Viro-immunological studies on the role of Epstein-Barr virus in the development of AIDS-related non-Hodgekin's lymphoma
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Chapter 4

Epstein-Barr virus (EBV) load in Human immunodeficiency virus-1 infection is not predictive of AIDS-related non-Hodgkin’s lymphoma

submitted for publication
Epstein-Barr virus (EBV) load in Human Immunodeficiency Virus-1 infection is not predictive of AIDS-related non-Hodgkin’s Lymphoma

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(1) Purpose: To study whether EBV load can predict the occurrence of AIDS-related non-Hodgkin’s lymphoma (AIDS-NHL) in (HIV)-1 infected individuals.

(2) Patients and Methods: A real time quantitative PCR assay was used to study EBV load in PBMC of HIV+ and HIV- homosexual individuals and to compare EBV load in the course of HIV-1 infection in PBMC and serum of HIV-infected individuals who progressed to AIDS-NHL, AIDS without lymphoma or who were long-term asymptomatic (LTA).

(3) Results: In a cross-sectional study, EBV load in PBMC of HIV+ homosexual men was found to be 100-fold higher than of HIV- homosexual men who had a higher EBV load than HIV- heterosexual individuals. These differences were not related to the degree of immunodeficiency or EBV type(s) present. In a longitudinal analysis, overall EBV load in PBMC of all individuals proved to be high and displayed considerable fluctuations over time. In most individuals tested EBV was also detectable in serum at some time points, but at a lower level. Overall, no correlation was found between EBV load in PBMC and serum. In 7 out of 9 AIDS-NHL patients EBV load showed a steady increase prior to lymphoma development, suggestive of decreasing immune control, whereas in LTA and progressors to AIDS bursts of EBV load were followed by a decrease to baseline, suggestive of EBV-control.

(4) Conclusion: Absolute EBV load in PBMC is not predictive of the development of AIDS-NHL. We postulate that the risk for AIDS-NHL is determined by the balance between EBV load and EBV immune control.

Introduction

Epstein-Barr Virus (EBV) is a widespread human gamma herpesvirus, 1; 2 which infects squamous epithelial cells in the oropharynx and B lymphocytes in the peripheral blood. 3; 4 After primary infection, which usually occurs asymptomatically, 1 the virus persists for life in a latent form in the B lymphocytes. 2 The viral genomes form episomes in the B cell nucleus, which can range from 5 to 500 per latently infected B cell. 5

The initial expansion and reactivation of these latently infected B lymphocytes is controlled by specific cytotoxic T lymphocyte (CTL) responses. 6 During immunodeficiency, a higher rate of reactivation of EBV-infection can lead to uncontrolled lymphoproliferation. 7

Thus, in human immunodeficiency virus
(HIV)-1 infected individuals, the incidence of diffuse large cell non-Hodgkin's lymphomas (AIDS-NHL) is considerably increased (5-10%). Lymphoproliferation in AIDS-NHL patients is accompanied by a decrease in EBV-specific T cell immunity and an increase in the number of infected B cells as measured in an in vitro EBV transformation assay.  

To study EBV load in normal and immunocompromised individuals, biological assays have been used based on the spontaneous outgrowth of EBV-transformed B cells. These assays depend on the transforming capacity of the EBV strain(s) present and are time-consuming. With the use of semi- and quantitative PCR techniques EBV load (number of DNA copies) can be measured more easily and with high precision. Cross-sectional studies using these PCR assays have shown that in individuals with lymphoproliferative disorders, such as transplant recipients and AIDS-NHL patients, EBV load at diagnosis was much higher (40000 to >1500000 and >200000 EBV copies/10⁶ PBMC, respectively) than in healthy EBV-seropositive individuals (5 to <0.1 EBV copies per 10⁶ PBMC). Although in most studies EBV load was investigated in peripheral blood mononuclear cells, several studies have shown that high EBV load in serum or plasma correlates with the occurrence of post-transplant lymphoma, Hodgkin's disease or the presence of a Nasopharyngeal Carcinoma (NPC). In only one study EBV load in PBMC was compared with EBV load in serum in a cross-sectional analysis. However, no studies were performed longitudinally to compare these two compartments.

