Dynamics of Epstein–Barr virus in HIV-1-infected subjects on highly active antiretroviral therapy

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Objective: Patients infected with HIV-1 are at high risk of developing Epstein–Barr virus (EBV)-associated lymphoproliferative disorders. This study evaluated the impact of highly active antiretroviral therapy (HAART) on EBV infection.

Methods: To measure EBV content in peripheral blood lymphocytes (PBL) and in plasma, we set up a quantitative analysis using the real-time PCR. EBV latent membrane protein 1 (LMP1) expression was determined by reverse transcriptase-PCR.

Results: EBV levels were determined in 33 HIV-1- and EBV-coinfected patients at the start of HAART, and during therapy. At baseline, EBV content in PBL samples ranged from 8 to 14 532 copies/μg DNA. EBV levels transiently increased in nine out of 17 patients in whom HIV-1 plasmaviraemia declined to undetectable levels (virological response) and CD4 cell counts increased (immunological response), while they remained fairly stable or decreased in the other eight virological and immunological responders, and in seven patients who showed a virological response only. Of interest, a significant increase in EBV load was observed in five out of nine patients who showed an increase in CD4 cell counts but lack of HIV-1 suppression during HAART. This EBV increase was accompanied by the detection of both LMP1 transcripts in PBL and EBV DNA in plasma, and was paralleled by an increase in immunoglobulin levels, a marker of B-cell stimulation.

Conclusions: These findings suggest that peripheral immune reconstitution during HAART without a reduction in HIV-1 replication may increase B-cell stimulation and the number of EBV-infected B cells.

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Introduction

Regardless of its congenital, iatrogenic, or infectious origin, immunodeficiency increases the risk of tumour development, and particularly of tumours aetiologically linked with viral agents [1]. It is striking that Kaposi's sarcoma (KS) and B-cell non-Hodgkin's lymphoma (NHL), which have a remarkably high incidence in immunocompromised hosts, are linked to human herpesvirus type 8 (HHV-8) and Epstein–Barr virus (EBV) infections, respectively. KS and NHL are also the most common malignancies in people infected with the HIV-1 [2]. EBV is consistently found in large cell-immunoblastic NHL which usually arises in the late

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stage of HIV-1 disease, in association with a low CD4 cell count and immunocompromised status [3–5].

EBV is a ubiquitous virus that can establish both latent and fully productive infections. In the imunocompetent host, after the primary infection which stimulates a vigorous T-cell immune response [6], the virus remains in its latent form in a few B cells, and no replication is detectable in the peripheral blood. Compared to healthy individuals, HIV-1-infected subjects usually have a higher content of EBV and/or EBV-infected cells both in the peripheral blood compartment and lymphoid tissues [7,8]. The finding that the presence of EBV in lymphoadenopathies correlated with a higher risk of developing lymphoma over time [9] suggested that EBV might constitute an early promoter in lymphomagenesis. The expansion of EBV-positive B cells is probably due to the chronic B-cell stimulation driven by HIV-1 antigens, and the impaired immunosurveillance against EBV [10]; the finding that the onset of large cell-immunoblastic NHL is preceded by a decrease in EBV-specific cytotoxic T lymphocytes and an increase in EBV load [11] supports the concept that impaired immunosurveillance against EBV is a critical step toward lymphomagenesis.

The introduction of highly active antiretroviral therapy (HAART), a combination treatment that makes use of reverse trancriptase and protease inhibitors, has greatly modified the natural course of HIV-1 infection. HAART brings about a significant and sustained decrease in peripheral blood HIV-1 RNA levels, as well as an increase in CD4 cells, and a significant decrease in HIV-1-associated opportunistic infections, thus indicating a restoration of immune functions [12]. The decline in HIV-1 replication, and the partial immune reconstitution might also have a favourable impact on HIV-1-associated malignancies. Indeed, a significant decrease in KS incidence was reported after HAART introduction [13]; moreover, complete remission of KS lesions, and a decrease in the HHV-8 burden in peripheral blood lymphocytes (PBL) have been described in patients under HAART [14–17]. How the antiretroviral therapy influences EBV-associated diseases in HIV-1-infected subjects is less clear; epidemiological reports suggest that HAART may have a less favourable impact on NHL than on other AIDS-defining illnesses, including KS [13,18,19]; furthermore, no data on the dynamics of EBV burden in patients on HAART are available.

We set up a quantitative method based on real-time PCR assay to quantify EBV DNA in cells and plasma, and determined EBV levels in peripheral blood samples from HIV-1-infected patients collected before and during antiretroviral treatment. Our findings show that the dynamics of the EBV load differ according to HIV-1 decline, and immune reconstitution.

