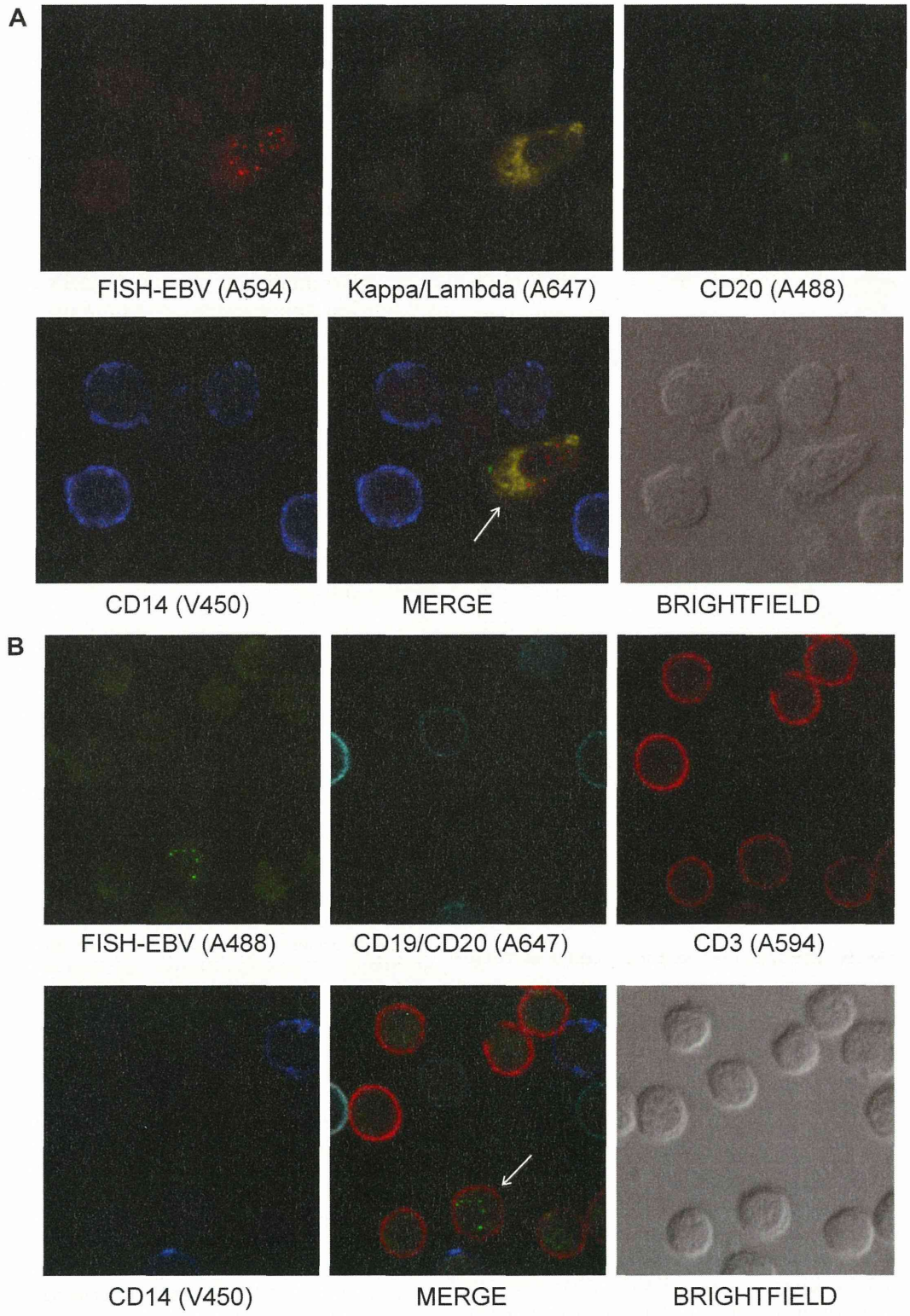


**Figure 3. EBV is detected in plasmablasts/plasma cells and T cells.** (A) Photomicrographs of PBMCs stained by Immuno-FISH from patient 4 shows EBV in plasmablasts/plasma cells identified using Alexa 647–conjugated anti-κ and anti-λ chain immunoglobulin antibodies. The EBV-positive cells are negative for CD20 using Alexa 488–conjugated anti-CD20 antibody. EBV DNA was identified with the biotinylated FISH probe and streptavidin-conjugated Alexa 594 and monocytes with V450-conjugated anti-CD14 antibody. Although most of the EBV DNA was detected in the nucleus, some viral DNA was detected in the cytoplasm, suggesting that EBV lytic infection was present in the cell. (B) Photomicrographs of PBMCs stained by Immuno-FISH from patient 12 shows EBV in $CD3^+$ T cells. EBV DNA was identified with biotinylated FISH probe and streptavidin-conjugated Alexa 488, B cells using a combination of Alexa 647–conjugated anti-CD19 and anti-CD20 antibodies, T cells with Alexa 594–conjugated anti-CD3 antibody, and monocytes with V450-conjugated anti-CD14 antibody. Arrows indicate EBV-positive cells.

gene expression.[37,38] It is possible that EBV infected T cells using CD21 in our patients and down-regulated the expression of CD21; alternatively, EBV may have used a different receptor than CD21 to enter the T cells.

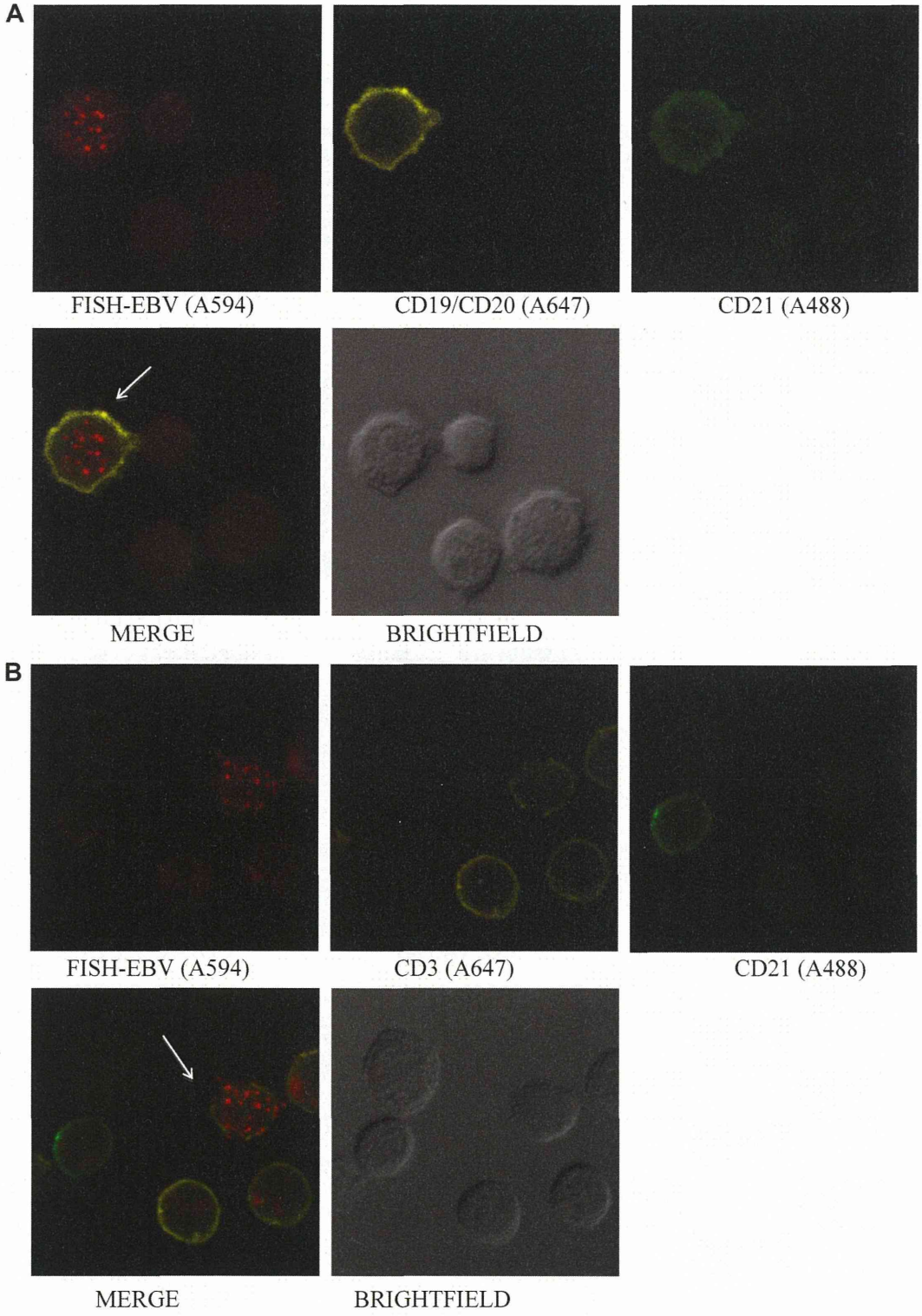


**Figure 4. EBV is detected in CD21$^+$ B cells and in CD21$^-$ T cells.**(A) Photomicrographs of PBMCs stained by Immuno-FISH from patient 12. EBV is detected in B cells that are CD21$^+$. (B) EBV is detected in CD3$^+$ T cells that do not express CD21. EBV DNA was identified with the biotinylated FISH probe and streptavidin-conjugated Alexa 594, B cells using a combination of Alexa 647–conjugated anti-CD19 and anti-CD20 antibodies, T cells with Alexa 647–conjugated anti-CD3 antibody, and CD21 with Alexa 488–conjugated anti-CD21 antibody. Arrows indicate EBV-positive cells.

We detected EBV in monocytes from 4 patients. EBV infection of peripheral blood monocytes has been described in patients with HIV based on sorting of cells by flow cytometry and EBV DNA PCR.[15] EBV has also been reported to infect monocytes in vitro and result in the activation of the viral lytic cycle.[39-41] Our observation that very few monocytes were infected by EBV, and that the viral copy number per cell was very low (ranging from 1 to 3 copies per cell), is consistent with the hypothesis that EBV infection of monocytes might be very transient with lytic replication, resulting in the rapid death of infected cells, or that monocytes have a shorter lifespan than B cells in the circulation.