The aim of this study was to determine whether the EBV load is a parameter that can predict the occurrence of NHL in immunocompromised 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 particles in both PBMC and serum in the course of HIV-1 infection in AIDS-NHL patients. For comparison, EBV load was measured in the course of HIV-1 infection in HIV-1 infected individuals who progressed to AIDS with Opportunistic Infections (AIDS-OI) or Kaposi's Sarcoma (AIDS-KS) or were long-term asymptomatic (LTA). In addition, we performed a cross-sectional study to compare EBV load in PBMC between HIV⁺ and HIV⁻ homosexual men and HIV⁻ heterosexual individuals.

Materials and methods

Study population

This study was performed on participants of the Amsterdam Cohort studies on AIDS and HIV-1 infection. Blood samples from these (homosexual) individuals at risk for HIV-1 infection were collected every three months for HIV-1 serology and immunological studies. In addition, at all time points PBMC were cryopreserved. In a cross-sectional analysis, we studied 20 HIV-1 seronegative heterosexual healthy volunteers, 36 HIV-1
seronegative homosexual men and 65 HIV-1 seropositive homosexual men. The HIV-1 seropositive homosexual men consisted of 22 long-term non-progressors or slow progressors and 43 progressors to AIDS. Both groups were matched by 1) initial CD4 count or year of seroconversion/study entry, 2) age at seroconversion/first HIV positive visit, 3) HIV status (seroconverter or seropositive at entry).

In a longitudinal analysis, we studied 21 HIV-1 infected individuals: 9 who progressed to AIDS related diffuse large cell NHL (AIDS-NHL); 7 who progressed to AIDS (classification of the Centers for Disease Control 1993) without a lymphoma (progressors, PROG). Of these progressors, 5 developed an opportunistic infection (AIDS-OI) and 2 developed Kaposi’s Sarcoma (AIDS-KS). Furthermore, 5 HIV-1 seropositive long-term asymptomatic individuals (LTA) with CD4+ T cell counts above 500/mm³ during more than 8 years of seropositive follow-up were studied.

Characteristics of the 3 different patient groups are summarized in Table 1.

<table>
<thead>
<tr>
<th>group</th>
<th>n†</th>
<th>median age* (range)</th>
<th>median follow-up† (range)</th>
<th>median CD4 counts* (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS-NHL</td>
<td>9</td>
<td>40 (35-55)</td>
<td>96 (25-149)</td>
<td>0.15 (0.04-0.85)</td>
</tr>
<tr>
<td>PROG</td>
<td>7</td>
<td>37 (33-47)</td>
<td>92 (53-134)</td>
<td>0.15 (0.09-0.23)</td>
</tr>
<tr>
<td>LTA</td>
<td>5</td>
<td>42 (35-48)</td>
<td>141 (95-167)</td>
<td>0.36 (0.24-0.50)</td>
</tr>
</tbody>
</table>

† number of patients; *median age in years at HIV-1 seroconversion or first seropositive visit; † median seropositive follow-up (months), from HIV-1 seroconversion or first seropositive visit until AIDS-diagnosis or last time point studied; * median CD4+ T cell numbers at AIDS-diagnosis or for LTA last time point studied; * patient group studied consisted of HIV-1 infected individuals who progressed to AIDS-related non-Hodgkin’s lymphoma (AIDS-NHL), AIDS with opportunistic infections or Kaposi Sarcoma (PROG) and Long-term asymptomatic individuals (LTA).
Lymphocyte isolation and DNA extraction

PBMC were isolated from heparinized blood by Ficoll-hypaque density centrifugation and 1x10^6 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 H₂O. To isolate DNA from serum the QIAamp blood kit was used according to the protocol as supplied by the manufacturer (QIAGEN, USA).

The B95.8 cell line and the Ag876 cell line were used as a source of EBV type 1 and EBV type 2, respectively. The EBV-negative B cell line BJAB was used as a negative control.