Patients and methods

Patients

Thirty-three patients attending the Department of Infectious Disease at Padova Hospital were studied. All of these patients were positive for EBV type 1 by PCR analyses of *EBNA2* and *EBNA3C* genes, performed as described previously [4]. Of the 33 patients 29 had previously been treated with antiretroviral regimens containing reverse transcriptase inhibitors. The HAART regimen consisted of a triple-drug combination, including two reverse transcriptase inhibitors (azidothymidine, dideoxyinosine, or dideoxycytidine) and one protease inhibitor (indinavir, ritonavir, or saquinavir), administered according to current guidelines [20,21]. Patients were followed over time for plasma HIV-1 RNA level and CD4 cell count. Virological responders were defined as persons in whom the HIV-1 RNA load decreased to undetectable levels (< 200 copies/ml). Immunological responders were defined as persons in whom the CD4 T-cell count increased $> 30\%$ compared with the baseline level, with an absolute value $> 100 \times 10^6$ cells/l. Median follow-up was 24 months (range, 6–33 months). EBV content was determined in PBL and plasma samples collected at HAART entry, and during treatment, at the time of virological and immunological responses (i.e. the first time at which plasma HIV-1 RNA levels dropped to undetectable levels, and the point when the CD4 cell count reached the maximum increase, respectively).

Sample preparation

PBL were obtained from EDTA-treated peripheral blood samples by Ficoll Hypaque gradient separation (Pharmacia Biotech, Uppsala, Sweden). PBL and plasma samples were cryopreserved and stored at −80 °C until use. Two million PBL were lysed for 1 h at 56 °C in 200 µl TE buffer (10 mM Tris–HCl pH8, 0.1 mM EDTA) containing 0.001% Triton X-100, 0.0001% sodium dodecyl sulfate, and 600 µg/ml proteinase K. After lysis, proteinase K was inactivated by heating the mixture for 15 min at 94 °C. DNA was extracted from cells and plasma by QIAamp DNA Mini Kit (Quiagen, Hilden, Germany), using the protocols for cells and blood, respectively, according to the manufacturer's recommendations. Four hundred microlitres plasma were used for DNA extraction per column, and the extracted DNA was resuspended in 50 µl AE buffer (provided in the kit).

Quantification of EBV-DNA

EBV DNA content was measured using a real-time quantitative PCR. As detailed elsewhere [22], this method measures the accumulation of PCR products by a fluorescence detector system, and allows quantification of the amount of amplified PCR products in the log phase of the reaction. Primers/probe combinations

for the *EBNA2* gene of EBV were developed using Primer Express software (PE Applied Biosystems, Foster City, California, USA); the forward and the reverse primers were 5′-CTGCCCACCCTGAGGATTTCC-3′ and 5′-CTGCCACCTGGCGGCAAC-3′. A fluorogenic probe, 5′-FAM-AATCCTCCTACCCTCTCTTTATGCCATGTGTGT-TAMRA-3′, with a sequence located between those recognized by the PCR primers and where the 6-carboxyfluorescein (FAM) serves as reporter fluorochrome and 6-carboxytetramethylrhodamine (TAMRA) as the quencher, was synthesized by PE Applied Biosystems. Cell lysate (15 μl, 150 000 cells) or extracted DNA from plasma (10 μl) was used for amplification. Each PCR was performed in a 50 μl reaction mixture containing 25 μl 2 × Taq Man Universal PCR master Mix (PE Applied Biosystems), 300 nM each primer, and 100 nM fluorogenic probe. Amplifications were carried out in a 96-well reaction plate (PE Applied Biosystems) in a spectrofluorimetric thermal cycler (ABI PRISM 7700 Sequence Detector, PE Applied Biosystems); after 2 min at 50°C to allow the uracil N-glycosylase to act, and a denaturation step of 10 min at 95°C, 45 cycles were run, each consisting of 15 s at 95°C and 60 s at 60°C. Each sample was run in duplicate; a threshold cycle (Ct) value for each duplicate was calculated by determining the point at which the fluorescence exceeded a threshold limit (10-fold the standard deviation of the baseline), and the mean of the two duplicates was used to calculate the EBV copy number in the samples. To quantify EBV, a reference curve was run in duplicate in each set of reactions. The curve was prepared by amplifying serial dilutions of DNA extracted from EBV-positive Namalwa cells, which contain two integrated EBV type 1 genomes/cell [23]; the Ct values, calculated by sequence detector system software version 1.6.3 (PE Applied Biosystems), were plotted against the DNA input, and a standard reference curve was obtained. The Ct values of the clinical samples were plotted against the reference curve, and the EBV copy number was then calculated. The conversion factor of 6.6 pg DNA/diploid cell was used to express EBV copy number/μg DNA. To normalize clinical samples for cell equivalents, and the efficiency of each PCR reaction, a 294 base pair (bp) β-actin fragment was amplified with the forward primer 5′-TCACCCACACTGTGCCCATCTTACGA-3′ and reverse primer 5′-CAGCGGAACCGCTCATTGCCAATGG-3′, and quantified using a fluorogenic probe 5′-VIC-ATGCCCTCCCCCATGCCATCCTGCGT-TAMRA-3′. PCR conditions were the same as those used for EBV quantification, except that primer concentrations were 100 nM (forward primer) and 600 nM (reverse primer), and the probe concentration was 200 nM. A reference curve to quantify β-actin was generated by amplifying serial dilutions of DNA extracted from Namalwa cells, as performed for the EBV reference curve. Appropriate negative controls (DNA from EBV-negative A301 cells, and distilled water) were included in each set of reactions. To quantify EBV DNA in plasma samples, 10 μl of the extracted DNA were used in the amplification reaction; PCR conditions were exactly the same as described above. DNA from Namalwa cells was serially diluted in 400 μl plasma samples from EBV-negative healthy donors; Ct values obtained by amplifications of DNA extracted from these samples were used to generate a reference curve to quantify EBV in plasma. The conversion factor × 12.5 was used to estimate the number of EBV copies/ml of plasma; negative results were then expressed as < 50 EBV copies/ml plasma.