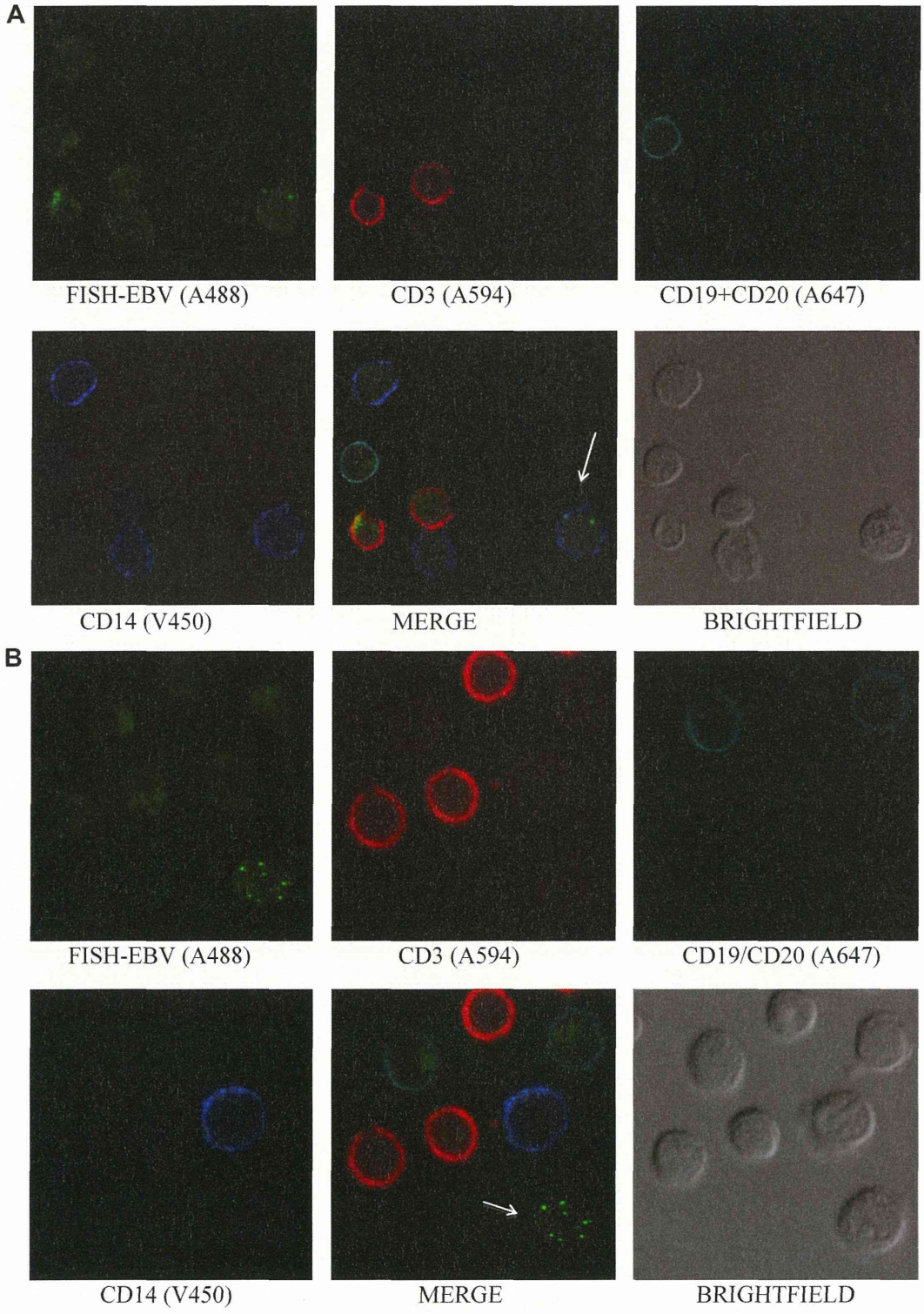


**Figure 5. EBV is detected in monocytes and in non-B, non-T, and non-monocyte cells.** (A) PBMCs stained by Immuno-FISH from patient 5 shows EBV in monocytes. EBV DNA was identified with the biotinylated FISH probe and streptavidin-conjugated Alexa 488, T cells using Alexa 594–conjugated anti-CD3, B cells using a combination of Alexa 647–conjugated anti-CD19 and anti-CD20 antibodies, and monocytes with V450-conjugated anti-CD14 antibody. Arrow indicates EBV-positive cell. (B) PBMCs stained by Immuno-FISH from patient 6 shows EBV in non-B, non-T, and non-monocyte cells. Arrow indicates EBV-positive cell.

We found that 69% (20 of 29) of the patients had EBV in circulating non-B, non-T, non-monocyte cells. These cells might represent one or more of several cell types. First, these non-B, non-T, non-monocyte cells might be plasmablasts/plasma cells. Approximately half (9 of 17) of the patients with EBV in non-B, non-T, non-monocyte cells had virus in plasmablasts/plasma cells ($CD20^-$, positive for κ and λ immunoglobulin light chains). Therefore, some of the non-B, non-T, non-monocyte cells may have been plasmablasts/plasma cells that were not stained with antibody to κ and λ immunoglobulin light chains. These cells, however, have a very prominent cytoplasm (Figure 3A), and most of the non-B, non-T, non-monocyte cells did not have a prominent

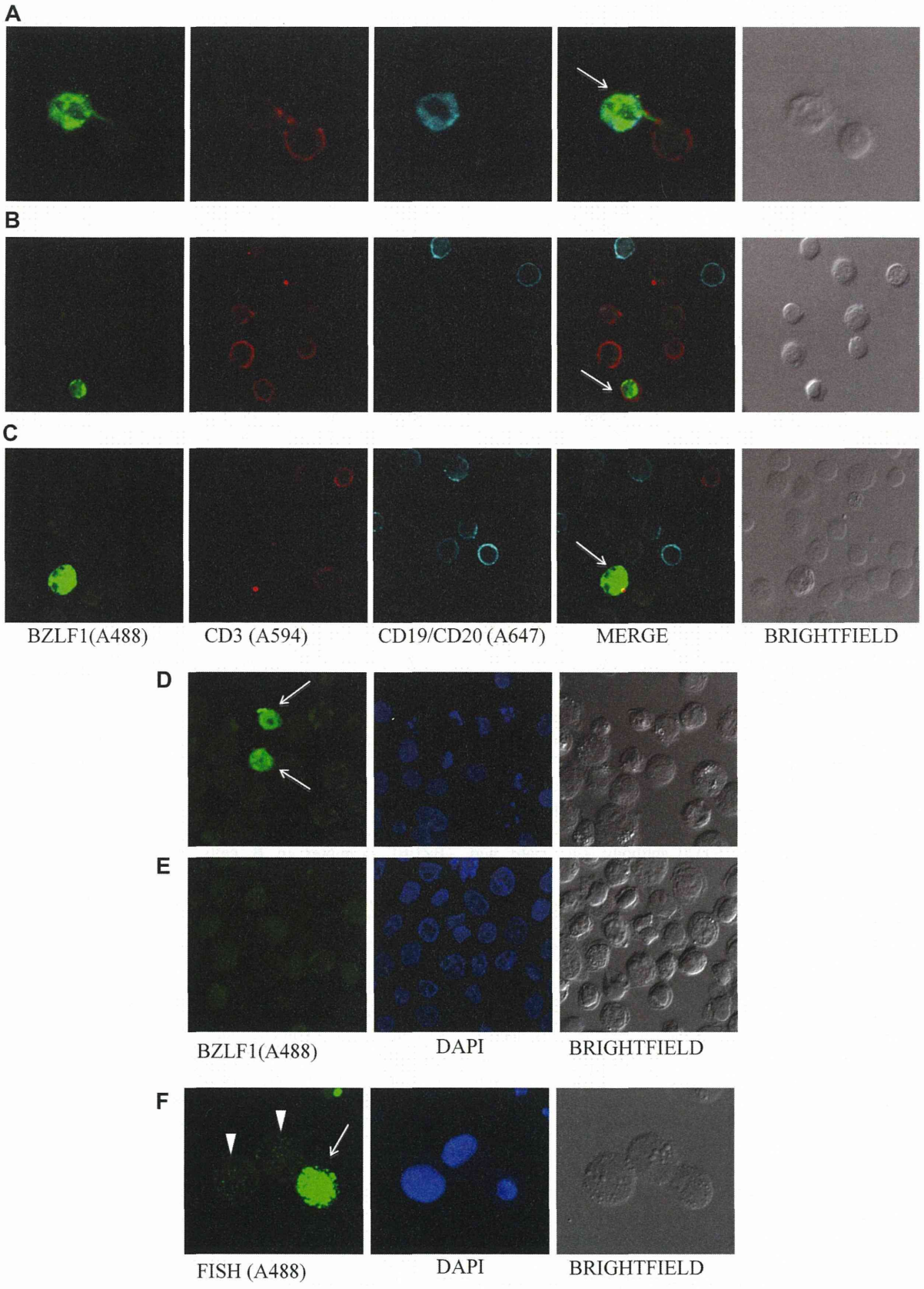


**Figure 6. EBV lytic protein BZLF1 is detected in PBMCs.** Photomicrographs of PBMCs stained for EBV BZLF1 shows virus lytic infection in B cells (patient 21, A), T cells (patient 12, B), and non-B, non-T cells (patient 26, C). BZLF1 was directly conjugated to Alexa 488. T cells were stained by using Alexa 594–conjugated anti-CD3, and B cells by using a combination of Alexa 647–conjugated anti-CD19 and anti-CD20 antibodies. BZLF1 staining of EBV-positive B95-8 (D) and EBV-negative BJAB (E) cells served as positive and negative controls, respectively, for EBV lytic infection. Arrows indicate BZLF1-positive cells. (F) B95-8 cells stimulated with butyric acid and TPA (phorbol 12-myristate 13-acetate) served as an additional control for lytic infection; cells undergoing lytic infection show a homogeneous pattern with high levels of EBV DNA (arrow), and latently infected cells show a punctate pattern (arrowheads).