Real-time quantitative PCR assay

DNA from 2x10^5 cells or 40 µl serum was amplified in duplicate using PCR primers selective for the EBV DNA genome encoding the non-glycosylated membrane protein BNRF1 p143. PCR amplification was performed as previously described in a 50 µl volume containing 2x TaqMan universal mastermix using 45 pmol/µl of the EBV/p143 forward primer 5’-GGA.ACC.TGG.TCA.TCC.TTT.GC-3’ and 2.5 pmol of reverse primer 5’-ACG.TGC.ATG.GAC.CGG.TTA.AT-3’ (Isogen Biosciences, Maarssen, The Netherlands) resulting in a 74 basepairs DNA product. In the PCR reaction 5 pmol of a fluorogenic probe was added (5’-C.AGG.CAC.TCG.TAC.TGC.CTT-3’, PE Biosystems, Nieuwekerk aan de IJssel), with a FAM reported molecule attached to the 5’ end and a TAMRA quencher linked at the 3’ end, to detect amplified DNA. After preparing the reaction tubes, the whole plateholder was centrifuged for 1 minute at 1000 g at room temperature in a swing out rotor (Hettich Rotina 48R, Tuttingen, Germany) to remove small air bubbles in the vessels.

Amplification and detection was performed with an ABI Prism 7700 Sequence Detection System (PE, Biosystems). Real time measurements were taken and a threshold cycle (Ct) value was calculated for each sample by determining the point at which the fluorescence exceeded a threshold limit of 0.04. Each run contained several 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 insert (range from 100 up to 10 million copies per ml). The standard curve was created automatically by the ABI 7700 Sequence Detection System software, by plotting the Ct values against each standard of known concentration.

The assay was shown to detect as few as 50 EBV copies per ml plasma, thus 2 copies per reaction, and has a linear range up to at least 10 million copies per ml. The assay is very accurate with a variability (both intra- and interassay) of less than 3% and only in the low copy number range a variability of up to 12%.
Polymerase Chain reaction (PCR) for EBV typing

Genomic DNA (1μg) was amplified in a nested-PCR in 50 μl reactions containing 5.0 μl 10x PCR buffer, 1.5 mM MgCl₂, 10mM dNTPs and 1 U DNA Taq polymerase as described recently using 100 ng of the non-type discriminating EBNA-2 primers, EBNA-2F (5'-TGG.AAA.CCC.GTC.ACT.CTC-3') and EBNA-2I (5'- TAA.TGG.CAT.CGG.TGG.AAT.G -3') in the first reaction. The region within the EBNA-2 gene discriminating between EBV type 1 and 2 was amplified with the type-specific nested primers EBNA-2C (5'-AGG.GAT.GCC.TGG.ACA.CAA.GA-3') and EBNA-2G (5'-GCC.TGG.GTT.GTG.ACA.GAG-3') for type 1 and EBNA-2C and EBNA-2B (5'-TTG.AAG.AGT.ATG.TCC.TAA.GG-3') for type 2. After PCR the identity of the amplified EBV fragment was confirmed by Southern blot analysis using the ³²P-(dATP)-labeled type-specific probes EBNA-2.1 (5'- TCC.AGC.CAC.ATG.TCC.CCC.CTG.TAC.GCC.CGA.CA-3') and EBNA-2.2 (5'-AAC.GTC.AAC. CTG.TCC.ACA.ACC.CTC.GCC.AGG.A G-3').

Statistical analysis

Mann-Whitney tests were used for the comparison of the EBV DNA copy numbers in each group analysed. Pearson and Spearman's Correlation Tests were used to correlate CD4⁺ T cell numbers with EBV load and EBV copies in PBMC with serum, respectively, using the software program SPSS 7.5 for Windows (SPSS Inc., Chicago, Illinois).

Results

Cross-sectional analysis of EBV load in PBMC in HIV⁻ and HIV⁺ individuals

We studied EBV load in 22 HIV-negative heterosexual individuals, 36 HIV-negative homosexual men and 65 HIV-positive homosexual men. EBV load in HIV⁺ homosexual men (median 1.2x10⁶/10⁶ PBMC, range 0 - 3.9x10⁶, 6.6x10⁶) was approximately 100 fold higher than in HIV⁻ homosexual men (median 1.9x10³/10⁶ PBMC, range 0 - 6.9x10³) (p<0.001, Mann-Whitney) who, in their turn had a higher EBV load than HIV⁻ heterosexual individuals (median 36.8/10⁶ PBMC, range 0 - 6.9x10³) (p<0.001, Mann-Whitney). (Table 2a)

