5 \times 10^6$ and $>2 \times 10^5$ EBV copies/ 10^6 PBMC, respectively) [6–9] than in healthy EBV-seropositive individuals (<0.1 to 5 EBV copies/ 10^6 PBMC) [6, 10]. Several studies have shown that high EBV load in serum or plasma correlates with the occurrence of posttransplantation lymphoma [11, 12].

The aim of this study was to determine whether EBV load can be used to predict the occurrence of AIDS-NHL in HIV-

infected individuals at an early stage. Therefore, we performed a longitudinal study, using a real-time quantitative PCR assay to determine the number of EBV virus DNA in both PBMC and serum for different groups of HIV-infected individuals.

Patients, Materials, and Methods

Study population. This study was performed on longitudinal PBMC and serum samples obtained from 21 HIV-1-infected homosexual men included in the Amsterdam Cohort Studies on HIV/AIDS [13]. We studied several time points during HIV-positive follow-up, from a few years after seroconversion or study entry through AIDS diagnosis or late in HIV infection. The individuals were selected to represent 3 distinct groups of HIV-infected individuals. For 9 individuals, AIDS-related diffuse large cell NHL (AIDS-NHL) was the first AIDS-defining event. Seven individuals progressed to AIDS (according to the classification of the Centers for Disease Control and Prevention [14]) without developing a lymphoma (“progressors”) but had duration of follow-up similar to that of the AIDS-NHL group (median duration, 94 months). Among the progressors, 5 developed an opportunistic infection, and 2 developed Kaposi sarcoma. Five individuals remained long-term asymptomatic (LTA) with $CD4^+$ T cell counts >500 cells/ mm^3 over the course of >8 years of HIV-seropositive follow-up (median duration, 141 months).

Lymphocyte isolation and DNA extraction. Cryopreserved PBMC (1×10^6 cells) were lysed by addition of L6 lysis buffer. Genomic DNA was extracted by precipitation with isopropanol, washed twice with 70% ethanol, and dissolved in distilled water. To isolate DNA from serum, the QIAamp blood kit (Qiagen) was used according to the protocol supplied by the manufacturer.

Real-time quantitative PCR assay. DNA from 2×10^5 PBMC or 40 μ L of serum was amplified in duplicate, using PCR primers selective for the EBV DNA genome that encodes the nonglycosylated membrane protein BNRF1 p143. PCR amplification was performed, as described elsewhere [15], in a 50- μ L volume con-

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Informed consent was obtained from each patient included in this study.

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taining $2\times$ TaqMan Universal Mastermix (PE Biosystems) with $45\ \mu\text{M}$ of the EBV/p143 forward primer 5'-GGA-ACC-TGG-TCA-TCC-TTT-GC-3' and $2.5\ \mu\text{M}$ of reverse primer 5'-ACG-TGC-ATG-GAC-CGG-TTA-AT-3' (Isogen Biosciences). In the PCR, 5 pmol of a fluorogenic probe was added (5'-CGC-AGG-CAC-TCG-TAC-TGC-TCG-CT-3'; PE Biosystems), with a FAM reported molecule at the 5' end and a TAMRA quencher at the 3' end, to detect amplified DNA.

Amplification and detection were performed with an ABI Prism 7700 Sequence Detection System (PE Biosystems). A threshold cycle (Ct) value was calculated by determination of the point at which the fluorescence exceeded a threshold limit of 0.04. Each run contained negative controls (no template or EBV-negative DNA), a positive control (a known amount of EBV copies), and a standard serial half-log dilution of plasmid DNA containing the PCR product as an insert (range, 1×10^2 – 1×10^8 copies/mL). The standard curve was created automatically with the ABI Prism 7700 software, by plotting of the Ct values against each standard of known concentration.

The assay was shown to detect as few as 50 EBV copies/mL of plasma (2 copies/reaction) and had a linear range as high as 1×10^7 copies/mL. The assay is very accurate, with a variability (both intra-assay and interassay) of $<3\%$; only in the low copy number range does variability reach 12%.

Statistical analysis. Spearman's correlation test was used to correlate EBV copies in PBMC with EBV copies in serum. The Wilcoxon signed rank test was used to compare EBV load at early and late time points for the different groups, using SPSS software (version 7.5 for Windows).