**Table 3. Distribution of the number of EBV genomes per cell**

| Patient | Diagnosis | EBV DNA load/$10^6$ cells | % of range of EBV genomes per cell | | | | Total EBV$^+$ cells counted |
|---|---|---|---|---|---|---|---|
| | | | 1-10 | 11-20 | 21-30 | > 30 | |
| 1 | HIV | 12 000 | 10 | 30 | 40 | 20 | 10 |
| 2 | HIV | 4300 | 34 | 44 | 22 | 0 | 9 |
| 3 | HIV | 18 000 | 31 | 31 | 38 | 0 | 13 |
| 4 | HIV | 130 000 | 50 | 38 | 4 | 8 | 24 |
| 5 | HIV | 9400 | 75 | 25 | 0 | 0 | 4 |
| 6 | HIV | 5100 | 43 | 43 | 7 | 7 | 14 |
| 7 | HIV | 22 000 | 58 | 42 | 0 | 0 | 19 |
| 8 | HIV | 6300 | 25 | 38 | 25 | 12 | 16 |
| 9 | Tx | NA | 75 | 25 | 0 | 0 | 4 |
| 10 | Tx | 30 000 | 54 | 38 | 8 | 0 | 24 |
| 11 | Tx | 6200 | 40 | 40 | 20 | 0 | 10 |
| 12 | LyG/HL | 170 000 | 64 | 27 | 6 | 3 | 77 |
| 13 | Severe primary EBV infection | 280 000 | 27 | 54 | 11 | 8 | 37 |
| 14 | PTLD | 1 300 000 | 36 | 29 | 9 | 16 | 33 |
| 15 | ATL EBV-LPD | 190 000 | 48 | 44 | 4 | 4 | 25 |
| 16 | LyG | 28 000 | 70 | 25 | 0 | 5 | 20 |
| 17 | ICL | 110 000 | 45 | 31 | 21 | 3 | 38 |
| 18 | AA | 64 000 | 69 | 23 | 8 | 0 | 13 |
| 19 | AA | 110 000 | 46 | 21 | 25 | 8 | 24 |
| 20 | AA | 6 500 000 | 36 | 37 | 18 | 9 | 55 |
| 21 | B-cell CAEBV | 15 000 | 65 | 30 | 5 | 0 | 20 |
| 22 | B-cell CAEBV | 30 900 | 31 | 54 | 9 | 6 | 35 |
| 23 | B-cell CAEBV | 310 000 | 54 | 33 | 13 | 0 | 15 |
| 24 | T-cell CAEBV | 54 666/μg | 75 | 24 | 1 | 0 | 88 |
| 25 | T-cell CAEBV | 67 612/μg | 71 | 29 | 0 | 0 | 21 |
| 26 | T-cell CAEBV | 230 000/μg | 56 | 41 | 3 | 0 | 78 |
| 27 | T-cell CAEBV | 4154/μg | 54 | 39 | 7 | 0 | 18 |
| 28 | IM | 1700 | 65 | 29 | 6 | 0 | 17 |
| 29 | IM | 10 651 | 57 | 29 | 14 | 0 | 14 |

Additional cell subsets (eg, "non-T, non-NK, non-monocytes") that are not represented in Tables 1 and 2 are included in the counts for this table.

cytoplasm. Second, some of the non-B, non-T, non-monocyte cells might be B cells with very low levels of CD20 that are undetectable by antibody staining. This seems unlikely, based on the sensitivity of Immuno-FISH to detect B cells after sorting for B cells (supplemental Table 1). Furthermore, only 1 of the 29 patients (patient 21) had received anti-CD20 antibody (rituximab), and EBV was still detected in his B cells. Third, EBV could be present in cells other than T, B, NK cells, and monocytes. For example, EBV infects pre-Langerhans cells in the peripheral blood.[16] However, we did not detect EBV DNA in pre-Langerhans cells (CD1a$^+$ CD11c$^+$, and CD14$^-$) or in basophils (CD123$^+$) in PBMCs from 4 patients who had EBV in non-B, non-T, non-monocyte cells (patients 12, 15, 16, and 20), using Immuno-FISH (data not shown). Infection of non-B, non-T, non-monocyte cells may be more common in immunocompromised persons. The only groups of patients for whom we did not detect EBV in these cells were patients with T-cell CAEBV and IM; the latter were otherwise healthy persons. In addition, the 1 HIV patient without EBV in non-B, non-T, and non-monocyte cells had the highest CD4 T-cell count (354 cells per μL); the other HIV patients all had CD4 T-cell counts less than 250 cells per μL.

We detected evidence of lytic EBV infection in nearly all of the patients for whom we performed immunofluorescence staining for BZLF1 protein. BZLF1 is the first viral protein expressed during the reactivation of EBV in B cells; it initiates a cascade of viral lytic gene expression, culminating with the death of the infected cells.[42] It is interesting to note that although some of the BZLF1-positive cells were CD19/C20$^+$, others were negative when stained with antibodies for B and T cells. These cells could be plasmablast/plasma cells (negative for CD19/CD20), because EBV-positive plasmablast/plasma cells were detected by Immuno-FISH in most of these patients. BZLF1 was previously detected in lymphocytes in tonsils of patients with IM; most of these cells were CD20$^-$ and VS38c$^+$ with morphologic features indicative of plasma cells, whereas only rare cells were CD20$^+$.[43] The BZLF1 promoter in B cells is activated only after they differentiate into plasma cells.[31] In the latter study, BZLF1 was detected almost exclusively in CD20$^{lo}$ plasma cells in human tonsils. Plasma cells in the bone marrow are CD20$^-$, whereas plasma cells in tonsils express low levels of CD20.[44]

Immuno-FISH allows one to determine both the EBV genome copy number and phenotypic characterization of individual cells in the peripheral blood. This procedure combines the highly sensitive, specific FISH technique with multicolor immunofluorescence using monoclonal antibodies directly conjugated to Alexa dyes. These fluorochromes are very stable at high temperatures and low pH and therefore maintain their activity during the FISH procedure. Moreover, the use of directly conjugated antibodies obviates the problems of the cross-reacting secondary antibodies and permits the detection of multiple surface markers in the same slide.

Other studies have analyzed EBV copy numbers in various cell populations by sorting cells with monoclonal antibodies to cell surface markers and then performing PCR on the sorted populations. Although this technique has been useful,[2,4] it has several limitations. First, the sensitivity has been estimated to be approximately 90%[2]; therefore, one cannot detect cell populations containing less than 10% of a given cell phenotype. Second, the specificity of cell sorting followed by PCR amplification has limitations; if very rare EBV PCR-positive cells are detected in sorted cells, this could be a result of a low level of

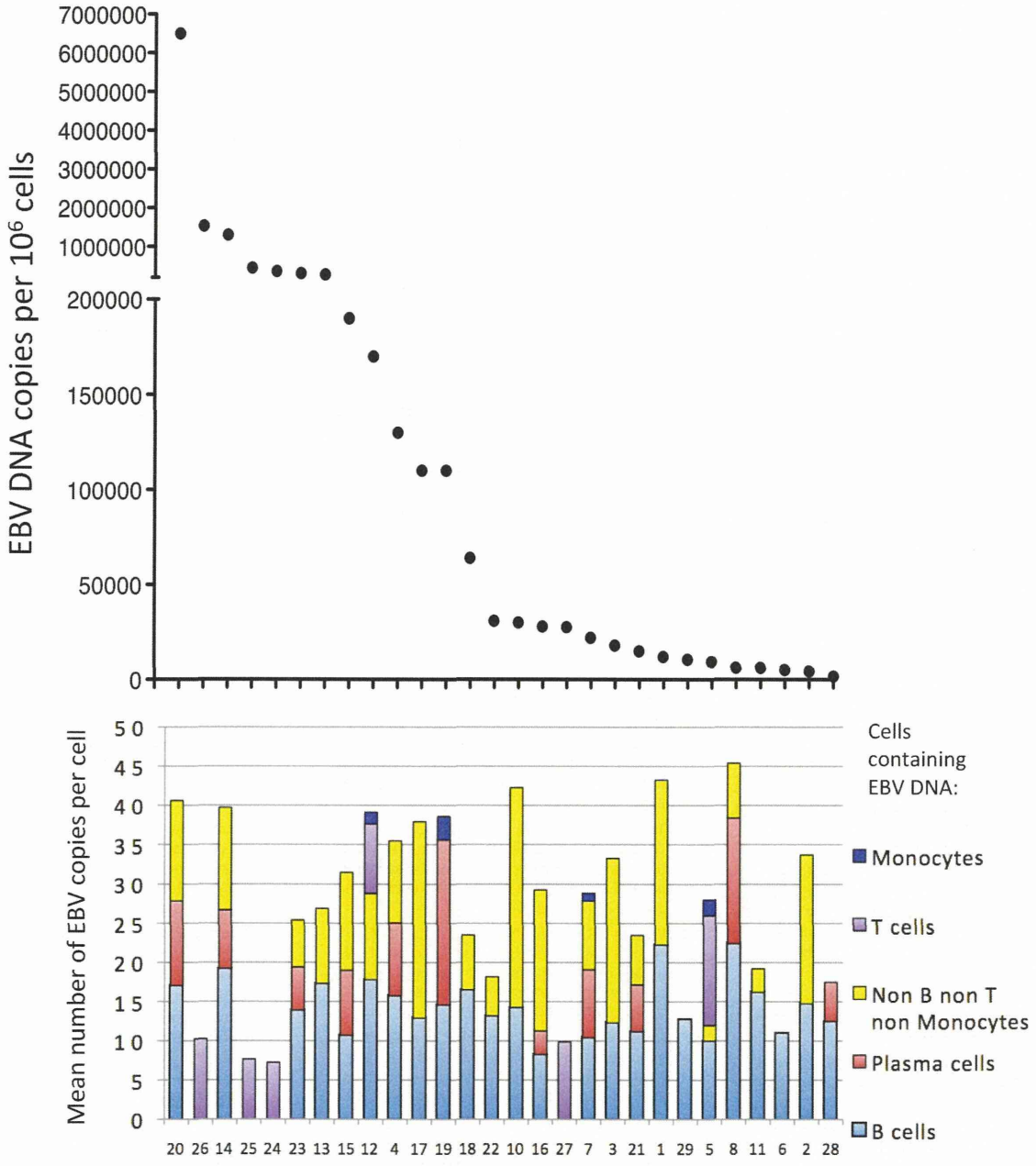


**Figure 7. Correlation between the EBV DNA load in peripheral blood and the mean number of EBV DNA copies per cell for each cell subpopulation for each patient.** Patient numbers are indicated at the bottom of the figure, and the vertical bars for each patient indicate the mean EBV DNA copy number per cell type.

EBV-positive B cells contaminating these sorted cells. Third, the number of EBV genomes per cell cannot be determined. Recently, combined cell-surface staining and in situ hybridization for EBER followed by flow cytometry has been used to identify EBV-positive T-cell subsets in the blood[18]; however, the number of the EBV genomes per cell could not be determined. Fourth, sorting cells requires a large number of cells and may not be feasible in patients with low numbers of PBMCs. Although labor intensive, the Immuno-FISH procedure has improved specificity, compared with the cell sorting and PCR procedure, and allows one to accurately measure the number of EBV genomes per cell. Using this procedure, we were able to determine the phenotype of EBV-infected cells with a specificity of more than 97% (supplemental Table 1; supplemental Figure 1).

