Tight Regulation of the Epstein-Barr Virus Setpoint: Interindividual Differences in Epstein-Barr Virus DNA Load Are Conserved after HIV Infection

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Healthy individuals carry a constant number of Epstein-Barr virus–infected B cells in the peripheral blood over time. Here, we show that interindividual differences in Epstein-Barr virus DNA levels are maintained after HIV infection, providing evidence for the existence of an individual Epstein-Barr virus setpoint. Immune activation may contribute to the increase in this setpoint after human immunodeficiency virus seroconversion.

In immunosuppressed individuals, Epstein-Barr virus (EBV) is associated with a wide array of lymphoid malignancies, making it one of the most important tumor-associated viruses [1]. EBV is a common γ-herpes virus that persists for life in 95% of the adult human population [2]. In healthy carriers, reactivation of the virus and proliferation of EBV-infected cells is regulated mainly by $CD8^+$ T cells [3], and infection is strictly restricted to the memory B cell compartment [4]. Under the influence of iatrogenic immunosuppression, such as organ or hematopoietic stem cell transplantation, it is clear that uncontrolled proliferation of EBV-infected B cells results from lack of immune control, and the absolute number of DNA copies in PBMC or plasma is a good indicator of the relative risk for lymphoproliferative disease [5, 6]. In contrast, in the context of coinfections with other pathogens, such as HIV or *Plasmodium falciparum*, it is much more complicated to discern the effects of (T cell) immunosuppression. This is because of major alterations in the immune system as a whole, including extensive perturbations of the normal B cell biology, which are characteristic of these infections [7]. This could be the main reason why the level of EBV DNA in HIV-infected individuals is often elevated [8] and not a good predictor of EBV-related lymphoproliferative disease [9]. In support of this, we recently observed a steep increase in EBV DNA load in PBMCs shortly after HIV infection, despite a functional EBV-specific $CD8^+$ T cell response [10] and no correlation with total $CD4^+$ T cell count. Therefore, we hypothesize that increased immune activation and stimulation of B cells could be a factor that leads to increased EBV DNA levels in the peripheral blood. Furthermore, long-term follow-up studies suggest that healthy individuals carry a surprisingly constant number of EBV-infected memory B cells in the peripheral blood for years, suggesting that there is an individual EBV setpoint [11]. In this study, we aim to address whether these interindividual differences in EBV setpoint are conserved after HIV infection and whether generalized immune activation may be a factor contributing to the increase in this setpoint after seroconversion for HIV.

METHODS

EBV load and immune activation markers on T cells were analyzed in PBMCs obtained from 36 EBV-sero-

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positive HIV seroconverters in the Amsterdam cohort studies on HIV and AIDS before HIV seroconversion ($n = 36$), at 1 year after HIV seroconversion ($n = 36$), and at 5 years after HIV seroconversion ($n = 13$) [10] and in PBMCs obtained from 10 HAART-treated individuals [12] Procedures performed were in accordance with the institutional medical ethical committee and the Helsinki Declaration of 1975, as revised in 1983. Written informed consent was obtained from the subjects at inclusion in the Amsterdam cohort studies.

Genomic DNA from 2×10^5 cells was amplified in duplicate using PCR primers specific for the nonglycosylated membrane protein BNRF1 p143. Real-time PCR amplification was performed [9] using a fluorogenic probe to detect the 74–base pair product. As a control for the input DNA, the β-albumin DNA level was also determined [10]. All individuals studied were seropositive for EBV at the time of sampling. Absence of viral DNA indicates an EBV DNA load less than the threshold value of 50 copies per 10^6 PBMCs.

Activation markers on PBMC were analyzed by 4-color fluorescence cytometry after staining with fluorochrome conjugated Mabs to CD4, CD8, HLA-DR, and CD38. To study the fraction of cells in cycle, cells were first stained for extracellular markers, permeabilized (FACS Permeabilizing Solution, BD), and stained intracellularly with a fluorescein isothiocyanate–conjugated Mab for Ki67.

RESULTS AND DISCUSSION

To study whether interindividual differences in EBV load setpoint are maintained after HIV seroconversion, we set out to analyze EBV DNA load over time in samples from both untreated and treated HIV-infected individuals. Within a subset of these samples, we investigated whether changes in EBV DNA levels over time in individual virus carriers might correlate with changes in immune activation.