Results

Longitudinal analysis of EBV load in PBMC of HIV-1-infected individuals. To investigate the kinetics of EBV load during HIV-1 infection, we determined EBV load in 9 AIDS-NHL patients with DLCLs, 5 LTA individuals, and 7 progressors. Figure 1 shows the results for 6 representative AIDS-NHL patients, 3 LTA individuals, and 3 progressors. Over time, most AIDS-NHL patients displayed bursts in EBV load (figure 1A) that could reach levels of $>1\times 10^6$ copies/ 10^6 PBMC (figure 1A, patient NHL8199). However, in most of these AIDS-NHL patients, EBV load did not reach levels $>2\times 10^4$ copies/ 10^6 PBMC (figure 1A, patients NHL0292, NHL0068, and NHL0118). EBV load at the time that AIDS-NHL was diagnosed was 1.4×10^3 – 3.1×10^6 copies/ 10^6 PBMC (figure 1A). This absolute amount of EBV DNA was not related to CD4⁺ T cell numbers or HIV RNA load at the time of diagnosis or to HIV-positive follow-up (data not shown).

Although in the LTA group, EBV load could be undetectable early in the course of HIV-1 infection (figure 1B, patient LTA1160), bursts of EBV load also were observed that reached levels of 7×10^4 copies/ 10^6 PBMC (figure 1B). Similarly, bursts of EBV load were observed in the group of progressors (figure 1C). EBV load peaks varied from 1×10^4 to 1.6×10^5 copies/ 10^6 PBMC but decreased again to baseline values in most LTA

individuals and progressors. Bursts of EBV load were not caused by increases in the number of B cells in the blood (data not shown).

We calculated the mean EBV load for each individual both early (approximately the first 4 years of follow-up) and late (approximately the last 4 years of follow-up) in infection. As shown in figure 2, there was no difference in absolute EBV load (mean) between AIDS-NHL and LTA individuals, either early or late in infection ($P = .44$ and $P = .7$, respectively; Mann-Whitney *U* test). The mean EBV load for AIDS-NHL patients and that for the group of progressors also did not differ ($P = .41$ early in infection and $P = 1$ late in infection; Mann-Whitney *U* test). In addition, no difference was found among the 3 groups when we compared the highest peaks in EBV load observed for each individual during the entire follow-up period (data not shown). Interestingly, whereas LTA individuals and progressors had a stable EBV load or a significant decrease ($P = .35$ for the LTA group and $P = .018$ for the progressor group; Wilcoxon test), 7 of 9 AIDS-NHL patients had an increase in EBV load during the course of HIV-1 infection ($P = .05$; Wilcoxon test). The change in EBV load for AIDS-NHL patients was significantly different from that for progressors ($P < .008$; Mann-Whitney *U* test).

EBV load in PBMC versus EBV load in serum. We subsequently compared the EBV load in PBMC with that in serum for 7 AIDS-NHL patients, 3 LTA individuals, and 1 progressor from whom serum samples were available at the same time points. Figure 1A and 1B shows the results for 4 AIDS-NHL patients (patients NHL0068, NHL0118, NHL0308, and NHL6006) and 2 LTA individuals (patients LTA0750 and LTA0036). In most individuals at some time points EBV could be detected in serum. Levels were much lower, however, than levels in PBMC, and the kinetics often were different. One AIDS-NHL patient had detectable levels of EBV in serum only at the last time point studied (figure 1A, patient NHL0068). Interestingly, a high peak in the serum load occurred when the load in PBMC was decreasing after initiation of highly active antiretroviral therapy (figure 1A, patient NHL0068) and following the initiation of successful chemotherapy after a diagnosis of lymphoma was made (figure 1A, patient NHL6006).

Overall, no correlation between EBV load in PBMC and EBV load in serum was found (data not shown).

Discussion

In the present study, we investigated the kinetics of EBV load in PBMC and serum in different groups of HIV-1-infected individuals, using a real-time quantitative PCR assay. We observed bursts in the EBV load in PBMC in all subgroups of HIV-infected patients that we studied and found that the absolute EBV load is not predictive of whether AIDS-NHL will develop. Furthermore, there is no clear correlation between EBV load in PBMC and EBV load in serum.

