Patients with human immunodeficiency virus type 1 (HIV-1) infection are at high risk of developing Epstein-Barr virus (EBV)–associated lymphoma. However, little is known of the EBV DNA loads in patients receiving highly active antiretroviral therapy (HAART). Using a real-time quantitative polymerase chain reaction assay, we demonstrated that significantly more HIV-1–infected patients receiving HAART than HIV-1–uninfected volunteers had detectable EBV DNA in blood (57 [81%] of 70 vs. 11 [16%] of 68 patients; $P < .001$) and saliva (55 [79%] of 68 vs. 37 [54%] of 68 patients; $P = .002$). The mean EBV loads in blood and saliva samples were also higher in HIV-1–infected patients than in HIV-1–uninfected volunteers ($P = .001$). The frequency of EBV detection in blood was associated with lower CD4+ cell counts ($P = .03$) among HIV-1–infected individuals, although no differences were observed in the EBV DNA loads in blood or saliva samples in the HIV-1–infected group. Additional studies are needed to determine whether EBV-specific CD4+ and CD8+ cells play a role in the pathogenesis of EBV in HIV-1–infected patients receiving HAART.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that infects most of the adult population worldwide. A primary mode of transmission is through saliva, by which the virus invades the oropharyngeal mucosa and infects B lymphocytes, establishing latency in the B cell compartment [1–3]. Studies from the pre-HAART era suggested that patients with HIV-1 infection had higher EBV genome loads in PBMCs than did healthy individuals [4–6]. In addition, HIV-1–infected patients had increased amounts of EBV in oropharyngeal secretions, compared with HIV-1–seronegative persons [7–12]. Immunodeficiency increases the risk of some types of cancer, especially malignancies that are etiologically linked to viral agents [13, 14]. EBV is suspected of playing a major causative role in primary CNS non-Hodgkin lymphoma (NHL) and Hodgkin lymphoma in HIV-1–infected patients, because most of those tumors contain EBV DNA and express viral genes [13–16]. EBV is also considered a cofactor in the development of a subset of cases of systemic NHL during HIV-1 infection [13–15, 17, 18].

The introduction of HAART and prophylaxis against opportunistic diseases have significantly improved the rate of survival of patients with HIV-1 infection [19, 20]. HAART can cause a significant and sustained decrease in peripheral blood HIV-1 RNA levels, as well as an increase in the number of CD4+ cells [21–24]. However, the influence of this therapy on EBV-associ-
ated malignancies is less clear [25, 26]. Recent data from different cohorts of adult HIV-1–infected patients from the United States and western Europe have shown no significant decreases in the incidence of NHL and Hodgkin lymphomas, in contrast to reductions in the incidence of Kaposi sarcoma and primary CNS NHL among patients treated with HAART [20, 27–35]. A meta-analysis of the incidence of cancer in selected cohorts [36] of HIV-1–infected patients from the period of 1992–1996 to 1997–1999 by the International Collaboration on HIV and Cancer suggested a small decrease in the rate of systemic diffuse large-cell lymphoma but no decreases in other types of systemic NHL. However, it is important to point out that the analysis of systemic NHL is limited by the lack of specific pathologic information from some of the cohorts included in the meta-analysis. Furthermore, a limited assessment of Hodgkin lymphoma was performed in that study. Thus, it is unclear whether the incidence of systemic lymphoproliferative disorders among HIV-1–infected patients has changed by the use of HAART.

Data on the EBV burden in HIV-1–infected patients receiving HAART are based on small studies [37–39]. More importantly, up to 52% of the patients included in those studies had discordant virological and immunological responses to HAART [37–39], suggesting noncompliance with the therapeutic regimen or the emergence of drug-resistant HIV-1 strains. Therefore, it is difficult to establish whether the EBV DNA levels in patients receiving HAART were different than those in HIV-1–uninfected individuals or whether EBV DNA loads correlate with CD4+ cell counts. The goals of the present investigation were to quantitatively evaluate the frequency of detection of EBV DNA in blood and saliva samples obtained from adult HIV-1–infected patients receiving HAART, to determine whether detection rates differ among HIV-1–infected patients and HIV-1–uninfected volunteers, and to ascertain whether EBV DNA loads in HIV-1–infected patients correlate with CD4+ cell counts.