Analysis of EBV variants and detection of *LMP1* gene transcription

To investigate EBV variants, 1 μg of sample DNA was amplified by PCR using a set of primers that immediately flank a portion of the 33 bp tandem repeats in the *LMP1* gene, exactly as reported previously [4]. Repeat number was assessed by comparison with a marker containing a mixture of four sequenced amplified products [4]. For analysis of *LMP1* mRNA, total RNA was extracted using the TRIZOL Reagent (Life Technologies, Paisley, UK), and cDNA was coamplified with primer pairs specific for *LMP1*, and β-actin genes, exactly as reported previously [4].

EBV serology

EBV serology tests for antibody to viral capsid antigen (VCA)-IgG, early antigen (EA)-IgG (D + R), and nuclear antigen (EBNA)-IgG were performed using standard titrered immunofluorescence methods, (EBV VCA IFA IgG, EBV EA IFA, and EBV EBNA ACF; MRL Diagnostics, Cypress, California), following the manufacturer's instructions. Antibody titre was expressed as the lowest plasma dilution giving a positive result.

Quantification of HIV-1 RNA

The plasma HIV-1 RNA copy number was determined using the Amplicor HIV-1 Monitor test version 1.5 (Roche Diagnostic System, New Jersey, USA), according to the manufacturer's instructions.

Statistical analyses

Data were compared using the non-parametric Wilcoxon signed-rank test for paired data, and the Mann–Whitney U test for unpaired data. All *P* values were calculated using the two-sided test. Correlation coefficient between continuous variables was obtained by using the Spearman test. Analyses were carried out using SAS/STAT software version 6.12 (SAS Institute, Cary, North Carolina, USA).

Results

Development of real-time PCR assay for quantifying EBV DNA

To determine the dynamic range of real-time quantitative PCR, duplicate five-fold serial dilutions of DNA from Namalwa cells, ranging from 12.8 to 200 000 pg, were amplified for both *EBNA2* and β-actin genes; the amplification curves obtained are shown in Fig. 1a and Fig. 1b, respectively. For each dilution, the fluorescent emission of reporter molecules (ΔRn) was plotted against the cycle number of PCR. As the reaction with fewer target molecules required more amplification cycles to produce a detectable amount of reporter molecules than the reaction with more target molecules, the amplification curve shifted to the right as the input target DNA was reduced; the system was sensitive enough to detect EBV genomes (Fig. 1a) and β-actin molecules (Fig. 1b) contained in 12.8 pg of DNA input. The Ct values derived from the amplification curves were plotted against the DNA input (Fig. 1c, d), and a linear correlation was obtained over the range of 12.8–200 000 pg DNA for both *EBNA2* (Fig. 1c) and β-actin (Fig. 1d) genes. As Namalwa cells contain two EBV genomes/cell [23], it was estimated that the assay detects from four EBV genomes (contained in 12.8 pg DNA input) to 60 600 EBV genomes (contained in 200 000 pg DNA input). The linearity of the graphs demonstrates the large dynamic range of the assay. In every set of experiments appropriate negative controls (200 000 pg DNA from EBV-negative A301 cells and H_2O) were included. The Ct of the negative controls was always 45 (Fig. 1).

Cell-associated EBV load in patients at HAART entry

The characteristics of the study population at baseline are shown in Table 1. The median HIV-1 RNA level was 156 000 copies/ml (range, 1050–1 000 000 HIV-1 RNA copies/ml), and median CD4 cell count was 54×10^6/l (range, 3×10^6–355×10^6/l); all but one subject had CD4 cell counts $< 200 \times 10^6$/l. The