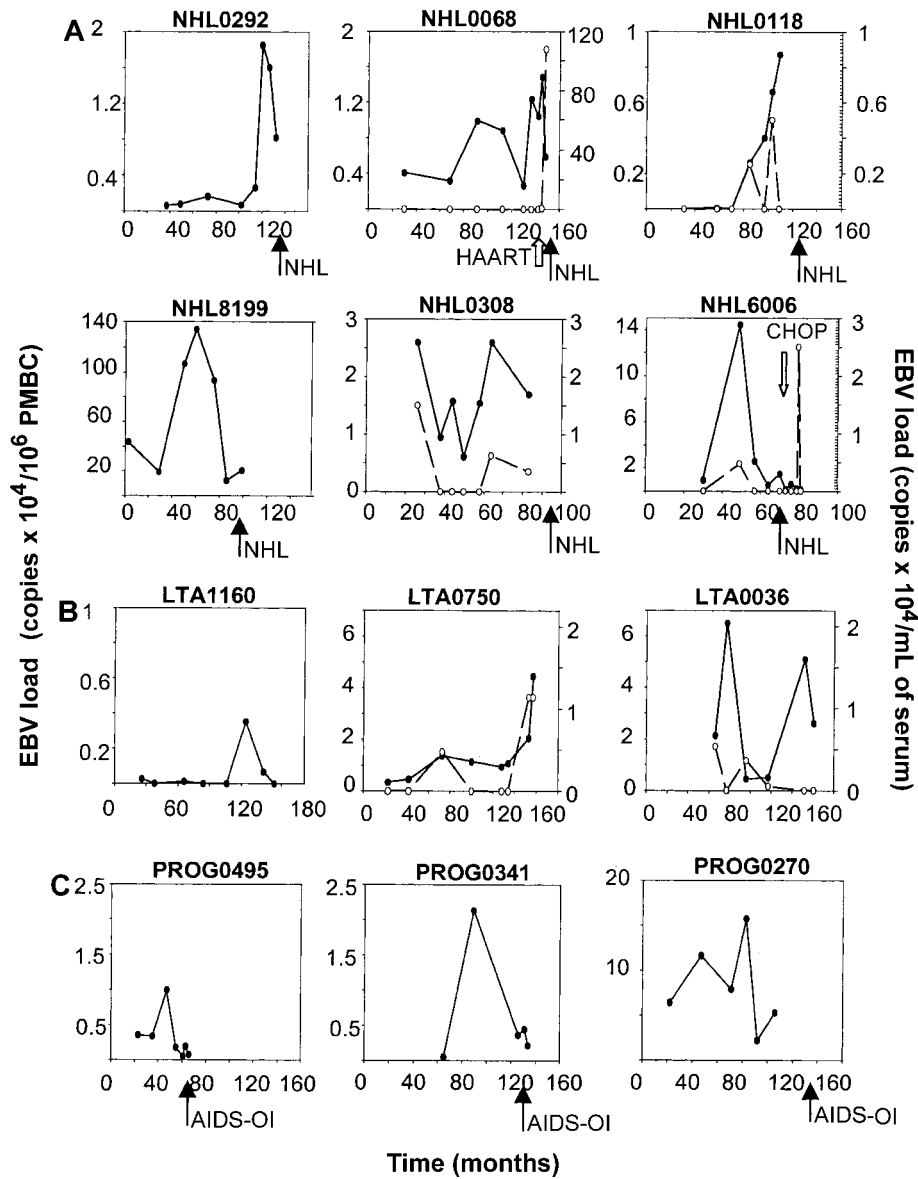


Figure 1. Epstein-Barr virus (EBV) load in peripheral blood mononuclear cells (PBMC; *solid lines*) and in serum (*dashed lines*) for 12 individuals with human immunodeficiency virus infection, measured by a real-time quantitative polymerase chain reaction assay. Measurements were made beginning a few years after seroconversion or on study entry and continued until (A) the diagnosis of AIDS-related non-Hodgkin lymphoma (NHL), for those who developed lymphoma ($n = 6$); (B) late in infection, for long-term asymptomatic (LTA) individuals ($n = 3$); or (C) the diagnosis of AIDS, for those who progressed to AIDS but did not develop a lymphoma (PROG; $n = 3$). Black arrows indicate diagnosis of NHL or AIDS-related opportunistic infection (AIDS-OI). White arrows indicate initiation of chemotherapy (CHOP) or highly active antiretroviral therapy (HAART).