METHODS

Patients. Seventy adult HIV-1–infected patients receiving care at the Thomas Street Clinic of the Harris County Hospital District in Houston, Texas, were enrolled prospectively from August 2000 through March 2001. Inclusion criteria at the time of enrollment included (1) positive results of ELISA and Western blot serological tests for HIV-1, (2) an HIV-1 RNA load of <1000 copies/mL and a CD4+ cell count of 200–700 cells/mm³, (3) receipt of a stable HAART regimen for ≥3 months (HAART was defined as 2 nucleoside reverse-transcriptase inhibitors and 1 protease inhibitor and/or 1 nonnucleoside reverse-transcriptase inhibitor), (4) no active opportunistic infection within the past 3 months, and (5) no receipt of antiviral medications with activity against herpesviruses for ≥2 weeks before enrollment. HIV-1–infected patients were excluded if they had any active malignancy or history of NHL or Hodgkin lymphoma. Sixty-eight HIV-1–uninfected individuals without any type of cancer also enrolled and served as the control group. Institutional review board approval was obtained for this study.

Obtainment and processing of blood and saliva samples. Blood and saliva specimens were obtained on the day of enrollment for each patient after receiving informed consent. Fifty milliliters of whole blood was collected into acid-citrate-dextrose collection tubes by peripheral venipuncture using antiseptic techniques. PBMCs were obtained after differential centrifugation through a ficoll-hypaque gradient (Accuspin System-Histopaque-1077 centrifuge tube; Sigma). The PBMCs were aliquoted at 1 × 10⁷ cells/cryovial and pelleted, and the pellets were stored at −70°C. Saliva samples were obtained from cotton plugs that had been chewed by each individual for 1–2 min. The plugs were placed into collection tubes (Sarstedt) and spun at low speed; the liquid was then transferred to cryovials and stored at −70°C.

Extraction and purification of DNA. All sample processing was performed in a laminar flow hood within a BL3 facility free from viruses and plasmids at the Department of Molecular Virology and Microbiology, Baylor College of Medicine (Houston). PBMC DNA was prepared using a QI Amp DNA Blood Mini-Kit (Qiagen) in accordance with the manufacturer’s protocol. Saliva samples of up to 500 μL were processed using a standard proteinase K-phenol procedure for DNA extraction [40]. Samples were incubated at 55°C for 5 h with proteinase K (Fisher), were phenol:chloroform extracted, and the aqueous layer was precipitated by isopropanol precipitation. The DNA pellets were then resuspended in TE buffer (pH, 8.0) and stored at −20°C.

PCR amplification and quantification of EBV DNA. EBV was detected using real-time quantitative PCR with a fluorogenic probe, as described elsewhere [41]. Primers and probe for the EBER gene of EBV were developed using primer express software (PE Applied Biosystems). The forward and reverse primers (EBER-FP/EBER-RP) were 5′-TGACGTAGTCTGCTCTTGAGGAGATG-3′ and 5′-CGTCTGCTCCCTAGCAAAACC-3′. The fluorogenic probe was VIC-TGCAAAACCTCAGGACCTTACGGTGCTG-TAMRA. The PCR reaction was performed using a Taqman PCR kit (PE Applied Biosystems). In brief, 500 ng of DNA extracted from PBMCs or saliva was added to a PCR mixture containing 10 mmol/L Tris (pH, 8.3); 50 mmol/L KCl; 10 mmol/L EDTA; 5 mmol/L MgCl₂; 100 μmol/L dATP, dCTP, dGTP, and dTTP; 0.2 μmol/L of each primer; 0.1 μmol/L fluorogenic probe; and 1.25 U of AmpliTaq Gold. Samples with a known template amount were run during the reaction to generate a standard curve of Ct versus LogN (N denotes the original copy number in the standard). The standards were prepared using IB4 cells, which contain 2 EBV genome copies/
cell. EBV-negative DG75 cells were used to equilibrate all standards to a total mass of 500 ng. A dilution series was made with decreasing amounts of IB4 DNA (100, 50, 10, 5, 1, 0.5, and 0 ng). These amounts correspond to 20,000, 10,000, 2000, 1000, 200, 100, and 0 copies of EBV, respectively. The limit of detection of the assay was ~10 EBV copies from 500 ng of total PBMC DNA. Because of the variability of DNA obtained from saliva samples and as a confirmation of sample quality, all samples were also tested for amplification of the β-actin gene. Primers and conditions for real-time PCR of β-actin were followed according to the manufacturer’s recommendations (PE Applied Biosystems).