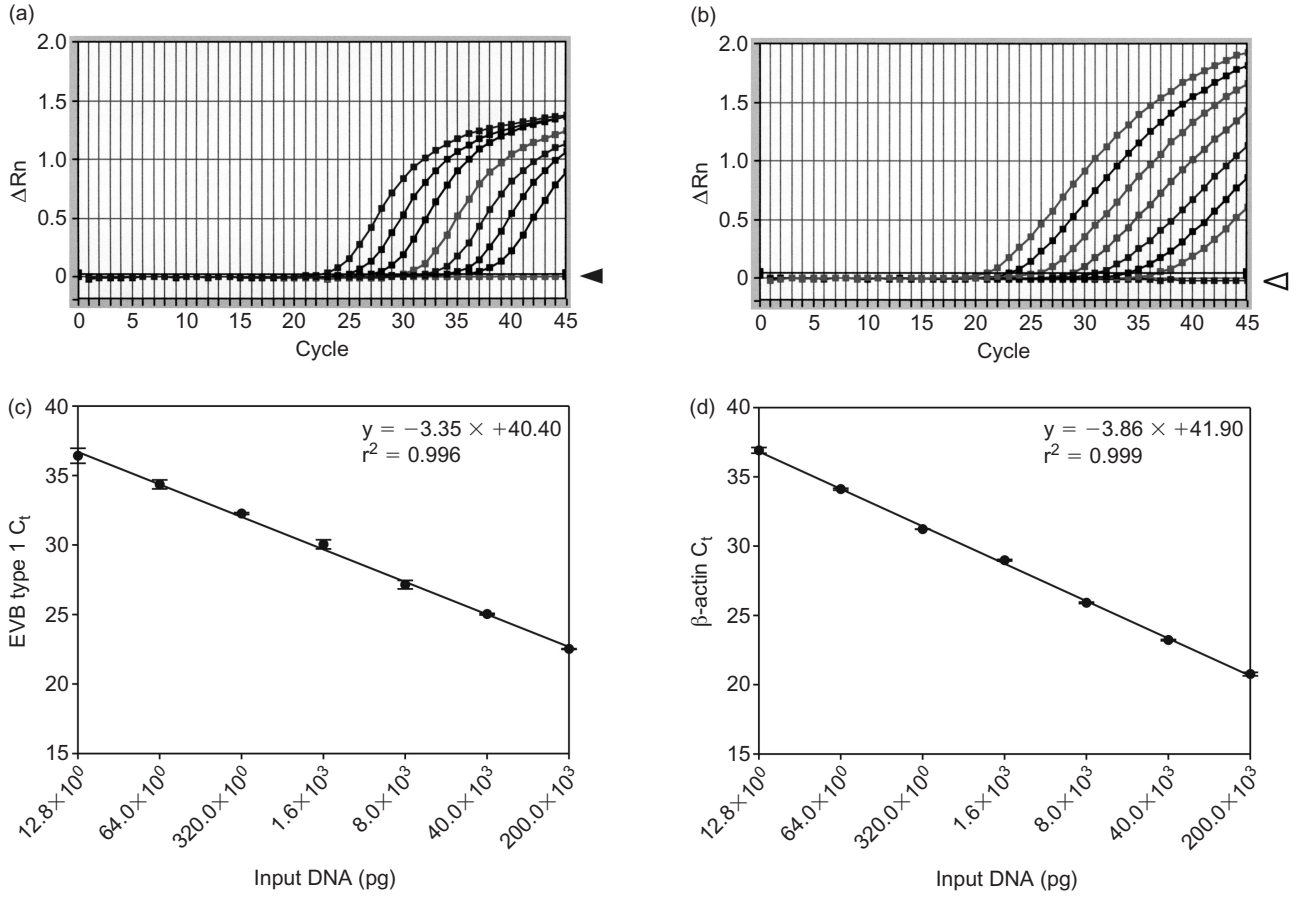


Fig. 1. Quantification of EBV by real-time PCR. Five-fold serial dilutions of Namalwa DNA samples (ranging from 12.8 to 200 000 pg) and appropriate negative controls (200 000 pg DNA from A301 EBV-negative cells for EBV (filled arrowhead), and H_2O for β-actin (open arrowhead), were amplified for (a) EBV *EBNA2* and (b) β-actin genes. For each dilution, fluorescent emission (ΔRn) is plotted against the cycle number of PCR. Standard curves were constructed for both (c) EBV *EBNA2* and (d) β-actin genes by plotting the input target DNA against the threshold cycle (Ct). Each dilution was run in duplicate; mean Ct values (filled circles) and standard error (bars) are shown.

Table 1. Main characteristics of patients at HAART entry.

	Total	Virological and immunological responders	Virological responders	Immunological responders
Male : female (n)	27 : 6	13/4	7 : 0	7 : 2
Age (years) [median (range)]	40 (32–74)	40 (32–60)	40 (38–74)	38 (32–52)
C3 : C2[a] (n)	32 : 1	16 : 1	7 : 0	9 : 0
Risk group (n)				
Injecting drug user	12	7	3	2
Heterosexual	6	3	1	2
Homosexual/bisexual	14	6	3	5
Transfusion recipient	1	1	0	0
Baseline CD4 cell count ($\times 10^6$/l) [median (range)]	54 (3–355)	45 (9–355)	77 (3–127)	60 (12–110)
Baseline HIV-1 RNA (copies/ml) [median (range)]	156 000 (1050–1 000 000)	193 000 (14 000–1 000 000)	32 000 (1050–423 000)	173 000 (1500–817 000)
Baseline cell-associated EBV (copies/μg DNA) [median (range)]	140 (8–14 532)	103 (8–14 532)	887 (19–3464)	222 (24–2016)

[a]According to Centers for Disease Control and Prevention criteria.

median EBV content at baseline was 140 copies/μg DNA (range, 8–14 532 EBV copies/μg DNA). According to the response to HAART, patients were classified as virological and immunological responders (i.e., patients showing both a drop in plasma HIV-1 RNA to undetectable levels and a CD4 cell count increase), virological responders (i.e., patients showing a fall in HIV-1 RNA without an increase in the CD4 cell count) or immunological responders (i.e., patients showing an increase in CD4 cell count without a substantial decrease in HIV-1 RNA). The three groups of patients showed no significant differences in plasma HIV-1 RNA, CD4 cell count, and EBV load at baseline (Table 1).