Immuno-FISH might be used to better characterize the cell types infected with EBV and viral copy number of individual cells in the peripheral blood of immunocompromised patients. Immuno-FISH does not require tissue biopsies and therefore might be used to noninvasively monitor transplant patients or other immunocompromised patients who have high EBV DNA loads. Although an elevated EBV DNA load is often predictive of EBV PTLD in recipients of T cell–depleted allogeneic hematopoietic cell transplants, EBV DNA loads in non-T cell–depleted allogeneic hematopoietic cell and solid organ transplant recipients may remain persistently elevated without disease and often does not require treatment with potentially toxic agents[5]; currently, there is no noninvasive procedure to predict which of these patients with high EBV DNA loads would develop PTLD. Determination of the number of EBV genomes per cell and the subtype of B (or non-B) cells infected with the virus might provide additional prognostic information for the development of PTLD in patients with high EBV DNA loads. Immuno-FISH should be useful to identify

EBV-infected cell populations in the blood of persons with CAEBV whose B, T, or NK cells are infected with the virus. Immuno-FISH might also be useful for studies of other virus infections in the blood.

In conclusion, using Immuno-FISH, we found that EBV is present in B cells as well as in plasmablasts/plasma cells and non-B, non-T, non-monocyte cells in the blood of patients with lymphoproliferative diseases. In patients with high EBV loads who are immunocompromised, the number of genomes per cell and the type of cells infected may provide additional prognostic information for the development of EBV lymphoproliferative diseases. Moreover, the identification of EBV-positive, CD20-negative cells in the blood may have important implications for rituximab therapy in these patients.

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## Authorship

Contribution: S.C. performed the experiments, analyzed the data, and wrote the manuscript; I.S., P.S., H.K., and R.W.C. provided PBMC samples and critically reviewed the manuscript; and J.I.C. designed the study, analyzed the data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Jeffrey I. Cohen, Laboratory of Infectious Diseases, National Institutes of Health, 50 South Dr, Building 50, Room 6134, Bethesda, MD 20892; e-mail: jcohen@niaid.nih.gov.

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# Immunologic and Virologic Analyses in Pediatric Liver Transplant Recipients with Chronic High Epstein-Barr Virus Loads

**Kensei Gotoh,[1] Yoshinori Ito,[1] Rieko Ohta,[1] Seiko Iwata,[2] Yukihiro Nishiyama,[2] Taro Nakamura,[3] Kenitiro Kaneko,[4] Tetsuya Kiuchi,[3] Hisami Ando,[4] and Hiroshi Kimura[2]**

Departments of [1]Pediatrics, [2]Virology, [3]Transplant Surgery, and [4]Pediatric Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan

***Background.*** Long-term Epstein-Barr virus (EBV) monitoring for potentially life-threatening posttransplant lymphoproliferative disorder (PTLD) has identified asymptomatic patients who maintain high EBV loads over long periods.

***Methods.*** Thirty-one pediatric liver transplant recipients were designated as 11 chronic high EBV load carriers (EBV DNA level >5000 copies/mL of whole blood for >6 months) and 20 control recipients. Serial quantification of EBV DNA, measurement of interleukin 10 (IL-10) concentrations, EBV-specific tetramer staining, and relative quantification of EBV gene expression in peripheral blood mononuclear cells were performed.

***Results.*** Most of the chronic high EBV load carriers were seronegative at transplant, the median time to resolution of a chronic high EBV load was 23 months, and no recipient developed late-onset PTLD. EBV DNA was detected predominantly in $CD19^+$ cells. The plasma concentration of IL-10 and the EBV-specific $CD8^+$ cell frequency did not differ significantly between the chronic high EBV load carriers and the control recipients. Analysis of gene expression showed that EBV-encoded small RNA 1, *Bam*HI A rightward transcripts, and latent membrane protein 2 were positive in peripheral blood mononuclear cells from chronic high EBV load carriers.

***Conclusions.*** EBV-infected cells in the blood of chronic high EBV load carriers expressed a highly restricted set of latency genes, suggesting that the EBV-infected cells escaped from a T cell response.

Epstein-Barr virus (EBV) is a ubiquitous virus that usually infects humans by early adulthood and can cause benign or severe disease. EBV often persists in infected cells, and all EBV-positive cells exhibit 1 of 4 latency types, distinguished by the pattern of expressed EBV antigens. In latency type 0, all antigens are suppressed, as in a healthy virus carrier. In latency type 1, only EBV-encoded nuclear antigen 1 (EBNA1) is expressed, as in Burkitt lymphoma. Latent membrane protein 1 (LMP1) and LMP2, as well as EBNA1, are expressed in latency type 2, as in Hodgkin disease. In latency type 3, highly immunogenic EBNA3 genes (EBNA3A, EBNA3B, and EBNA3C) are expressed, together with other EBV-latent antigens, as in posttransplant lymphoproliferative disorder (PTLD). The noncoding EBV-encoded small RNAs (EBERs) and the *Bam*HI A rightward transcripts (BARTs) are expressed in all latency types [1–3]. EBV-specific immune control is mediated by innate and adaptive immune responses. Disruption of the balance of antiviral immunity may lead to the development of EBV-associated disease [4].

EBV-related PTLD is a significant cause of morbidity and mortality after solid-organ transplant in children. It has been reported that elevated levels of EBV DNA are a predictive factor for PTLD, and monitoring EBV loads in the peripheral blood of transplant recipients by polymerase chain reaction (PCR) can help identify patients at risk for developing PTLD before the onset of clinical signs [5–7]. Recently, serial viral load monitoring has identified a population of children who subsequently develop and maintain very high EBV loads

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Reprints or correspondence: Dr Yoshinori Ito, Dept of Virology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466–8550, Japan (yoshi-i@med.nagoya-u.ac.jp).

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in the absence of clinical symptoms [8–12]. This persistent presence of EBV DNA may increase the risk of late-onset PTLD. Previous reports have described the clinical courses among these patients after liver transplant, and a small number of them developed late-onset PTLD [10, 12]; however, no pathophysiological analysis has been reported.

## METHODS

***Patients.*** Thirty-three pediatric liver transplant recipients who underwent living-donor liver transplant at Nagoya University School of Medicine from 1999 through 2007 were registered for this study. The primary diseases were biliary atresia ($n = 25$), fulminant hepatitis ($n = 4$), Alagille syndrome ($n = 1$), congenital absence of portal vein ($n = 1$), metastatic liver tumor ($n = 1$), and ornithine transcarbamylase deficiency ($n = 1$). We performed a prospective analysis of EBV load in whole blood over the course of 6 months. Blood samples for the quantification of EBV DNA were obtained weekly until hospital discharge (median, 10 weeks after transplant; range, 6–37 weeks after transplant) and subsequently at each outpatient visit (monthly for 6 months) thereafter or until the EBV load resolved. Two patients were excluded from this study because they continued to be EBV seronegative throughout the study period. Chronic high EBV load was defined as the continuous presence of EBV loads >5000 copies/mL of whole blood for a minimum of 6 months after asymptomatic EBV infection or after complete resolution of symptomatic EBV infection; 11 patients met this criteria. Twenty pediatric liver transplant recipients who were not chronic high EBV load carriers were studied as control recipients. Blood samples for cell sorting, measurement of interleukin 10 (IL-10) concentration, tetramer staining, and real-time reverse-transcription PCR (RT-PCR) assay were obtained over the time of chronic high EBV load states. At the time of blood sampling, all patients were clinically asymptomatic. The results of these analyses were compared with those from 2 patients who developed EBV-related PTLD after a bone marrow transplant, 15 pediatric immunocompetent patients with acute-phase infectious mononucleosis (IM), and 25 healthy EBV-seropositive adult carriers.

The standard immunosuppressive regimen consisted of tacrolimus and prednisolone, as reported elsewhere [13]. Target tacrolimus trough levels in plasma were as follows: 12–15 ng/mL for the first 2 weeks after transplant, 10 ng/mL for the second through fourth weeks, 5–8 ng/mL for the first through sixth months, 5 ng/mL for the sixth through 12th months, and 2–3 ng/mL after the 12th month. When a liver transplant recipient who was positive for EBV developed clinical symptoms or the whole-blood EBV load increased to >5000 copies/mL, immunosuppression with tacrolimus was gradually decreased and kept at the minimum considered safe. Oral acyclovir (30–60 mg/kg/day) was administered until the EBV load decreased to <5000 copies/mL. No patient received antiviral prophylaxis in this study, and a preemptive approach was adopted to treat patients at risk for PTLD; indeed, no disease occurred.

Informed consent was obtained from all patients or their parents. This study was approved by the University of Nagoya Institutional Review Board.

***Quantification of EBV DNA.*** Viral DNA was extracted from either 200 $\mu$L of whole blood or $10^6$ peripheral blood mononuclear cells (PBMCs), using QIAamp DNA blood kits (Qiagen). A real-time quantitative PCR assay was performed, as described elsewhere [14, 15]. The minimum detection level was 2 copies per reaction, which is equivalent to ~20 copies/$\mu$g of DNA for PBMCs and 100 copies/mL for whole blood.

***Determination of EBV-infected cells.*** To determine which cells harbored EBV, PBMCs were fractionated into $CD3^+$, $CD19^+$, and $CD56^+$ cells by means of Dynabeads (Invitrogen). The fractionated cells were analyzed by real-time quantitative PCR. EBV-infected cell fractions were defined as having larger amounts of EBV DNA than of unfractionated PBMCs [14].

***Plasma concentration of IL-10.*** The plasma concentration of IL-10 was measured using enzyme-linked immunoassay kits (Quantikine HS Human IL-10 Immunoassay; R&D Systems). Normal plasma concentrations in healthy persons are 5 pg/mL or less. The samples for measurement of IL-10 were the same as those used for EBV load measurements. The mean times of blood sampling ± standard deviation (SD) were 1.6 ± 1.2 years after the onset of high EBV load status (3.0 ± 1.8 years after liver transplant) among chronic high EBV load carriers and 4.3 ± 2.3 years after liver transplant among control recipients.

***Tetramer staining.*** Fresh PBMCs from HLA-A2–positive and HLA-A24–positive persons were stained with a phycoerythrin-labeled major histocompatibility complex (MHC) class I tetrameric complex (Medical & Biological Laboratories) and a fluorescein isothiocyanate–labeled anti-CD8 monoclonal antibody (clone B9.11; Immunotech) and analyzed using a FACSCalibur flow cytometer (Becton Dickinson). For each analysis, $CD8^+$ T cells were gated, and 30,000–150,000 events were acquired. The limit of detection was 0.01% of $CD8^+$ T cells. The mean times of blood sampling ± SD were 3.0 ± 1.8 years after the onset of a high EBV load state (1.6 ± 1.2 years after liver transplant) among chronic high EBV load carriers and 4.3 ± 2.3 years after liver transplant among control recipients. The timing of sampling for tetramer staining was after the manipulation of immune suppression in all of the chronic high EBV load carriers. Five of 10 healthy control subjects and 6 of 11 chronic high EBV load carriers were analyzed at least twice during high EBV load periods to determine the stability of subpopulation frequencies.