The EBV load found in HIV-1 infected individuals in the present study was comparable with that reported elsewhere for pediatric bone marrow transplant recipients with lymphoproliferative disorders [6–8, 16] and for patients with AIDS-NHL [9]. The latter study did show a relationship between high EBV load and the development of a lymphoma, but no other groups of HIV-infected individuals were investigated. Although EBV load

measurements seemed to be useful for predicting the development of a lymphoma in transplant recipients, this did not appear to be the case for HIV-infected individuals. Of course, the first study group was more homogeneous; all patients received similar immunosuppressive treatment regimens (e.g., similar dosages) [6–8, 16], whereas, in the present study, the HIV-infected individuals all had different virologic and immunologic status. Because, on

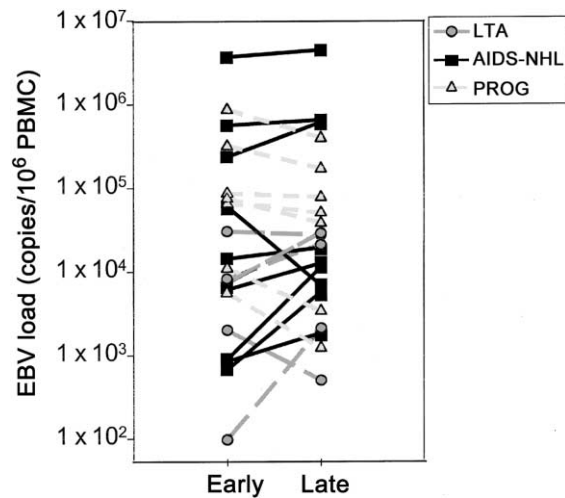


Figure 2. Mean Epstein-Barr virus (EBV) load in peripheral blood mononuclear cells (PBMC) both early (approximately the first 4 years of follow-up) and late (approximately the last 4 years of follow-up) in infection for 21 individuals with human immunodeficiency virus infection, measured by a real-time quantitative polymerase chain reaction assay. Data for long-term asymptomatic (LTA) individuals ($n = 5$) are represented by dark gray dashed lines; for those who developed AIDS-related non-Hodgkin lymphoma (AIDS-NHL; $n = 9$), by solid lines; and for those who progressed to AIDS without developing a lymphoma (PROG; $n = 7$), by light gray dashed lines.

the one hand, EBV load was high in all HIV-infected individuals, and, on the other, some of the AIDS-NHL patients did not have an EBV load $>2 \times 10^4$ copies/ 10^6 PBMC, other factors must be involved in the development of NHL in HIV-infected individuals.

Although EBV load alone may not be predictive of AIDS-NHL, the combination of measurements of EBV load and of cellular immunity against EBV may provide more information on the balance between virus and host and, thus, may have predictive value. As shown in our previous study [17], the combination of a low EBV load with collapsing cellular immunity against EBV results in a higher risk of AIDS-NHL than does the combination of a high EBV load with stable numbers of EBV-specific CD8⁺ T lymphocytes [17]. Similarly, the return of EBV load from peak to baseline values in LTA individuals and patients with AIDS who develop opportunistic infections suggests that EBV control is occurring. Furthermore, when the mean EBV load for early and for late follow-up was calculated, the load remained stable in LTA individuals, whereas AIDS-NHL patients show a significant increase in EBV load during the course of HIV-1 infection.

We found that the kinetics of EBV load in PBMC differs from that in serum. Overall, EBV load in serum was lower than load in PBMC and was often undetectable. In only 1 of 9 AIDS-NHL patients was EBV load in serum high at the time that AIDS-NHL was diagnosed. In addition, an increase in EBV load in serum was usually observed after a peak in EBV load

in PBMC. This may be due to elimination or apoptosis of EBV-infected B cells, resulting in the release of free EBV DNA into serum. When a lymphoma is present, the number of cell-free EBV DNA copies can be high because of the frequently observed spontaneous apoptosis of tumor cells or because of tumor lysis resulting from chemotherapy.

In conclusion, among HIV-1 infected individuals, absolute EBV load is high and is not predictive of the development of AIDS-NHL, although an overall increase in EBV load seems to parallel the occurrence of AIDS-NHL. This suggests that the risk for AIDS-NHL is not solely determined by EBV load.

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