Dynamics of cell-associated EBV DNA in patients during antiretroviral therapy

Seventeen patients were classified as virological and immunological responders to HAART. HIV-1 RNA dropped to undetectable levels in a median time of 6 months (range, 1–17 months) after HAART initiation; this decrease was accompanied by an increase in CD4 cell counts that reached a plateau in a median time of 9 months (range, 2–19 months). The median CD4 cell count was significantly higher [290 (range, 126–610) $\times 10^6$/l] at the time of the immunological response than at the virological response [198 (range, 95–489) $\times 10^6$/l; $P = 0.0002$, Wilcoxon signed-rank test] and at baseline (45 (range, 9–355) $\times 10^6$/l; $P < 0.0001$, Wilcoxon signed-rank test]. Besides individual variability over time, two different patterns of EBV were identified. In nine patients (Fig. 2a, closed circles) EBV increased along with the initial increase in CD4 cell count and the decrease in plasma HIV-1 RNA. At the time of the virological response, EBV had increased by 2–13-fold over baseline values and its median level was significantly higher than at baseline [218 (range, 100–3270) EBV copies/μg DNA versus 87 (range, 35–930) EBV copies/μg DNA; $P = 0.004$, Wilcoxon signed-rank test]. Thereafter, along with the further increase in CD4 cell counts, EBV values decreased and at the time of the immunological response [median, 112 (range, 4–842), EBV copies/μg DNA] did not differ significantly from those observed at baseline. In the other eight patients (Fig. 2a, open circles) EBV decreased with the HIV-1 decline, and at the time of the virological response its median value was lower than at baseline [104 (range, 4–2104) EBV copies/μg DNA versus 121 (range, 8–14 532) EBV copies/μg DNA; $P = 0.05$, Wilcoxon signed-rank test]. After that the EBV content remained fairly stable and at the time of the immunological response its median value was 130 EBV copies/μg DNA (range, 4–8566 EBV copies/μg DNA). These two groups showed no differences in risk factors for HIV-1 infection, HIV-1 load and CD4 cell count at baseline, or in the CD4 cell count increases at the times of both the virological and the immunological responses (data not shown).

Seven patients were classified as virological responders to HAART. Indeed, no significant CD4 cell count increase took place during HAART, even though HIV-1 plasma viraemia fell to undetectable levels; at the time of the virological response, achieved in a median time of 4 months (range, 2–17 months) after HAART initiation, the median CD4 cell count was 80×10^6/l (range, $25–139 \times 10^6$/l). EBV load remained fairly stable or decreased along with the drop in HIV-1 RNA levels, and at the time of the virological response its median value was lower than at baseline [288 (range, 4–3205) EBV copies/μg DNA versus 887 (range, 19–3464) EBV copies/μg DNA; $P = 0.37$, Wilcoxon signed-rank test] (Fig. 2b).

Nine patients were classified as immunological responders. Despite the lack of a substantial HIV-1 decline,

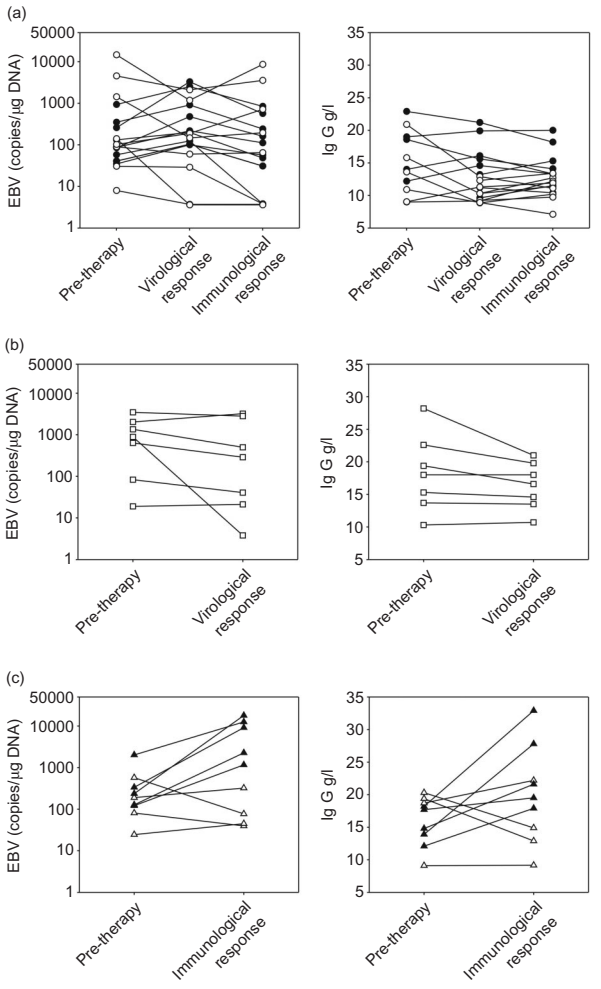


Fig. 2. Cell-associated EBV load and IgG levels at baseline and during HAART. Patients were subgrouped (see text) into (a) virological and immunological, (b) virological and (c) immunological responders. EBV contents (left panels) and IgG levels (right panels) were evaluated at commencement of HAART, and at the time of virological and immunological responses. Closed circles indicate patients who showed a transient increase in EBV load at the time of virological response (a); closed triangles indicate patients who showed an increase in EBV load at the time of immunological response (c).