**HIV-1 RNA load and CD4+ cell count.** The HIV-1 RNA load in plasma was measured by quantitative RNA-PCR assay (AmpliCor HIV Monitor Test, Roche Diagnostics), which has a lower limit of 400 copies/mL, as part of the clinical follow-up for patients at the Thomas Street Clinic of the Harris County Hospital District. The CD4+ cell counts for patients were determined in fresh blood specimens obtained on the day of enrollment by standard flow cytometry techniques [42, 43].

**Statistical analysis.** EBV loads were transformed to \( \log_{10} \) scale before analysis. Values that were less than the limit of detection (<10 EBV copies per 500 ng of total PBMC or saliva DNA) were set at 1.0, so that the \( \log_{10} \) value was 0.0 for those values. The correlation between EBV DNA load and CD4+ count in HIV-1–infected patients receiving HAART was determined according to the categories established by the Centers for Disease Control and Prevention in their classification system for HIV-1 infection and their expanded AIDS surveillance definition for adolescents and adults [44]. The continuous variables were not normally distributed, so a nonparametric test was used for the testing. The level of significance was set at \( P = .05 \).

**RESULTS**

The demographic characteristics and the WBC count of the 138 adult HIV-1–infected patients and HIV-1–uninfected volunteers at the time that blood and saliva samples were obtained are shown (table 1). The mean ages (± SD) of the infected and uninfected groups were similar (41.6 ± 8.0 and 37.5 ± 11 years, respectively). There were no significant differences in the mean WBC count, total lymphocyte percentage, or mean B cell count among HIV-1–infected patients and HIV-1–uninfected volunteers (table 1). The mean CD4+ count (± SD) of HIV-1–infected patients receiving HAART was 444 ± 164 cells/mm³ (range, 172–787 cells/mm³). Four patients (6%) had CD4+ cell counts of <200 cells/mm³ (range, 172–196 cells/mm³). Sixty-one (87%) of 70 HIV-1–infected patients had plasma virus loads that were less than the level of detection (i.e., <400 copies/mL), and 9 subjects (13%) had a median virus load of 680 copies/mL (range, 426–934 copies/mL).

**EBV DNA detected by PCR of blood and saliva samples.** PBMCs and saliva specimens obtained from both groups of patients were examined for viral sequences by PCR amplification using primer sets specific for the EBV EBER gene (EBER-FP/EBER-RP). PCR products were generated from PBMCs obtained from 57 HIV-1–infected patients (81%) and 11 HIV-1–negative subjects (16%). Two saliva specimens obtained from HIV-1–infected patients were excluded from the EBV analysis because they did not amplify the β-actin gene. EBV sequences were detected in saliva specimens obtained from 55 (79%) of the HIV-1–infected patients and 37 (54%) of the HIV-1–negative subjects. The mean EBV levels in PBMC and saliva samples among HIV-1–infected patients and HIV-1–negative individuals are presented in table 2.