The 65 HIV⁺ homosexual men were subdivided into 22 long-term non- or slow-progressing individuals and 43 rapid progressors to AIDS. As shown in Table 2b no difference in EBV load was found between these groups (median 6.4x10⁴ and 20x10⁴, respectively, p = 0.163, Mann Whitney test). In addition, no difference in EBV load was found between 31 HIV⁺ homosexual men with CD4⁺ T cell numbers below 400/mm³ and 34 with CD4⁺ T cell numbers above 400/mm³ (median 1.3x10⁵ and 1.2x10⁵, respectively, p = 0.958, Mann-Whitney test). Likewise, a similar EBV load was observed in HIV⁺ homosexual men with CD4⁺ T cell numbers below 200/mm³ (n=11) and above 200/mm³ (n=54) (p = 0.372, Mann-
Table 2a. cross-sectional analysis of EBV load in HIV- and HIV+ individuals

<table>
<thead>
<tr>
<th>group</th>
<th>n</th>
<th>EBV load/10⁶ PBMC (median)</th>
<th>range</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV hetero</td>
<td>22</td>
<td>36.8</td>
<td>0 - 6.9x10⁶</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HIV homo</td>
<td>36</td>
<td>1926</td>
<td>0 - 6.6x10⁶</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HIV+ homo</td>
<td>65</td>
<td>1.2x10⁶</td>
<td>0 - 3.9x10⁶</td>
<td></td>
</tr>
</tbody>
</table>

Table 2b. EBV load in different subgroups of HIV-1* homosexual men

<table>
<thead>
<tr>
<th>HIV+ homo</th>
<th>n</th>
<th>EBV load/10⁶ PBMC (median)</th>
<th>range</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-progressors</td>
<td>22</td>
<td>6.4x10⁴</td>
<td>0 - 1.4x10⁶</td>
<td></td>
</tr>
<tr>
<td>Progressors</td>
<td>43</td>
<td>20x10⁴</td>
<td>0 - 3.9x10⁶</td>
<td>0.163</td>
</tr>
<tr>
<td>CD4≤400</td>
<td>31</td>
<td>1.3x10⁵</td>
<td>0 - 3.9x10⁶</td>
<td>0.958</td>
</tr>
<tr>
<td>CD4&gt;400</td>
<td>34</td>
<td>1.2x10⁵</td>
<td>0 - 9x10⁵</td>
<td></td>
</tr>
<tr>
<td>CD4≤200</td>
<td>11</td>
<td>1.2x10⁵</td>
<td>0 - 3.5x10⁶</td>
<td>0.372</td>
</tr>
<tr>
<td>CD4&gt;200</td>
<td>54</td>
<td>1.1x10⁵</td>
<td>0 - 3.9x10⁶</td>
<td></td>
</tr>
</tbody>
</table>

n = number of individuals tested; non-progressors = HIV-1 infected individuals who were long-term non-progressors or slow progressors to AIDS; progressors = progressors to AIDS; CD4 = CD4+ T cell numbers/mm³

Whitney test). (Table 2b) Moreover, there was no correlation between EBV load and CD4+ T cell numbers in HIV+ homosexual men (p=0.442, Pearson correlation test). (data not shown) Although in LTA EBV load could be undetectable early in HIV-1 infection (Fig.2a), bursts of EBV load were also observed and could reach levels of 7x10⁶ copies/10⁶ PBMC (Fig.2a and b). Only 1 out of 5 LTA had a progressive increase in EBV load which did not return to baseline levels as was the case for the other LTA (Fig.2c).

Longitudinal analysis of EBV load in PBMC of HIV-1 infected individuals

To investigate the kinetics of EBV load in the course of HIV-1 infection, we determined EBV load in 9 AIDS-NHL patients, 5 LTA and 7 progressors to AIDS without lymphoma. Over time, most AIDS-NHL patients displayed increases in EBV load, which could reach levels of over 1 million copies/10⁶ PBMC and were not caused by increases in the number of B cells. (Fig.1 and data not shown) In five AIDS-NHL patients the increase in EBV load peaked around
AIDS-diagnosis. (Fig.1a, b and c) However, in 4 of these 5 EBV load did not reach levels higher than 20,000 copies/10^6. (Fig. 1a and b) In the other 4 AIDS-NHL patients bursts in EBV load were followed by decreases, although load levels remained higher than baseline, (Fig. 1d) and ranged from 5x10^3 to 3x10^6 copies/10^6 PBMC at AIDS-NHL diagnosis.