To address the first question, we correlated EBV load before and after HIV seroconversion (in 36 individuals at 1 year and in 13 individuals at 5 years after HIV seroconversion). In these 36 samples from an earlier study [10], EBV load increased significantly, from a median of 205 copies per 10^6 PBMC (range, 0–4603 copies per 10^6 PBMCs) before HIV seroconversion to 1002 copies per 10^6 PBMCs (range, 0–50,416 copies per 10^6 PBMCs) at 1 year after HIV seroconversion ($P < .001$, by Wilcoxon signed rank test); in the subset of 13 samples, EBV load increased from 1827 copies per 10^6 PBMCs to 2478 copies per 10^6 PBMCs between 1 and 5 years after HIV seroconversion, but this increase was not statistically significant ($P = .530$). Interestingly, there was a significant correlation between EBV DNA load before HIV seroconversion and EBV load both at 1 year ($n = 36$; $R = 0.555$; $P < .001$) (figure 1*A*) and at 5 years after HIV seroconversion ($n = 13$; $R = 0.599$; $P = .031$) (figure 1*B*). Comparable results were found in 10 individuals included in a longitudinal study on the effects of HAART [12]. EBV DNA load early in HIV infection (at 20 months) strongly correlated both with EBV load later in HIV infection (at 7 years; $R = 0.782$; $P = .008$) (figure 1*C*) and EBV load after 5 years of effective antiretroviral treatment ($R = 0.661$; $P = .038$) (figure 1*D*). Thus, interindividual differences in EBV load appear to be maintained after HIV infection. Importantly, this may explain why the absolute level of EBV load is not a definite correlate of loss-of-control over EBV, independent of the mechanisms that lead to an increase of EBV load in an individual.

Although changes in EBV load over time after HIV seroconversion were not statistically significant, we set out to test whether the changes that do occur would correlate with immune activation. Therefore, we analyzed data from 25 HIV-infected men who have sex with men at 1 and 5 years after HIV seroconversion (an enlarged subset including the individuals described in the study by Piriou et al. [10]), for whom consecutive samples were available for immune activation marker analyses. In these 25 individuals, EBV DNA load increased from a median of 1049 copies per 10^6 PBMCs (range, 0–50,416 copies per 10^6 PBMCs) at 1 year to 1381 copies per 10^6 PBMCs (range, 0–190,625 copies per 10^6 PBMCs) at 5 years after HIV seroconversion ($P = .114$). As an indication of changes in immune activation, the percentage of $CD38^+HLA\text{-}DR^+$ T cells increased from 3.14 (range, 0.59–7.87) at 1 year to 4.71 (range, 0.93–16.23) at 5 years for $CD4^+$ T cells ($P = .030$) and from 2.58 (range, 0.54–11.37) at 1 year to 3.42 (range, 0.11–24.36) at 5 years for $CD8^+$ T cells ($P = .638$). We calculated correlations between changes in EBV DNA load and T cell immune activation markers (both indexed to the value at 1 year after HIV seroconversion, because there was a wide range in both EBV DNA load and proportions of activated T cells). Interestingly, although changes in EBV load did not correlate with changes in total $CD4^+$ or $CD8^+$ T cell counts ($R = -0.244$ and $R = 0.223$, respectively, by Spearman's correlation test; data not shown), changes in EBV load correlated positively with a number of T cell activation markers on both $CD4^+$ T cells (for $CD38^+$HLA-DR, $R = 0.489$ and $P = .013$; for Ki67, $R = 0.527$ and $P = .007$; for CD70, $R = 0.490$ and $P = .013$) (figure 1*E;* data not shown) and $CD8^+$ T cells (for $CD38^+HLA\text{-}DR$, $R = 0.452$ and $P = .023$; for Ki67, $R = 0.548$ and $P = .005$; for CD70, $R = 0.457$ and $P = .022$) (figure 1*F;* data not shown). This indicates that changes in EBV load, especially in the individuals in whom these are most important, are more strongly associated with changes in immune activation than with generalized immunodeficiency.

In our opinion, these data show that, although it remains unclear how HIV affects the biology of EBV, interindividual differences in EBV DNA load are in great part conserved, even during antiretroviral treatment. Importantly, these interindividual differences—whether intrinsic differences in EBV-spe-