CD4 cell counts showed a significant increase; at the time of the immunological response, achieved in a median time of 14 months (range, 6–24 months), HIV-1 RNA levels [median, 27 500 (range 3940–523 000) HIV-1 RNA copies/ml] did not differ significantly from those observed at baseline, whereas CD4 cell counts were significantly higher [median, 220 (range, 141–365) versus median 60 (range, 12–110) $\times 10^6$/l; $P = 0.004$, Wilcoxon signed-rank test]. Along with the increasing CD4 cell counts, EBV remained fairly stable in four patients (Fig. 2c, open triangles), but increased greatly in five (Fig. 2c; closed triangles).

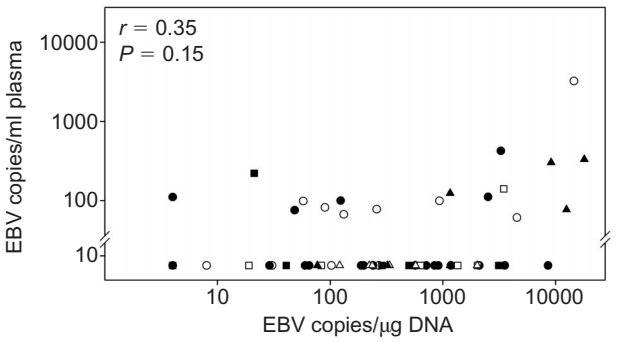


Fig. 3. Relationship between cell-free and cell-associated EBV load before commencement of therapy (open symbols) and during HAART (closed symbols) in virological and immunological responders (circles), virological responders (squares), and immunological responders (triangles).

At the time of immunological response, the EBV content in these five subjects had increased from 6- to 76-fold, and was significantly higher than baseline values [median, 9129 (range, 1160–17 964) EBV copies/µg DNA versus median, 236 (range 121–2016) EBV copies/µg DNA; $P = 0.06$, Wilcoxon signed-rank test] (Fig. 2c); high EBV levels persisted during a subsequent 6 months follow-up (data not shown).

Immunoglobulin levels in patients during antiretroviral therapy

B-cell stimulation, a hallmark of HIV-1 infection, may lead to an expansion of EBV-infected B cells [24]. As the serum Ig level is a marker of B-cell stimulation [25], we analysed IgG levels in plasma samples collected at baseline and during HAART.

At baseline, the median IgG level in all the samples was 15.8 g/l, with a range of 9.0–28.2 g/l (normal range, 8–18 g/l). It is noteworthy that in most of the virological and immunological responders (Fig. 2a), as well as in the virological responders (Fig. 2b) there was a decline in the IgG level along with the HIV-1 decline. Of interest, a slight transient IgG increase was observed in two immunological and virological responders who showed a transient increase in EBV load during therapy (Fig. 2a, closed symbols). Furthermore, among the immunological responders, IgG levels increased significantly in the five patients in whom an increase in the EBV load was observed (Fig. 2c, closed circles) [median, 21.6 (range, 17.9–32.9) g/l at the immunological response versus median, 14.8 (range, 12.1–18.2) g/l at baseline; $P = 0.06$, Wilcoxon signed rank-test], whereas they remained stable or decreased in the other four patients (Fig. 2c, open symbols).

EBV-specific antibodies were studied. At baseline all but three patients were positive for EBNA antibodies; 52% were positive for EA antibodies (median EA IgG

titre, 200; range 40–1280), and all were positive for VCA antibodies (median VCA IgG titre, 2048; range, 256–8192). No differences in EBV antibody profile and titres were observed among the three groups of patients at baseline. During the follow-up no significant differences in the EA and VCA antibody titres were observed among immunological and virological responders, or in virological responders; indeed VCA titres decreased or increased within a twofold range, and EA titres decreased in three cases. Of interest, a 2–10-fold increase in VCA IgG titres along with an increase in EA IgG titres was observed in three of the five immunological responders showing an increase in EBV load (see Fig. 4c).

Plasma EBV DNA levels in patients during antiretroviral therapy

The increase in the cell-associated EBV load might reflect an expansion of EBV latently infected B cells and/or an increased number of newly infected cells by replicating infectious virus. To see whether the variation in the cell-associated EBV content was correlated with the cell-free EBV level, we determined EBV load in plasma samples collected at baseline, and at the time of the virological and/or immunological responses. At baseline and during follow-up, 65%, and 71%, respectively, of the plasma samples tested had undetectable EBV levels, even when the corresponding cell sample contained a high number of EBV copies; overall, no correlation emerged between EBV values in plasma and cells (Fig. 3). At baseline the median cell-free EBV load in the positive samples was 90 copies/ml plasma (range, 61–3234 EBV copies/ml plasma). It is noteworthy that a transient increase in plasma EBV load was observed during HAART in three immunological and virological responders along with a transient increase in the cell-associated EBV content; however, plasma EBV levels, at the end of follow-up in virological and immunological responders and in virological responders, did not exceed baseline values. By contrast, an increase in plasma EBV levels was observed in four immunological responders along with the increase in cell-associated EBV content (Fig. 3, closed triangles, and Fig. 4c).