Similarly, bursts of EBV load were observed in 4 out of 7 progressors to AIDS without lymphoma. (Fig 2d, e and f) EBV load peaks varied from 10,000 to 160,000 EBV copies/10^6 PBMC, but decreased again to baseline values. Two individuals initially presented with an EBV load above 2x10^5 copies, but their EBV load decreased in the course of HIV-1 infection (data not shown). Bursts of EBV load were not caused by increases in the number of B cells in the blood, since correction for the number of B cells, did not change the patterns observed. (data not shown)

For each individual we calculated the average EBV load both from the first ~ 4 years (early) and the last ~ 4 years (late) of follow-up. Patient NHL0345, who had an extremely high EBV load (range 2 - 7x10^6 copies) during the entire follow-up was excluded from these analyses. As shown in figure 3, there was no difference in absolute load (average) between AIDS-NHL, other progressors and LTA individuals, neither early nor late in infection. Also no difference was found between the 3 groups when we compared the highest peak in EBV load observed during the entire follow-up (data not shown). Interestingly, whereas most progressors remain stable or show a decrease, 7 out of 9 AIDS-NHL patients show an increase in EBV load in the course of HIV-1 infection. When the change in EBV load was compared a significant difference was found between AIDS-NHL patients and progressors without lymphoma (p<0.008, Mann-Whitney test).

![fig.1. EBV load/PBMC in 4 AIDS-NHL patients](image)

**EBV load/10^6 PBMC as measured by real-time Q-PCR in the course of HIV-1 infection in a/b) two AIDS-NHL patients who show an increase in load, but do not reach levels higher than 20,000 copies and c/d two AIDS-NHL patients who show peaks in EBV load, which reach levels higher than 1x10^6 copies**
Fig. 2. EBV load/PBMC in 3 LTA and 3 progressors
EBV load/10^6 PBMC as measured by real-time Q-PCR in the course of HIV-1 infection in a) three long-term asymptomatic individuals and b) three progressors to AIDS.

![Graph showing EBV load in PBMC in LTA and progressors to AIDS.](image1)

**Fig. 3. Average EBV load/PBMC early and late for all individuals**
Average EBV load/10^6 PBMC from early (first 4 years of follow-up) and late (last 4 years of follow-up) as measured by real-time Q-PCR for all individuals with lower than 50,000 copies (left panel) and higher than 50,000 EBV copies (right panel). LTA individuals are represented by the solid middle gray lines, progressors to AIDS-NHL by dashed black lines and other progressors by semi-dashed light gray lines.

**EBV load in PBMC versus EBV load in serum**
Because EBV load in serum has been reported to be highly predictive of lymphoma development in solid organ, bone marrow and stem cell transplant recipients, we compared EBV load in PBMC with serum in 7 AIDS-NHL...
patients, 3 LTA and 1 progressor in the course of HIV-1 infection. In most individuals at some time points EBV could be detected in serum. (Fig. 4) The level was, however, (much) lower than in PBMC and kinetics were often different (Fig. 4a). Two out of four lymphoma patients with an increase in EBV load in PBMC had detectable EBV load in serum only at the last time point studied. Interestingly, this peak, which was extremely high in patient NHL0068, occurred when EBV load in PBMC was decreasing after initiation of highly active antiretroviral therapy (HAART). (Fig. 4a) Kinetics of EBV load in PBMC and serum could sometimes run in parallel, as shown for NHL0308 and the early peak load in PBMC and serum in NHL6006 (fig. 4b). Interestingly, in the latter patient EBV load in serum increased after start of successful chemotherapy (CHOP) after lymphoma diagnosis. In only 1 LTA (LTA0750) the increase in EBV load in PBMC was paralleled by an increase in EBV load in serum. However, in the other LTA and 1 progressor an increase in EBV load in PBMC was accompanied by a decrease in EBV load in serum and vice versa (Fig.4c).

Overall, no correlation between EBV load in PBMC and EBV load in serum was found. (Figure 5)

![Image of EBV load in PBMC versus serum](image)

fig.4. EBV load in PBMC versus serum (dashed line)
EBV load/10^6 PBMC (solid lines) and EBV load/ml serum (dashed lines) as measured by real-time Q-PCR in the course of HIV-1 infection in a) two AIDS-NHL patients with low load and steady increase in PBMC, who show different kinetics and b) two AIDS-NHL patients with higher peak loads in PBMC, who show similar kinetics and c) two LTA individuals, who show opposite kinetics. The white arrow indicates start of chemotherapy (CHOP).