The frequency of detection of EBV DNA in blood samples obtained from HIV-1–infected patients was significantly different than that for uninfected persons (table 2). The frequency of detection of EBV DNA in saliva samples was also significantly different for HIV-1–infected patients versus HIV-1–uninfected volunteers (table 2). Of note, there were significant differences in the mean EBV loads in blood samples (10^1.29 vs. 10^0.27 copies/500 ng DNA; \( P = .001 \)) and saliva samples (10^3.45 vs. 10^1.43 copies/500 ng DNA; \( P = .001 \)) obtained from HIV-1–infected patients and HIV-1–uninfected individuals, respectively.

**Correlation between EBV DNA loads and CD4+ cell

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV-1–infected patients ( (n = 70) )</th>
<th>HIV-1–uninfected volunteers ( (n = 68) )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>41.6 ± 8.0</td>
<td>37.5 ± 11</td>
<td></td>
</tr>
<tr>
<td>No. of men/no. of women</td>
<td>46/24</td>
<td>36/32</td>
<td></td>
</tr>
<tr>
<td>Laboratory value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC count, cells/mm³</td>
<td>6036 ± 1835</td>
<td>6321 ± 1777</td>
<td>.42</td>
</tr>
<tr>
<td>Total lymphocyte percentage</td>
<td>39 ± 9</td>
<td>40 ± 8</td>
<td>.73</td>
</tr>
<tr>
<td>CD19 B cell count, cells/mm³</td>
<td>334 ± 218</td>
<td>361 ± 179</td>
<td>.50</td>
</tr>
</tbody>
</table>

**NOTE:** Data are mean ± SD, unless otherwise indicated.
counts. Among the EBV DNA–positive HIV–1–infected patients receiving HAART, there was a significantly increased rate of detection of EBV in the blood of those who had CD4+ cell counts of 200–500 cells/mm³, compared with those who had CD4+ cell counts of >500 cells/mm³ (table 3). However, when the EBV DNA loads in PBMCs were compared by CD4+ cell count, no significant differences in the mean number of EBV copies per 500 ng of PBMC DNA were observed among the 3 groups of patients (table 3). The analysis of EBV DNA loads in saliva specimens obtained from HIV–1–infected patients, by CD4+ cell count, was restricted to patients with CD4+ cell counts of 200–500 cells/mm³ (33 patients) and >500 cells/mm³ (17 patients), because only 4 patients with CD4+ cell counts of <200 cells/mm³ had detectable EBV DNA. No significant differences in the mean EBV load in saliva specimens was observed among these patients (3.71 vs. 3.04 copies/500 ng DNA; P = .3).

**DISCUSSION**

This investigation showed high EBV DNA loads in the PBMCs and saliva specimens obtained from HIV–1–infected patients, despite receipt of treatment with HAART, evidence of HIV–1 suppression, and absence of EBV-related malignancies. Moreover, there were more EBV DNA–positive HIV–1–infected patients than EBV DNA–positive HIV–1–negative volunteers, and the number of EBV copies detected in blood and saliva specimens obtained from HIV–1–infected persons was significantly higher than that for HIV–1–seronegative individuals. The differences in EBV positivity rates and viral DNA loads may be related to an impaired immune surveillance against EBV during HIV–1 infection, even among individuals with nondetectable HIV–1 replication and high CD4+ cell counts. This is an important finding, because recent data [34] indicate that 35% of HIV–1–infected patients with systemic NHL receiving HAART had both HIV–1 RNA loads that were less than the level of detection and high CD4+ cell counts. In this same study group, there was also evidence of more frequent shedding of JC virus by the HIV–1–infected patients receiving HAART, which supports the interpretation that their control of viral infections has not returned to normal [45].