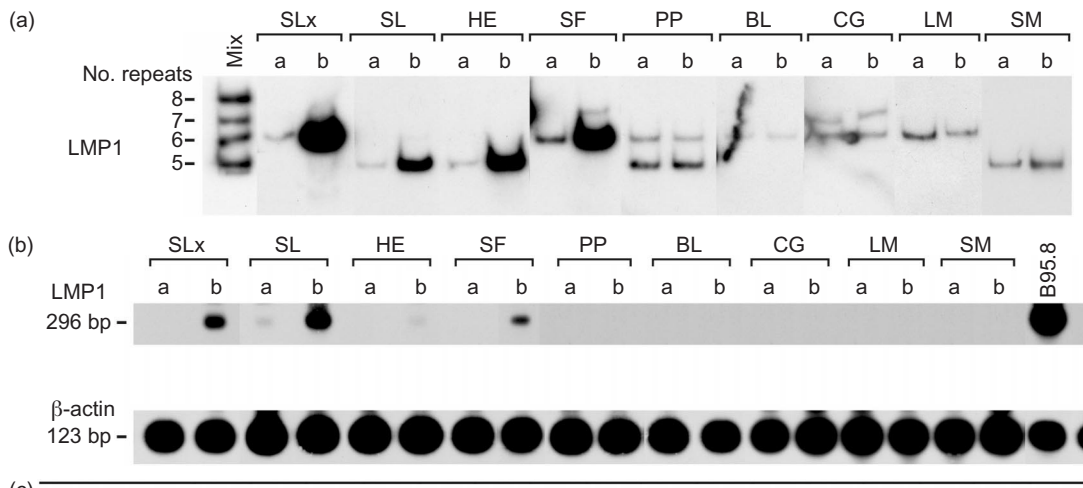
LMP1 gene expression

LMP1, a latent protein of EBV, is a key effector of EBV-mediated transformation of B cells [26]; its expression is consistently found in most of the EBV-related diseases, including large cell-immunoblastic lymphoma and lymphadenopaties arising in HIV-1-infected subjects [4,27,28]. *LMP1* expression has been found in PBL of infectious mononucleosis patients undergoing primary EBV infection, but never in healthy long-term EBV virus carriers [29]. We thought it of interest to analyse *LMP1* gene expression in PBL samples from the five immunological responder patients who showed a consistent increase in EBV content during HAART (patients SLx, SL, HE, SF, PP; Fig. 4); we included the four patients showing no significant EBV increase (BL, CG, LM, SM; Fig. 4) in the analysis for comparison. As shown in Fig. 4a, analysis of the *LMP1* gene containing the 33 bp repeats revealed that two patients harboured two EBV variants whereas all the others had a single EBV variant at baseline (Fig. 4a). An additional EBV variant, differing in the 33 bp repeat, emerged during follow-up in patient SF. As these variants could be generated by EBV reinfection and/or recombination during EBV replication [30–32], this finding together with the increase in EA antibody titre might support the idea that EBV reactivation took place during HAART in this subject. *LMP1* gene expression at baseline was positive in one case (patient SL), and along with the EBV-DNA increase it became positive in three more cases. *LMP1* expression was not found in patients not showing an EBV increase (Fig. 4b).

Discussion

Following the introduction of HAART, the incidence of opportunistic infections and KS in HIV-1-infected subjects underwent a dramatic decline. Decreased rates of herpes virus-associated diseases, such as cytomegalovirus retinitis and KS, were associated with a reduction in the burden of cytomegalovirus [33] and HHV-8 [14–17], respectively. Although epidemiological data suggest that HAART is less effective in preventing NHL than other AIDS-related conditions, a decline in a subset of NHL, i.e., systemic large cell-immunoblastic lymphoma and primary nervous system lymphoma, was observed [13]. As these tumours are EBV-associated, we thought it interesting to study the dynamics of EBV content during HAART. Our study demonstrated that cell-associated EBV levels remained fairly stable or declined in patients who showed a fall in HIV-1, while they increased significantly in most of the patients in whom CD4 cell counts increased, despite persistence of HIV-1 replication. Moreover, we found that cell-associated EBV expansion was paralleled by an increase in plasma EBV and IgG levels.

At baseline, about half of our patients had hypergammaglobulinaemia, a marker of B-cell stimulation. That IgG levels decreased during HAART to normal range values in patients who achieved a substantial decline in HIV-1, regardless of immune restoration, strongly supports the notion that B-cell stimulation is driven mainly by HIV-1 antigens [24]. However, that IgG levels increased significantly in subjects who underwent immune restoration despite the persistence of HIV-1 replication is of particular interest. Indeed, it was shown recently that B-cell stimulation is an independent predictor marker of NHL in HIV-1-infected



(c)

	SLx		SL		HE		SF		PP		BL		CG		LM		SM	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
EBV/DNA copies; copies/μg DNA	366	9.129	127	2.260	121	1.160	236	17.964	2.016	12.500	573	77	222	322	81	40	24	45
EBV/DNA copies; copies/ml plasma	<50	302	ND	ND	<50	124	<50	322	<50	77	<50	<50	<50	<50	ND	ND	ND	ND
EBV-VCA IgG	8.192	4.096	8.192	16.384	8.192	16.384	256	1.024	2.048	8.192	512	512	1.024	4.096	512	1.024	512	512
EBV-EA IgG	1.280	1.280	Neg	40	1.280	1.280	40	320	Neg	40	40	80	1.280	1.280	320	160	Neg	Neg
IgG; g/l	13.9	27.8	12.1	17.9	18.2	32.9	14.8	21.6	17.7	19.5	19.4	12.9	18.7	22.2	9.11	9.16	20.3	14.9
HIV-1 RNA; copies/ml ($\times 10^3$)	173	12.2	137	7.8	218.9	131.5	5.8	15.7	817	73.4	106.8	427.7	214	523	280	27.5	1.5	3.9
CD4+ count; cells/μl	12	256	14	141	83	220	79	360	60	364	49	190	37	220	89	215	110	201