***RNA purification and real-time RT-PCR.*** RNA was extracted from $2 \times 10^6$ PBMCs, using a QIAmp RNeasy Mini kit (Qiagen). Viral mRNA expression was quantified by 1-step

**Table 1. Characteristics of Chronic High Epstein-Barr Virus (EBV) Load Carriers**

| Patient (sex) | Age at LTx, years | Post-LTx follow-up, years | EBV serology[a] | Post-LTx time to initial EBV,[b] months | EBV-related symptoms | Maximum EBV load, copies/mL | Sustained high EBV load period,[c] months |
|---|---|---|---|---|---|---|---|
| 1 (M) | 0.5 | 0.8 | $R^-/D^+$ | 1.0 | Elevated liver enzyme | $1.0 \times 10^5$ | 12[d] |
| 2 (M) | 1.0 | 2.8 | $R^-/D^+$ | 1.5 | ... | $1.6 \times 10^5$ | 12 |
| 3 (F) | 1.7 | 2.3 | $R^-/D^+$ | 1.2 | Elevated liver enzyme | $1.2 \times 10^5$ | 29 |
| 4 (F) | 0.6 | 3.5 | $R^-/D^+$ | 0.8 | ... | $2.6 \times 10^5$ | 24 |
| 5 (M) | 0.6 | 4.3 | $R^-/D^+$ | 4.5 | ... | $1.4 \times 10^5$ | 8 |
| 6 (F) | 1.4 | 3.8 | $R^-/D^+$ | 10.4 | Fever, diarrhea | $1.1 \times 10^5$ | 36[d] |
| 7 (M) | 5.3 | 1.2 | $R^-/D^+$ | 1.0 | ... | $6.8 \times 10^4$ | 15[d] |
| 8 (F) | 1.6 | 5.0 | $R^-/D^+$ | 1.5 | ... | $5.8 \times 10^4$ | 14 |
| 9 (M) | 1.2 | 6.0 | $R^-/D^+$ | 2.4 | Cervical lymphadenopathy | $3.7 \times 10^4$ | 23 |
| 10 (M) | 1.3 | 6.5 | $R^-/D^+$ | 2.9 | Fever, diarrhea | $1.1 \times 10^6$ | 73[d] |
| 11 (F) | 1.8 | 7.4 | $R^+/D^+$ | 53.2 | Atypical lymphocytosis | $1.6 \times 10^5$ | 33 |

**NOTE.** LTx, liver transplant.

[a] EBV serostatus of recipient/donor (R/D) at transplant (+, positive; −, negative).

[b] Time when EBV DNA in whole blood was positive for the first time.

[c] Continuous detection in whole blood of >5000 EBV DNA copies/mL.

[d] Chronic high EBV load carrier state is ongoing without any symptoms.

multiplex real-time RT-PCR, using the Mx3000P real-time PCR system (Stratagene) as described elsewhere [16]. The stably expressed housekeeping gene $\beta_2$-microglobulin (*β2M*) was used as an endogenous control and reference gene for relative quantification [17]. The detection limits for EBNA1, EBNA2, LMP1, LMP2, EBER1, BARTs, *Bam*HI Z leftward reading frame 1 (BZLF1), and gp350/220 were $10^3$, $10^3$, $10^2$, $10^2$, $10^1$, $10^2$, $10^4$, and $10^4$ EBV-positive LCL cells of $10^6$ EBV-negative BJAB cells, respectively. All chronic high EBV load carriers were analyzed at least twice over a 12-month period, and the results in all patients were similar.

***Statistical analyses.*** Statistical analyses were conducted using StatView software (version 5.0; SAS Institute). The Fisher exact test or the Mann-Whitney *U* test was used for comparisons of 2 groups of patients. For comparisons of >3 groups, the Kruskal-Wallis test was used; if the result of the Kruskal-Wallis test was significant, the Tukey-Kramer test was used as a post hoc test. For the negative samples, the default value, defined as the lowest level of expression for a particular gene, was used for the calculation. The default values for the undetected genes were $10^{-8}$. Differences with $P < .05$ were deemed to be statistically significant.

## RESULTS

***Clinical characteristics and viral loads in peripheral blood from chronic high EBV load carriers.*** Characteristics of the 11 chronic high EBV load carriers are shown in Table 1. In six patients, the onset of the high-load carrier state was preceded by EBV-related symptoms. These symptoms were transient and resolved after the reduction of immunosuppression in all patients. During the study period, the chronic high EBV load resolved without progression to PTLD in 7 (64%) of the 11 carriers, whereas the chronic high EBV load state continued with no symptoms in the other 4 carriers (36%). The median time to resolution in those whose high EBV load resolved was 23 months. Longitudinal analysis of EBV load in whole blood

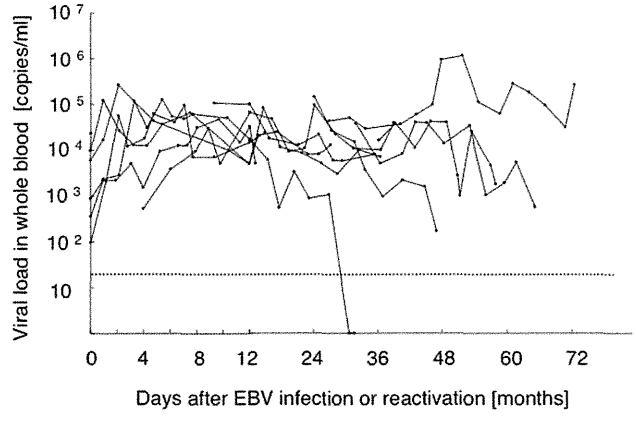


**Figure 1.** Time courses of Epstein-Barr virus (EBV) loads in whole blood from chronic high EBV load carriers. EBV load was serially measured by real-time polymerase chain reaction. Each line indicates the viral load of an individual patient. The dotted line indicates the detection limit.

**Table 2. Comparisons of Clinical Features between Chronic High Epstein-Barr Virus (EBV) Load Carriers and Control Recipients**

| Feature | Chronic high EBV load carriers ($n$ = 11) | Control recipients ($n$ = 20) | $P$[a] |
|---|---|---|---|
| Sex | | | .45 |
| Male | 6 | 7 | |
| Female | 5 | 13 | |
| Age at transplant, years | 1.5 ± 1.3 | 5.9 ± 5.4 | .13 |
| Follow-up period after transplant, years | 4.0 ± 2.0 | 4.9 ± 2.5 | .43 |
| EBV serostatus before transplant | | | **.02** |
| Positive | 1 | 11 | |
| Negative | 10 | 9 | |
| Rejection episodes | | | .13 |
| Yes | 5 | 15 | |
| No | 6 | 5 | |
| Trough levels of tacrolimus, ng/mL | | | |
| 1 month after transplant | 9.2 ± 1.4 | 9.0 ± 1.6 | .96 |
| 3 months after transplant | 6.2 ± 1.8 | 6.1 ± 1.0 | .94 |
| 6 months after transplant | 5.2 ± 1.3 | 6.6 ± 1.5 | **.03** |
| 12 months after transplant | 4.0 ± 1.5 | 4.7 ± 1.7 | .32 |
| 18 months after transplant | 3.4 ± 1.4 | 4.7 ± 2.6 | .27 |
| Features at the time of blood sampling for EBV monitoring | | | |
| Time after transplant, years | 2.9 ± 1.8 | 4.3 ± 2.3 | .14 |
| EBV load in whole blood, copies/mL | 23,800 ± 31,600 | 100 ± 200 | **.01** |
| Trough levels of tacrolimus, ng/mL | 2.5 ± 1.2 | 2.6 ± 1.4 | .64 |
| Percentage of $CD4^+$ lymphocytes within the lymphocyte population | 38.9 ± 8.7 | 35.5 ± 4.2 | .71 |
| Percentage of $CD8^+$ lymphocytes within the lymphocyte population | 20.9 ± 9.1 | 24.1 ± 6.5 | .31 |

**NOTE.** Data are means ± standard deviations, unless otherwise indicated. Boldface type indicates statistically significant differences.

[a] The Fisher exact test or the Mann-Whitney $U$ test was used to compare factors.

from each chronic high EBV load carrier is shown in Figure 1. EBV DNA was not detected in most of the selected plasma samples from chronic high EBV load carriers throughout the chronic high EBV load state (data not shown). In some carriers, viral loads gradually decreased, whereas they continued to be elevated in others. For comparison, the median EBV load was $2.5 \times 10^4$ copies/mL (range, $1.9 \times 10^3$–$3.1 \times 10^6$ copies/mL) of whole blood for 15 patients with IM and $4.0 \times 10^5$ copies/mL of whole blood for 2 patients who developed PTLD after a bone marrow transplant. EBV DNA was detected in 4 of 25 healthy control subjects (range, 0–$2.7 \times 10^2$ copies/mL).

Next, we compared clinical features between the chronic high EBV load carriers and control recipients (Table 2). Most of the chronic high EBV load carriers were seronegative for EBV before transplant, indicating that primary infection was a risk factor for the chronic high EBV load carrier state. Trough levels of tacrolimus were not significantly different, except 6 months after transplant, at which point the dose of tacrolimus was reduced because of the high EBV load.

***Dominant EBV-infected cell compartments.*** Viral DNA was dominantly found only in a population of $CD19^+$ cells of the peripheral blood from all chronic high EBV load carriers (Figure 2).

***Measurement of IL-10 concentration.*** Because several reports have demonstrated that levels of IL-10 might be predictive of the development of PTLD [18], plasma concentrations of IL-10 were compared among chronic high EBV load carriers ($n$ = 11), control recipients ($n$ = 20), and patients with IM ($n$ = 14). The mean levels of IL-10 in both chronic high EBV patients and control recipients were not elevated (mean ± SD, 2.1 ± 2.7 and 2.7 ± 5.7 pg/mL, respectively), and no significant difference was found between the 2 groups, although the levels in both groups were significantly lower than that in patients with IM (mean ± SD, 10.1 ± 6.5 pg/mL).