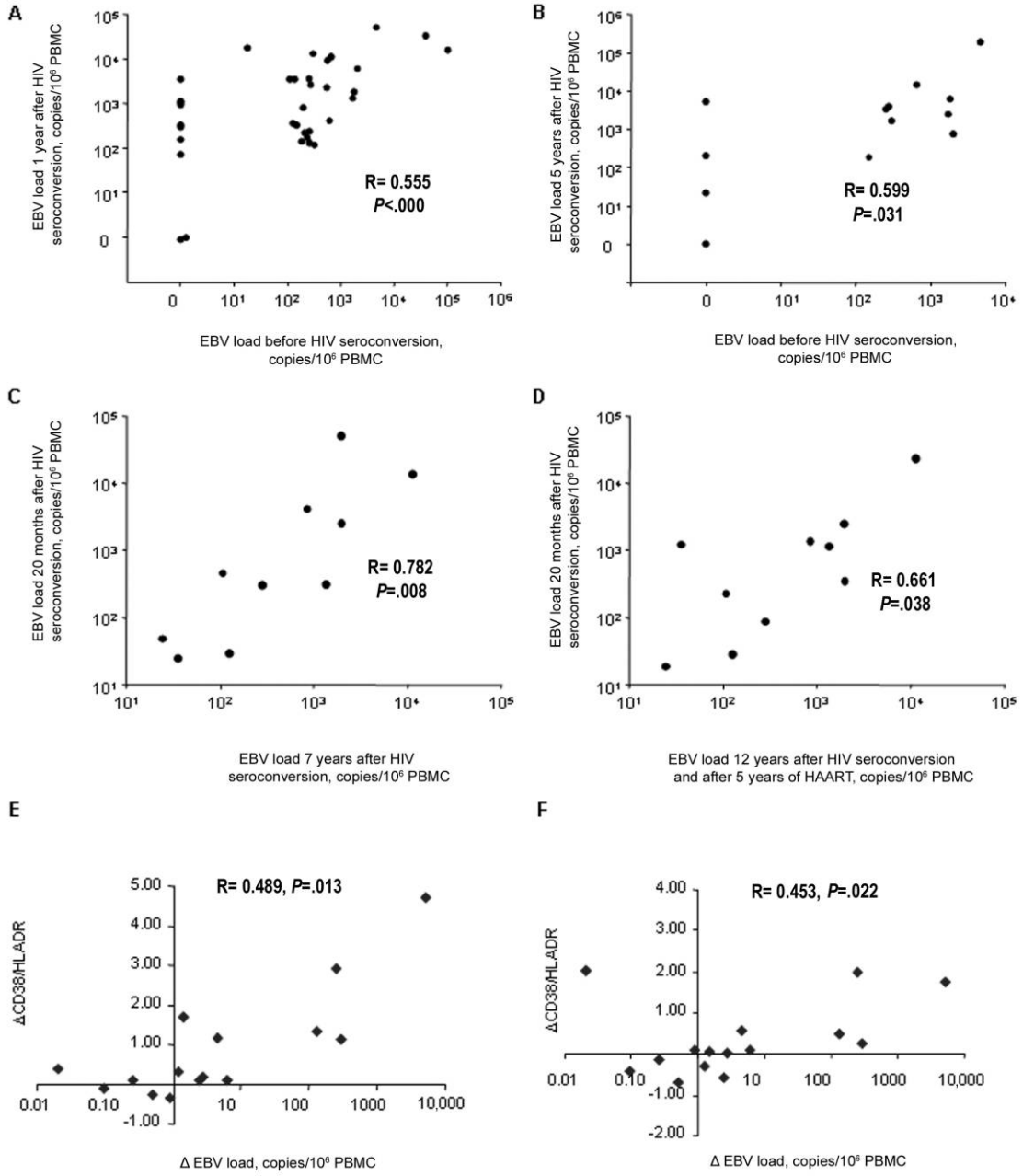

Figure 1. *A–D,* Correlations between Epstein-Barr virus (EBV) load in PBMC at different times before and after HIV seroconversion and before and after antiretroviral treatment of HIV infection. *A,* Correlation between EBV load before HIV seroconversion (median time before HIV seroconversion, 30 months) and 1 year after HIV seroconversion in 36 individuals. *B,* Correlation between EBV load before and 5 years after HIV seroconversion for 13 individuals. *C* and *D,* EBV load early in HIV infection (at 20 months) in 10 individuals correlated with EBV load after 7 years of HIV infection and before antiretroviral treatment *(C)* and after 12 years of HIV infection and 5 years of effective antiretroviral treatment *(D). E* and *F,* Correlations between changes in EBV load and immune parameters during HIV infection. Changes in EBV load (indexed to first time point, on the x-axis), were correlated to the change in CD38 and HLA-DR coexpression on $CD4^+$ T cells *(E)* and $CD8^+$ T cells *(F).* Correlation coefficient and significance are indicated in the figure (Spearmans' correlation test).

cific immune control, chronic immune activation, virulence of the particular EBV strain(s) carried, or any other individual factor—could mask the predictive value of EBV DNA load. It has been shown that HIV infection is associated with high levels of immune activation [13]. When studying T cell immune activation markers and taking the interindividual differences in EBV DNA load into account, it appears that immune activation may well be an essential factor leading to an increase of EBV DNA load in HIV-infected individuals. How exactly immune activation leads to an increase in cell-associated EBV DNA load

remains to be determined. It is known that HIV infection greatly affects the normal biology of both T and B cells [7, 14, 15]. An important alteration of the regulation of B cell memory generation and more frequent stimulation of these cells is very likely to affect the normal life-cycle of EBV, which may lead to an increase in the number of EBV-infected B cells. Stimulation of EBV-infected memory B cells and subsequent differentiation into plasma cells has been shown to lead to EBV reactivation [16]. Whether HIV can directly reactivate EBV from infected B cells—an analogy that was recently shown for *P. falciparum* [17]—is unknown at this point.

These data should encourage the organization of detailed longitudinal studies of EBV in the context of predominant coinfections, including determination of numbers of infected cells, niche of the virus (memory B cells, other B cells, or other cells), and EBV strains present over time. This would provide a better understanding of the coevolution of this virus in the human lymphoid system and how both host and pathogen have adapted to allow for an extremely well-regulated interaction, even in the presence of major coinfections.

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