![Image of Correlation EBV load serum/PBMC](image)

fig.5. Correlation EBV load serum/PBMC
For all individuals in which EBV load was measured both in PBMC and serum at all time points, EBV load in PBMC was plotted against EBV load in serum. Filled circles represent AIDS-NHL patients, open circles LTA individuals and filled squares other progressors to AIDS.
EBV load in relation to EBV type

There are two EBV types, 1 and 2. HIV-1 infected individuals have been found to be frequently infected with EBV type 2 and a high percentage of the AIDS patients is coinfected with type 1 and 2. It has been suggested that EBV load in EBV type 2 infected individuals is lower than in type 1 infected individuals. To investigate this, we determined the EBV type(s) present in PBMC from 93 of the 101 homosexual men (59 HIV+ and 34 HIV-). Of these 93 men 83 were positive in a type-specific nested PCR. Of these 83 men 41 were only infected with EBV type 1 and 42 were infected with EBV type 2 (single n=16) or superinfected with EBV type 1 and 2 (n=26). EBV load in superinfected individuals was higher (median 13500, fig.6 range 0-3.5x10^6) than EBV load in type 1 infected individuals only (median 3430, range 0-3.9x10^6, p<0.067, Mann-Whitney). However, no difference was found between single type 1 and single type 2 infected individuals (p<0.118, Mann-Whitney). (Figure 6)

Discussion

In this study we compared EBV load in HIV-positive and HIV-negative homosexual men using a real time quantitative PCR assay and studied the kinetics of EBV load in PBMC and serum in the course of HIV-1 infection in different groups of HIV-1 infected individuals.

Major conclusions from our study are 1) EBV load in HIV+ homosexual men is significantly higher than in HIV-homosexual men. 2) EBV load in HIV-homosexual men is higher than in HIV-heterosexual individuals. 3) There is no clear correlation between EBV load in PBMC and serum. 4) Bursts of EBV load in PBMC occur in course of HIV-infection in all subgroups. 5) Absolute levels of EBV are not predictive for the development of AIDS-NHL. 6) There is no difference in EBV load between EBV type 1 and type 2 infected individuals. Our finding that EBV load in HIV+ homosexual individuals is higher than in HIV+ homosexual individuals has also been reported in a study comparing HIV+ homosexual men with HIV-
heterosexual individuals using a semi-quantitative PCR assay. In addition, we observed a difference in EBV load between HIV-negative homosexual and HIV+ heterosexual men. The first finding might be explained by the difference in immune status. However, we found no difference in EBV load between non-progressors and progressors and no relation with CD4+ T cell counts. A second possible explanation might be the higher proportion of EBV type 2 (superinfection) in HIV+ individuals. Although EBV load in EBV type 1+2 superinfected individuals is higher than in single infected individuals (fig.4) and EBV superinfection occurs more often in HIV+ individuals, these data can not account for the 100-fold higher EBV load in HIV+ individuals. In addition, we also found a substantial fraction of EBV type 1 and 2 superinfected individuals within the HIV+ homosexual group.

A third possible explanation for the observed inter group differences in EBV load might be chronic antigenic stimulation leading to a higher rate of EBV reactivation. Indeed, it has been shown that increases in EBV DNA in blood coincided with elevated anti-ZEBRA antibodies in patients with Hodgkin's disease, suggesting ongoing EBV-reactivation. Although some EBV load peaks coincided with peaks in HIV RNA load and/or periods of fever in HIV-infected individuals (data not shown), this was only observed in LTA. Finally, more frequent sexual contacts in homosexual men as compared to heterosexual individuals may also result in increases in EBV load either directly due to superinfection with other EBV subtypes or indirectly due to higher levels of stimulation by other antigens (e.g. HIV in HIV-infected individuals) in these individuals.