***Precursor frequency of EBV-specific $CD8^+$ T cells in peripheral blood.*** HLA-A2 and HLA-A24 tetramers were used to analyze the precursor frequency of EBV- and cytomegalovirus (CMV)–specific $CD8^+$ T cells in the 4 groups of subjects who had HLA-A2 or HLA-A24: chronic high EBV load carriers (A24, $n$ = 8; A2, $n$ = 4), control recipients (A24, $n$ = 13; A2, $n$ = 10), healthy control subjects (A24, $n$ = 10), and patients with IM (A24, $n$ = 6). EBV-specific $CD8^+$ T cells were detected

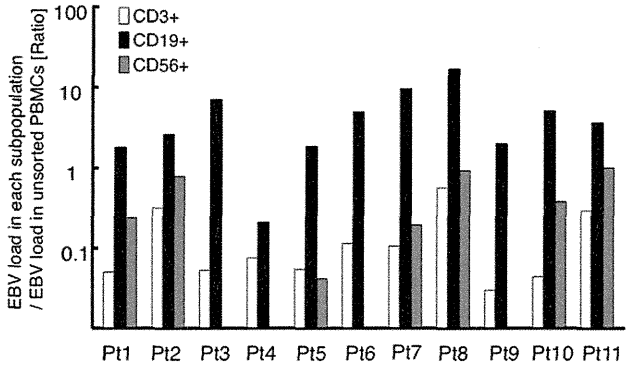


**Figure 2.** Epstein-Barr virus (EBV) load in each subpopulation of peripheral blood mononuclear cells (PBMCs) from chronic high EBV load carriers. Each subpopulation was positively selected with antibody-coated magnetic beads from PBMCs obtained from chronic high EBV load carriers. Viral load in each compartment was quantified by real-time polymerase chain reaction.

with 1 or more of the relevant tetramers in 9 of 10 chronic high EBV load carriers, in 18 of 19 control recipients, and in all subjects in the other groups. The frequency of EBV- and CMV-specific $CD8^+$ T cells in the 4 groups are summarized in Figure 3*A* and 3*B*. The frequency of $CD8^+$ T cells specific for lytic-cycle antigens (*Bam*HI R leftward reading frame 1 [BRLF1] and *Bam*HI M leftward reading frame 1 [BMLF1]) in patients with IM was significantly higher than in other groups. Regarding the 3 latent antigens (EBNA3A, EBNA3B, and LMP2), no statistical difference was found in the frequencies of EBV-specific $CD8^+$ T cells among the groups, although the frequency of LMP2-specific $CD8^+$ T cells were lower than those for the other epitopes in all groups. CMV pp65–specific $CD8^+$ T cells were measured for comparison, and no difference was found in the frequencies among the groups.

***Patterns of EBV-related gene expression in PBMCs.*** To investigate the levels and patterns of EBV gene expression, 6 latent and 2 lytic genes were quantified by multiplex real-time RT-PCR in all study groups. Representative quantitative results for chronic high EBV load carriers for each gene are shown in Figure 4*A*. EBER1 and BARTs were detected in all samples, and LMP2 was detected in 6 samples. EBER1 had the highest relative expression level, followed by BARTs and LMP2. The mean expression levels ± SD of EBER1, BARTs, and LMP2 were $10^{-4.0\pm0.9}$, $10^{-4.5\pm1.7}$, and $10^{-6.4\pm1.6}$, respectively. In contrast, more EBV-related genes were detected in patients with IM ($n = 15$) and PTLD ($n = 2$) (Figure 4*B* and 4*C*). In patients with IM, the mean expression levels ± SD of EBNA1, EBNA2, LMP1, LMP2, EBER1, BARTs, and BZLF1 were $10^{-7.0\pm1.6}$, $10^{-5.8\pm2.0}$, $10^{-6.7\pm1.1}$, $10^{-5.6\pm1.9}$, $10^{-4.2\pm1.3}$, $10^{-4.7\pm2.0}$, and $10^{-7.8\pm0.6}$, respectively (Figure 4*D*). Additionally, EBER1 and BARTs with or without LMP2 were detected in only 2 of 20 control recipients, an expression pattern similar to that observed in the chronic high EBV load carriers (data not shown). With regard to healthy control subjects, EBER1 was detected in 2 of 23, and no other gene was detected in any of them.

## DISCUSSION

Serial monitoring of EBV load for early diagnosis has become standard practice in the management of transplant recipients [5, 19–21]. This monitoring has led to the identification of a group of asymptomatic patients with chronic high EBV loads over long periods [10–12]. The incidence of chronic high EBV load, clinical features, and the risk of late-onset PTLD in liver transplantation have been reported by a few groups. D'Antiga et al [10] stated that 14 (41%) of 34 pediatric liver transplant recipients showed positive RT-PCR results for viral capsid antigen immunoglobulin M or early antigen immunoglobulin G lasting >6 months. Viral loads >500 copies/$10^5$ PBMCs occurred in most of them. All 14 of these patients were seronegative for immunoglobulin G before transplant. Three patients developed late-onset PTLD. Green et al [12] reported that 36 (18%) of 196 children who had undergone liver transplant had >16,000 copies/mL of whole blood or 200 copies/$10^5$ PBMCs in at least 50% of samples over a minimum period of 6 months after EBV infection. Three-quarters of these children were negative for EBV before transplant. Only 1 patient developed PTLD. In the present study, 11 (35%) of 31 patients were found to be chronic high EBV carriers, and 10 of these 11 patients were seronegative for EBV before liver transplant, consistent with previous reports. No recipient with chronic high EBV loads developed late-onset PTLD. Because the majority of chronic high EBV load carriers were negative for EBV before transplant, primary EBV infection while receiving immunosuppressive drugs is key to understanding this chronic high EBV load carrier state.

Bingler et al [11] reported that 9 (45%) of 20 pediatric heart transplant recipients with chronic high EBV loads (defined as the presence of >16,000 copies/mL or 200 copies/$10^5$ PBMCs in at least 50% of samples over a minimum period of 6 months, developed late-onset PTLD. The incidence among heart recipients in this report was higher than those among liver recipients stated above. This difference may result from the aggressiveness of immunosuppression during the posttransplant period. The incidence of PTLD varies significantly between different types of organ transplantation [22, 23]. This variation is thought to be related to the degree and duration of immunosuppression and the number of EBV-positive donor lymphocytes in the graft. Additional studies are required to examine the association between the incidence of late-onset PTLD among those with chronic high EBV loads and the method of immunosuppression.

In the present study, a chronic high EBV load carrier state was defined as the continuous presence of EBV loads >5000 copies/mL of whole blood over a minimum period of 6 months

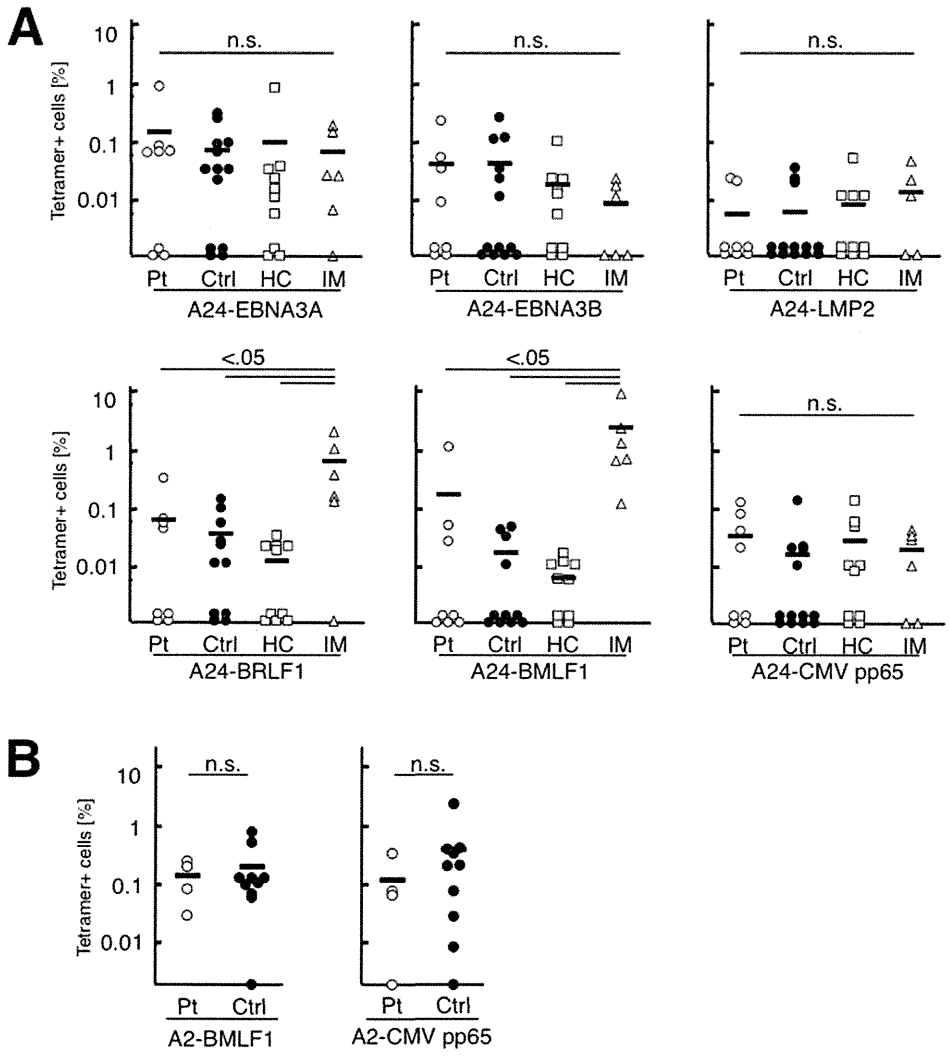


**Figure 3.** Comparison of the frequency of Epstein-Barr virus (EBV)–specific $CD8^+$ T cells among peripheral blood mononuclear cells (PBMCs). The precursor frequency of EBV-specific $CD8^+$ T cells in each study group was measured using major histocompatibility complex (MHC) peptide tetramers. EBV tetramer–positive cells are shown as a percentage of total $CD8^+$ T cells. *A*, HLA-A24 tetramer–positive cells among total $CD8^+$ cells in PBMCs from chronic high EBV load carriers (Pt [*white circles*]; $n = 8$), control recipients (Ctrl [*black circles*]; $n = 13$), healthy EBV-positive control subjects (HC [*squares*]; $n = 10$), and patients with infectious mononucleosis (IM [*triangles*]; $n = 6$). Horizontal bars denote the mean value in each group of subjects. *B*, HLA-A2 tetramer–positive $CD8^+$ cells among PBMCs from chronic high EBV load carriers (Pt [*white circles*]; $n = 4$) or control recipients (Ctrl [*black circles*]; $n = 10$).

after asymptomatic infection or after complete resolution of symptomatic EBV disease. Previously, we reported that, in hematopoietic stem cell transplantation, 90.6% (3/32) of all asymptomatic patients had an EBV load $<10^{2.5}$ copies/$\mu$g of DNA (82.4% [14/17] of asymptomatic patients with a positive EBV load had $<10^{2.5}$ copies/$\mu$g of DNA), 83% (5/6) of the patients with clinical symptoms had between $10^{2.5}$ and $10^4$ copies/$\mu$g of DNA, and 100% (5/5) of patients who developed PTLD had $>10^4$ copies/$\mu$g of DNA [24]. Recently, we measured the EBV load in whole blood in order to monitor liver transplant patients, and our conversion factor from copies per microgram of DNA to copies per milliliter of whole blood was 10–20 (authors' unpublished data). This conversion factor is close to that reported in another review [25]. Using this factor, $10^{2.5}$ (equal to 316) copies/$\mu$g of DNA is similar to 3160–6320 copies/mL of whole blood, and we decided that 5000 copies/mL of whole blood was the value expected to produce EBV-related clinical symptoms but not PTLD in our system for quantifying the EBV load.