Fig. 4. Detection of LMP1 EBV variants (a) and LMP1 transcripts (b) at baseline (lanes a), and at the time of immunological response (lanes b) in PBL of patients whose main characteristics are reported in (c). (a) LMP1 variants were assessed by comparison with a marker containing a mixture of four sequence amplified products. (b) Detection of LMP1 mRNA was performed by multiplex reverse transcriptase–PCR using primer pairs specific for LMP1 and β-actin. ND, Not done; neg; negative.

people [25]; moreover, it may lead to the expansion of the pre-existing pool of EBV-positive B cells [24,34]. The evidence that EBV levels remained stable or decreased along with a reduction in IgG levels, and increased with an increase in IgG levels supports this notion. However, why patients who underwent peripheral CD4 cell repopulation in the presence of HIV-1 replication showed this increase in B-cell stimulation is unclear. Very few studies have addressed the effect of HAART on the B-cell compartment [35,36], and none was conducted in patients with discordant virological and immunological responses to therapy. In agreement with our observations, a reduction in IgG levels along with the HIV-1 decline has been reported [35]; furthermore, a significant increase in the peripheral B-cell count has been observed during HAART both in children [37] and adults [38,39]. Our finding are consistent with the possibility that the B-cell repopulation occurring in the presence of HIV-1 replication might be associated with enhanced B-cell stimulation. This latter might be driven directly by HIV-1 antigens [24] in a context of a partial restoration of T-cell-dependent B-cell responses associated with the CD4 cell count increase. Nevertheless, the immunological response in this patient subset is very probably defective with regard to cell-mediated immunity, as suggested by the persistence of HIV-1 replication and LMP1 expression by circulating EBV-infected B cells. Further studies will help to elucidate these intriguing issues.

Besides an increase in the pre-existing pool of EBV latently infected B cells, EBV reactivation and the infection of new cells by replicating infectious virus might contribute to the EBV expansion we observed in these patients. Reactivation of EBV has often been reported in HIV-1-infected subjects [40], and is also

suggested by high VCA and EA antibody titres found in our patients at baseline. The finding that, at least in one case, the increase in EBV was accompanied by the emergence of a new LMP1 variant and by an increase in VCA and EA antibody levels may support this possibility [41]. We also found that, although most of the plasma samples were negative for EBV detection, the increase in cell-associated EBV was accompanied by a positive detection of EBV in plasma. The oropharyngeal epithelium is a major site of EBV replication, and there is no evidence of viral replication in the peripheral blood compartment [42] where most of the circulating B lymphocytes harbour the virus in its latent form, and express only the latent proteins EBNA1 and LMP2A [29,43]. Nevertheless, EBV has been consistently detected in the plasma and serum of transplant recipients with lymphoproliferative disorders [44,45], and in patients with nasopharyngeal carcinoma [46]. Furthermore, lytic replication was reported to occur in cells of EBV-associated B-cell lymphomas [47], and EBV DNA has been detected in plasma samples from patients with EBV-positive Hodgkin's lymphoma [48]. Although the origin of EBV DNA in plasma is not clear at present, EBV replication in circulating lymphocytes cannot be excluded [49]. The recent finding that PBL of transplant recipients expressed the *BZLF* gene, which drives the downstream steps of lytic replication [50], may support this hypothesis.

The detection of *LMP1* gene expression in patients' PBL samples along with the increase in EBV DNA content is of particular interest, as transcription of *LMP1* in PBL has so far been reported only in infectious mononucleosis patients [29]. LMP1 promotes B-cell survival and proliferation by up-regulating the expression of anti-apoptotic Bcl-2 and A20 proteins, [51–53], and is consistently expressed in a variety of EBV-associated malignancies, including large cell-immunoblastic NHL arising in HIV-1-infected subjects [4,27,28]. While LMP1-positive circulating B cells can be eliminated by specific cytotoxic T lymphocytes in healthy EBV-infected carriers [54], it is conceivable that in a context of impaired immunosurveillance, LMP1-expressing cells might be favoured in their survival and proliferation.

In conclusion, our study demonstrated that HIV-1- and EBV-coinfected patients showing a significant increase in peripheral CD4 cells but lack of substantial HIV-1 suppression during HAART underwent an increase in B-cell stimulation, and an expansion of EBV-positive B cells expressing LMP1. As both parameters are predictive of NHL [3,25], and LMP1 is a key viral protein in EBV-mediated transformation, these findings suggest that patients with such a paradoxical response to HAART, about 15% of cases [55,56], might represent a risk group for lymphoma development over time, and support the need to change antiretroviral therapy when HIV-1 replication is not substantially suppressed, even though an increase in CD4 cell count takes place.

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