The results of the present study are in agreement with a recent report that suggested that HAART has no influence on PBMC EBV loads and that the range of EBV copies in patients who are and are not receiving HAART are not significantly different [38]. In addition, we observed a high number of EBV copies in blood samples obtained from HIV–1–infected patients receiving HAART, which may explain, in part, the accumulating data from different cohorts showing no significant decrease in the incidence of systemic NHL and Hodgkin lymphoma [20, 27–35]. An elevated EBV load in blood suggests that there is an increased number of circulating EBV–infected B lymphocytes. This may increase the risk of malignant overgrowth of

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample</th>
<th>n/N (%)a</th>
<th>P</th>
<th>Mean EBV DNA load ± SDb</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV–1–infected patients</td>
<td>PBMC</td>
<td>57/70 (81)</td>
<td>.001</td>
<td>1.79 ± 1.10</td>
<td>.001</td>
</tr>
<tr>
<td>HIV–1–uninfected volunteers</td>
<td>PBMC</td>
<td>11/68 (16)</td>
<td></td>
<td>0.27 ± 0.71</td>
<td></td>
</tr>
<tr>
<td>HIV–1–infected patients</td>
<td>Saliva</td>
<td>55/68 (81)</td>
<td>.002</td>
<td>3.45 ± 2.26</td>
<td>.001</td>
</tr>
<tr>
<td>HIV–1–uninfected volunteers</td>
<td>Saliva</td>
<td>37/68 (54)</td>
<td></td>
<td>1.43 ± 1.62</td>
<td></td>
</tr>
</tbody>
</table>

a No. of samples with positive results/no. of samples tested (%).

b Mean no. of log₁₀ copies/500 ng DNA ± SD.

Table 3. Epstein–Barr virus (EBV) DNA loads, by CD4+ cell count, in PBMC samples obtained from HIV–1–infected patients receiving HAART.
those EBV-infected cells and subsequent development of some lymphomas. Indeed, one model for the pathogenesis of EBV-associated systemic lymphoma (Burkitt’s type) during HIV-1 infection postulates that these malignancies may result from EBV infection and transformation of B lymphocytes [46, 47]. However, longitudinal data suggest that the absolute level of EBV DNA in PBMCs obtained from HIV-1–infected patients is not predictive of diffuse large cell–type systemic NHL [48]. The EBV load in PBMCs was high and displayed considerable fluctuation over time in those patients, and the absolute level of EBV DNA was not related to CD4+ cell count or HIV-1 RNA load at the time of systemic NHL diagnosis. EBV was detectable in serum at some time points but at a lower level than in PBMCs. These results substantiate theories that the development of lymphomas in HIV-1–infected individuals is a complex multistep process not determined solely by EBV load [13, 14, 34].

The present study shows that EBV DNA loads in blood and saliva do not correlate with CD4+ cell counts in HIV-1–infected patients receiving HAART, which expands and confirms recent reports [37–39, 48] that have indicated no clear correlation between EBV load in PBMCs and CD4+ T cells. Assessing EBV-specific T cell responses in relation to EBV DNA loads in PBMCs rather than measuring the total CD4+ T cell population may provide insights into EBV pathogenesis during HIV-1 infection. Recently, Van Baarle et al. [49] indicated that individuals co-infected with HIV and EBV had low numbers of HIV-specific CD27+ T cells, in contrast to increased numbers of EBV-specific CD27+/CD8+ T cells. However, HIV-1–infected patients developing EBV-associated NHL had very low numbers of EBV-specific CD27+/CD8+ T cells. This observation suggests that virus-specific CD27+ T cells may play a role in controlling chronic viral infections. This is important, because data suggest that the continuing efficacy of present antiretroviral therapy may allow more HIV-1–infected patients to survive with long-term, mild-to-moderate immunosuppression, thereby placing such patients at risk for development of lymphoproliferative disorders, such as Hodgkin lymphoma and systemic NHL [34, 35].

In conclusion, the presence of EBV in the blood of HIV-1–infected patients receiving HAART was significantly associated with lower CD4+ cell counts; however, the EBV DNA loads in blood and saliva samples obtained from those patients who were EBV positive were high and did not correlate with CD4+ cell counts. Future studies that evaluate the dynamics of EBV infection in patients receiving HAART are needed, because evidence suggests that there is a persistent risk of some EBV-related lymphomas in those individuals.

References