$CD8^+$ cytotoxic T cells are known to be important in controlling EBV-associated PTLD [4]. The combination of increased EBV load and the absence of EBV-specific $CD8^+$ T cells can predict EBV lymphoproliferative disease [26]. Regarding the frequency of $CD8^+$ T cells specific for 2 lytic antigens

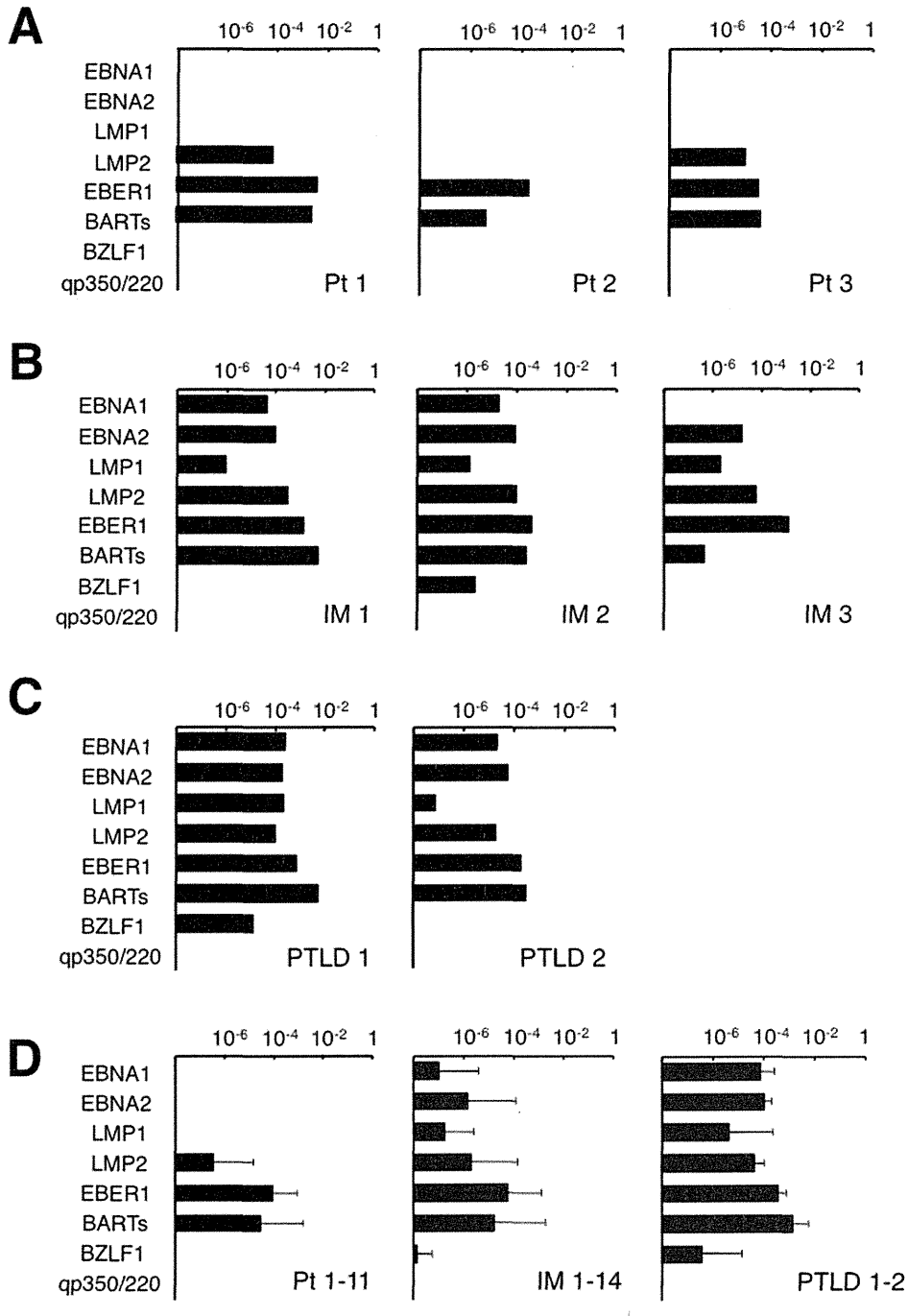


**Figure 4.** Comparison of the expression patterns of Epstein-Barr virus (EBV)–related genes in peripheral blood mononuclear cells (PBMCs). Relative quantification of EBV genes was performed in each study group by multiplex real-time reverse-transcription polymerase chain reaction. *A*, Representative data from chronic high EBV load carriers (Pt 1, 2, and 3). *B*, Representative data from 3 patients with infectious mononucleosis (IM 1, 2, and 3). *C*, Relative quantification of EBV gene expression in 2 patients with posttransplant lymphoproliferative disorder (PTLD 1 and 2). *D*, Levels of EBV gene expression in 11 chronic high EBV load carriers, 15 patients with IM, and 2 patients with PTLD. Data are means ± standard deviations.

(BRLF1 and BMLF1) and 3 latent antigens (EBNA3A, EBNA3B, and LMP2), we found no significant difference between chronic high EBV load carriers and control recipients. Macedo et al [27] reported that the frequency of EBV-specific $CD8^+$ T cells in "stable" (quiescent) transplant recipients was equal to or higher than that in healthy control subjects, which may agree with our results. In solid-organ transplant recipients, chronic administration of immunosuppressive drugs causes the impairment of cellular immune surveillance and allows EBV-infected B cells to proliferate. Smets et al [28] reported that

recipients with high EBV loads showing low activity of specific $CD8^+$ T were at high risk for PTLD, using an enzyme-linked immunospot assay in pediatric solid-organ transplant recipients. Such a functional impairment may be present and influence the chronic high EBV load state.

Analyzing the expression profile of EBV-related genes is useful in clarifying the pathogenesis of EBV-associated diseases [16]. In each chronic high EBV load carrier, EBER1 and BARTs were abundantly detected, and LMP2 was found in half of the carriers. The mean expression level of LMP2 was low, compared with those of EBER1 and BARTs. No transcript specific for latency type 3 was detected in chronic high EBV load carriers. This restricted pattern is latency type 0, which is found in EBV-infected memory B cells [8, 29, 30], suggesting that the pattern of infected B cells in those with chronic high EBV loads may be the same as that in healthy EBV carriers. Qu et al [31] reported that peripheral blood lymphocytes in those with chronic high EBV loads after solid-organ transplant were divided into 2 types of infected cells: lymphocytes expressing LMP2 and carrying a low number of copies of EBV, and others coexpressing LMP1 and LMP2 and having a high number of copies of EBV. In the present study, expression of LMP1 was not observed in all chronic high EBV load carriers. Quantification in the blood sample may not have been sufficiently sensitive to detect a small population of infected cells expressing the LMP1 gene. Additionally, the expression pattern may vary with the time at which the blood sample is obtained. Transcripts specific for latency type 3 were detected in some samples from chronic high EBV load carriers obtained within 4 months after EBV infection (data not shown), and the pattern of EBV gene expression subsequently changed to latency type 0. Dynamic changes in the immune response, such as an increased precursor frequency of EBV-specific $CD8^+$ T cells after reduced immune suppression, may influence expression patterns in chronic high EBV load carriers; however, this change was not evaluated in the present study.

After primary infection, EBV persists as a latent infection in memory B cells. Then, memory B cells occasionally differentiate into plasma cells that undergo lytic infection and produce new virus. Newly infected naive B cells become transformed but are controlled by $CD8^+$ T cells specific for EBV. This cycle is necessary to maintain a latent infection in vivo [21, 32]. Thus, we suggest a novel mechanism for the maintenance of high viral loads in the blood of chronic high EBV load carriers. First, a large number of memory B cells latently infected with EBV survive after the primary or reactivated EBV infection occurring after transplant. Second, the latently infected memory B cells readily differentiate into plasma cells to produce the virus. Immunosuppression may influence these processes. Treatment with acyclovir or ganciclovir for a relatively prolonged period may modulate the chronic high EBV load state. However, our results did not provide mechanistic or therapeutic information.


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ORIGINAL ARTICLE

# Multicenter evaluation of prototype real-time PCR assays for Epstein-Barr virus and cytomegalovirus DNA in whole blood samples from transplant recipients

Yoshinori Ito[1], Shunji Takakura[2], Satoshi Ichiyama[2], Mitsuharu Ueda[3], Yukio Ando[3], Kazuyuki Matsuda[4], Eiko Hidaka[4], Kaname Nakatani[5], Junji Nishioka[5], Tsutomu Nobori[5], Naoki Kajiyama[6] and Hiroshi Kimura[7]

Departments of [1]Pediatrics and [7]Virology, Nagoya University Graduate School of Medicine, Nagoya 466-8550, [2]Clinical Laboratory Medicine, Kyoto University Graduate School of Medicine, Kyoto 606-8501, [3]Department of Diagnostic Medicine, Kumamoto University Graduate School of Medicine, Kumamoto 860-8556, [4]Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto 390-8621, [5]Department of Molecular and Laboratory Medicine, Mie University Graduate School of Medicine, Tsu 514-8507 and [6]Roche Diagnostics K.K., Tokyo 105-0014, Japan

## ABSTRACT

**Quantitative PCR is becoming widespread for diagnosing and monitoring post-transplantation diseases associated with EBV and CMV. These assays need to be standardized to manage patients in different facilities. Five independent laboratories in Japan compared home-brew assays and a prototype assay system to establish a standard quantitative procedure for measuring EBV and CMV. Reference standards and a total of 816 (642 EBV and 174 CMV) whole blood samples from post-transplantation recipients were used for this multicenter evaluation. The prototype reference standard for EBV was compared to a panel of samples, with a theoretical expected value made using EBV-positive cells containing two virus genome copies per cell. The mean ratio of the reference standard at each site to the standard of the prototype assay was ≤4.15 for EBV among three different sites and ≤3.0 for CMV between two laboratories. The mean of the theoretical expected number of the EBV genome : prototype reference was close to 1.0. The correlation coefficients between the viral copy numbers determined using the prototype assay and those using each home-brew assay were high (EBV, 0.73–0.83, median = 0.78; CMV, 0.54–0.60, median = 0.57). The dynamics of the EBV and CMV loads in transplant recipients were similar between the assay types. There was an inter-laboratory difference among the quantification results, indicating that a unified protocol and kit are favorable for standardizing the quantification of EBV and CMV. Such standardization will help to standardize the diagnosis and monitoring of diseases associated with EBV and CMV.**

**Key words** cytomegalovirus, Epstein–Barr virus, real-time PCR, standardization, viral load.

Herpes viruses are widespread pathogens in the human population and often become reactivated in latently infected immunocompromised patients. These viruses thus frequently occur after hematopoietic stem cell and solid organ transplantation, and occasionally result in symptomatically severe disease (1, 2). EBV and CMV are representative viruses causing disease after transplant recipients. Life-threatening PTLD is known to be caused by the EBV virus (3). Frequent clinical manifestations of CMV are pneumonia and gastrointestinal disease (4). Because these viruses replicate without any clinical symptoms, quantitative methods are required to distinguish asymptomatic infection from impending diseases. Routine monitoring of these viruses and pre-emptive

**Correspondence**
Yoshinori Ito, Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Tel: 81-52-744-2294; fax: 81-52-744-2974; email: yoshi-i@med.nagoya-u.ac.jp

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**List of Abbreviations:** EBV, Epstein-Barr virus; CMV, cytomegalovirus; PTLD, post-transplant lymphoproliferative disorder.

intervention for virus-associated diseases are therefore important (5, 6).