Our study is the first longitudinal study comparing EBV load in PBMC with serum. We found that kinetics of EBV load in PBMC differs from EBV load kinetics in serum. Overall, EBV load is serum was lower than in PBMC and was often even undetectable. In only 1 out of 9 AIDS-NHL patients EBV load in serum was high at AIDS-NHL diagnosis. In addition, a rise in EBV load in serum was mostly observed after a peak in EBV load in PBMC, notably in LTA individuals in which an increase in EBV load in PBMC was accompanied by a decrease in EBV load in serum and vice versa. This may be due to elimination or apoptosis of EBV infected B cells causing release of free EBV DNA in serum. In case of a lymphoma, the number of cell free DNA copies can be high because of the frequently observed spontaneous apoptosis of tumor cells (notably in Burkitt's lymphoma) or by (chemo) therapy resulting in tumor lysis. We observed bursts of EBV load in PBMC in all HIV+ subgroups studied and found that the absolute EBV load is not predictive for the development of AIDS-NHL. However, it might be that the total number of EBV copies (or average number of EBV copies per B cell), as measured by PCR assays, is less predictive of a malignant outgrowth of B cells than the number of EBV-infected B cells, as measured by in vitro transformation assays. This is unlikely because in two previous studies addressing the correlation between EBV load and EBV-specific immunity, both
Fig. 7. Schematic model of EBV load kinetics in HIV-infected individuals

A model of EBV load kinetics in time for healthy controls, HIV-infected long-term asymptomatic individuals (HIV-LTA), HIV-infected progressors to AIDS without lymphoma (HIV-progressor) and HIV-infected individuals progressing to AIDS-NHL (AIDS-NHL). The dashed line indicates AIDS-NHL patients with lower but increasing numbers of EBV copies.

assays yielded similar results. Furthermore, in bone marrow (BM) transplant recipients both total EBV load (copies) and the number of infected B cells did predict the occurrence of lymphoma. EBV load in HIV-1 infected individuals in this study was comparable to what has been reported for paediatric (BM) transplant recipients with lymphoproliferative disorders and for patients with AIDS-related non-Hodgkin's lymphomas. The latter study did show a relation between high EBV load and lymphoma diagnosis, but no other HIV-infected individuals were investigated. Although in the post-transplant setting EBV load measurements seem useful for predicting the development of a lymphoma, in HIV-infected individuals this does not seem to be the case. Of course, the first study group is more homogeneous, in that all patients receive similar (dosages of) immunosuppressive treatment, whereas in our study the HIV-infected individuals all have a different virological and immunological status. Because on the one hand EBV load was high in all HIV+ individuals and on the other some of the AIDS-NHL patients did not reach EBV levels higher than 20,000 copies/10^6 PBMC, other factors must be involved in the development of NHL in HIV-infected individuals.

EBV load alone may not be predictive for AIDS-NHL because load is only 'half the story'. A combination of EBV load measurements with cellular immunity against EBV might provide more information on the balance between virus and host and may allow for a predictive value. Thus, low EBV load
with collapsing cellular immunity against EBV may result in a higher risk than high EBV load with stable numbers of EBV-specific CD8+ T lymphocytes. In line with this, EBV load peaks that returned to baseline in LTA and AIDS-OI patients are suggestive for EBV control as was shown in our previous study. In post-transplant patients initially EBV immunity is totally absent. Therefore, in these patients EBV load is the ‘whole story’, i.e. the only determinant of the risk for NHL.

In conclusion, in HIV-1 infected individuals overall EBV load is high and increases in EBV load are not predictive of the development of AIDS-NHL. Because EBV load in combination with other parameters of EBV-infection may be more predictive than EBV load alone, we propose the following scenario: For healthy controls, after EBV infection a “viral set point” will be reached. Upon infection with HIV, high antigen-stimulation leads to a new set point, which is at a higher level than in healthy controls. (Fig.7) Both in non-progressors and progressors, as long as there is adequate EBV-specific immune control, reactivation-induced peaks in EBV will return to set point levels (LTA and early in infection in progressors). With deteriorating cellular immunity against EBV and more frequent episodes of EBV reactivation (progressors>LTA), it will be increasingly difficult to suppress EBV load, leading to progressive accumulation of EBV particles in the B cell pool, which also increases the risk for secondary/tertiary genetic events. When there is substantial loss of immune control (in NHL patients), EBV load peaks do no longer return to baseline, but show a gradual increase. (Fig.7) Based on our observations we postulate that the risk for AIDS-NHL is independent of the absolute level of EBV, but is determined by the balance between EBV load and EBV immune control.

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