Recently, quantitative real-time PCR assays have become widespread methods for diagnosing and monitoring EBV-associated diseases after transplantation (5, 7). The CMV pp65 antigenemia assay has been widely used to evaluate the viral load of CMV-associated diseases and is considered the gold standard. However, quantitative PCR is increasingly used in diagnosing and monitoring transplant recipients because of its speed, reproducibility, and ease of use (6, 8). Currently, laboratories rely on their own home-brew quantitative PCR assay system. These home-brew assay systems need to be standardized because discrepancies existing between laboratories lead to site-specific patient management algorithms.

Five independent laboratories comprised the working group in this study and compared the quantitative results of each home-brew assay and a prototype assay system to establish a standardized quantitative procedure for measuring EBV and CMV. Distributed reference standards and whole blood samples from solid organ transplantation and hematopoietic stem cell transplantation recipients were used in this multicenter evaluation.

## MATERIALS AND METHODS

### Study group and samples

Five independent laboratories comprised the working group of the Japan Molecular Center of Excellence sponsored by Roche Diagnostics K.K, each using a different quantitative home-brew EBV PCR assay. Each laboratory provided details of its home-brew testing procedure (Table 1). The prototype assay kit (JMCoE EBV primer probe standard set, CMV primer probe standard set, DNA master mix set; Nihon Gene Research Laboratories, Sendai, Japan) and the reference standard for EBV and CMV were developed by Roche Diagnostics K.K. (Tokyo, Japan) and distributed among the participating laboratories. In total, 642 (EBV) and 174 (CMV) whole blood samples from solid organ and hematopoietic stem cell transplantation recipients as part of routine follow up after transplantation were studied retrospectively. The sample set for comparison was different among the participating sites: for EBV, 100 samples were used in site A, 100 in B, 240 in C, 72 in D, and 130 in E; for CMV, 103 in A and 71 in E. No samples were redundant among the participating sites. Each site carried out quantitative EBV and CMV testing on all reference standards and clinical samples using both their own home-brew procedure and the prototype test. The study design and purpose, approved by the institutional review board of each university hospital, were fully explained to all of the patients and/or guardians, and informed consent was obtained.

### Preparation of the reference standard for the prototype assay

As a reference standard for the prototype assay, a plasmid that contained the EBV *BALF5* gene and one containing CMV *IE* gene were constructed from pGEM-T vector (Promega, Madison, WI, USA) (9, 10). The copy number of the plasmids was calculated on the basis of its absorbance at 260 nm.

### Preparation of the panel samples with the EBV-positive cell line

To evaluate the value of the reference standard plasmid for the prototype assay, EBV-positive samples in which the actual EBV copy number could be estimated were prepared. Namalwa cells containing two EBV genome copies per cell were used as a source of EBV DNA. BJAB cells, known to be EBV negative, were used to prepare a background cellular matrix. Three types of sample were constructed: $5 \times 10^6$ Namalwa cells (defined as Namalwa 100%); $5 \times 10^5$ Namalwa cells with $4.5 \times 10^6$ BJAB cells (defined as Namalwa 10%); and $5 \times 10^4$ Namalwa cells with $4.95 \times 10^6$ BJAB cells (defined as Namalwa 1%). The theoretical expected value of the whole Namalwa 100% sample was $1 \times 10^7$ copies. When DNA was extracted from the Namalwa 100% sample, 58.4 $\mu$g/200 $\mu$l distilled water was obtained. In

**Table 1.** Comparison of procedural characteristics among different laboratories

| Laboratory | Target gene | | Sample volume ($\mu$l) | Extraction kit | Elution volume ($\mu$l) | DNA input ($\mu$l) | Reaction volume ($\mu$l) | Platform Platform |
|---|---|---|---|---|---|---|---|---|
| | EBV | CMV | | | | | | |
| A | BALF5 | IE | Whole blood (200) | QIAamp | 50 | 5 | 25 | Mx3000P |
| B | BNFR1 | – | Whole blood (200) | QIAamp | 200 | 5 | 25 | ABI 7700 |
| C | BALF5 | – | Whole blood (200) | QIAamp | 150 | 5 | 25 | ABI 7700 |
| D | BXLF1 | – | Whole blood (200) | QIAamp | 50 | 5 | 25 | LightCycler 2.0 |
| E | BALF5 | IE | Whole blood (200) | QIAamp or MagNA Pure | 100 | 10 | 50 | ABI 7700 |
| Prototype | BALF5 | IE | Whole blood (200) | QIAamp | 200 | 50 | 100 | Cobas Taqman48 |

the case of the prototype assay, 2 $\mu$g extracted DNA from 200 $\mu$l whole blood was transferred to a single assay well. Therefore, 2 $\mu$g of 58.4 $\mu$g of DNA was used as a sample to evaluate the value of the reference standard. Two micrograms of DNA from Namalwa 100% were expected to contain $3.42 \times 10^5$ ($1 \times 10^7 \times 2/58.4$) copies of the EBV genome. To evaluate different concentrations of DNA as an assay template, 0.2 $\mu$g of 58.4 $\mu$g was also measured in the prototype assay. The results from other Namalwa constructs were assessed in the same way.

### Prototype assay system

Viral DNA was extracted from 200 $\mu$l whole blood using QIAamp DNA blood kits (Qiagen, Hilden, Germany) and eluted in 200 $\mu$l distilled water. The specific primers and fluorogenic probes for EBV and CMV were as follows: EBV forward: CGGAAGCCCTCTGGACTTC, EBV reverse: CCCTGTTTATCCGATGGAATG, EBV probe: FAM-TGTACACGCACGAGAAATGCGCC-TAMRA (9); CMV forward: GACTAGTGTGATGCTGGCCAAG, CMV reverse: GCTACAATAGCCTCTTCCTCATCTG, CMV probe-1: FAM-AGCCTGAGGTTATCAGTGTAATGAA GCGCC-TAMRA (10), CMV probe-2: FAM-AGCCTGAGGTTATCAATATCATGAAGCGCC-TAMRA. Because a variation was reported within the sequence that would be amplified with the CMV-specific primers (11), two different probes were mixed and used for CMV quantification. Fifty microliters of a 200-$\mu$l DNA extraction solution was added as a reaction mixture containing the master mix reagent, specific primers, and probes. A real-time PCR reaction was carried out with a model Cobas TaqMan 48 (Roche Diagnostics K.K., Tokyo, Japan). All samples and standards were run in duplicate. Regarding the prototypic assay for EBV, the standard curves obtained were linear from 10 to $10^5$ copies/reaction with an average slope of $-3.50$. The standard curves of the CMV assay were also linear from 10 to $10^5$ copies/reaction with an average slope of $-3.87$.

### Statistical analysis

The concordance was analyzed by kappa statistics. The number (copies/ml) was converted to its logarithmic value. Regression analysis was carried out by simple regression on the home-brew assay to the prototype test.

## Results

### Procedural characteristics of home-brew assays among the five laboratories

Specific primers and probes, DNA extraction kit, DNA elution volume, real-time PCR reaction volume, and the real-time PCR platform were varied among participating laboratories (Table 1). The sequences of the primers and the probe for EBV were identical at sites A, C and E. The sequences of the primers and the probe for CMV at sites A and E were consistent. A reference standard for the home-brew assay was prepared in each laboratory.

### Evaluation of reference standards for EBV and CMV

The copy numbers of the standards in three (for EBV) or two (for CMV) home-brew systems using the same primer and probe set were measured based on the copy number of the reference standards for the prototype assays. The ratios of the reference standard in each site to the prototype assay standard at different copy numbers are shown in Table 2. The mean ratio was $\leq$4.15 for EBV among three different sites and $\leq$3.0 for CMV between two laboratories.

To evaluate the value of the EBV reference standard plasmid for the prototype assay, EBV-positive samples with an expected theoretical value were prepared using Namalwa cells known to contain two EBV genome copies per cell. When the prototype real-time PCR assay was carried out with 2 $\mu$g DNA extracts from these samples per reaction mixture, the mean of the theoretical expected number of EBV genome : quantitative result ratio was 0.62. In the

**Table 2.** Quantitative result of each home-brew reference standard based on the value of the prototype reference

| Laboratory | | Value of each home-brew assay reference standard (ratio) | | | | | Ratio (mean) |
|---|---|---|---|---|---|---|---|
| | | $10^2$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ | |
| EBV | A | ND | $9.9 \times 10^2$ (0.99) | $9.3 \times 10^3$ (0.93) | $8.8 \times 10^4$ (0.88) | $6.4 \times 10^5$ (0.64) | 0.86 |
| | C | ND | $3.4 \times 10^3$ (3.4) | $4.9 \times 10^4$ (4.9) | $4.2 \times 10^5$ (4.2) | $4.2 \times 10^6$ (4.2) | 4.15 |
| | E | ND | $7.9 \times 10^2$ (0.79) | $6.3 \times 10^3$ (0.63) | $6.4 \times 10^4$ (0.64) | $6.7 \times 10^5$ (0.67) | 0.68 |
| CMV | A | $1.7 \times 10^2$ (1.7) | $2.0 \times 10^3$ (2.0) | $2.2 \times 10^4$ (2.2) | $2.1 \times 10^5$ (2.1) | ND | 2.00 |
| | E | $4.0 \times 10^2$ (4.0) | $3.3 \times 10^3$ (3.3) | $3.0 \times 10^4$ (3.0) | $1.8 \times 10^5$ (1.8) | ND | 3.03 |

The average copy numbers were calculated from 12 wells at each concentration.