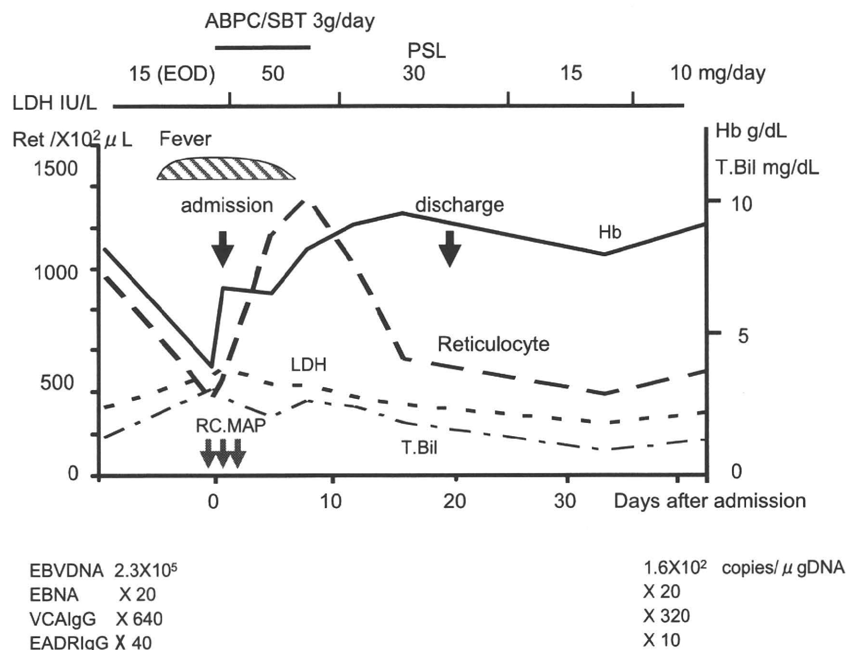

Figure 1. Clinical course. ABPC/SBT: ampicillin sulbactam, EOD: every other day, PSL: prednisolone, Ret: Reticulocyte, Hb: hemoglobin, T. Bil: total bilirubin, RC. MAP: red cell concentrates mannitol adenine phosphate

Materials and Methods

A sample from a patient with infectious mononucleosis (IM) was used as control to measure CTL activity.

Establishment of spontaneous LCL

EBV-infected spontaneous lymphoblastoid cell lines (LCL) were established from CD19-positive B cells in the peripheral blood of this patient and the control patient in the acute phase IM. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. Then CD19-positive cells were separated from these cells using anti-CD19 antibody conjugated magnetic beads (IMag Human CD19 Particles-DM; BD Biosciences, San Jose, CA). We cultured the cells in 10% FCS-RPMI to establish an LCL. The EBV infection in the LCL was confirmed by RT-PCR.

Measurement of EBV-specific CTL activity

ELISPOT assay was performed with the IMMUNOCYTO IFN-γ ELISPOT kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) following instructions supplied by the manufacturer as described previously (6). Briefly, CD8-positive T cells were isolated from PBMCs with the IMag anti-human CD8 Particles-DM (BD Biosciences). Samples were obtained from this patient in remission and the control IM patient after recovery phase, respectively. The viral loads at harvest were similar in the two; 8.0×10^2 copies/μgDNA in the patient, and 5.0×10^2 copies/μgDNA in the control. Mixture of these CD8-positive T cells and the LCL were incubated with IL-2 in microplates coated with antibody to IFN-γ for 17h. Captured IFN-γ was detected by biotinated antibody to IFN-γ and alkaline phosphatase conjugated streptavidin, and visualized by reaction with the BCIP/NBT Chromogen Substrate.

The study was approved by the ethical committee of Tokyo Medical and Dental University and written informed consent was obtained from this patient and the control IM patient.

Case Report

An 88-year-old man with no remarkable history was diagnosed with AIHA of unknown cause 9 years prior to admission. On two occasions (1 and 7 years before admission) his anemia had worsened. The triggers of developing anemia of both occasions could not be detected. Oral PSL of 30 mg/day (0.5 mg/kg) was started during his hospitalizations on both occasions, and he recovered soon. During the first hospitalization, PSL was discontinued successfully after AIHA improved; however, during his second hospitalization anemia relapsed as the PSL was tapered; therefore, he had been administered 15 mg of PSL every other day until this admission.

The clinical course of this admission is shown in Fig. 1. Two days before admission, the patient developed fever. As his symptoms became worse, he was admitted to the previous hospital. Although no focus of infection could be detected, sulbactam/ampicillin (ABPC/SBT) was initiated at 3 g/day. His hemoglobin (Hb) concentration was 8.6 g/dL on admission, but on the second hospital day, it drastically decreased to 3.6 g/dL, following which he was transferred to our hospital for further investigation and treatment of ane-

Table 1. Viral Laboratory Findings on Admission

Multiplex PCR Assay of Whole blood
EBV 2.3×10^5 copies/μg DNA
(plasma 2.4×10^6 copies/mL)

HSV1 : negative	VZV : negative
HSV2 : negative	CMV : negative
HHV6 : negative	JC Virus : negative
HHV7 : negative	Parvo virus B19 : negative
HHV8 : negative	

EBV antibody
anti-EBNA: ×20, anti-VCAIgG: ×640,
anti-VCAIgM: ×10,
anti-EA-DRIgG: ×40, anti-EA-DRIgA: ×10

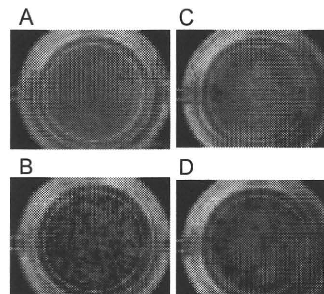

Figure 2. IFN-Enzyme-linked immunosorbent spot (ELISPOT) assay. EBV-specific CTL activity was measured by ELISPOT assay. A: Autologous LCL of infectious mononucleosis (IM) control. B: Autologous LCL and CD8-positive cells of IM control. C: Autologous LCL of the present patient. D: Autologous LCL and CD8-positive cells of the present patient. Spots were significantly decreased in D compared to B.

mia.

On admission, his body temperature was 37.9℃. On physical examination, he had severe anemia visible in the conjunctiva, however no jaundice was detected. His chest was normal. He had no lymphadenopathy or hepatosplenomegaly. He had marked normocytic normoclonic anemia; red blood cell (RBC), 128×10^4/μL; Hb, 3.9 g/dL; mean corpuscular volume, 93.8 fl; mean corpuscular hemoglobin concentration, 32.5%. The number of reticulocytes was lower than previous blood examinations. His peripheral blood leukocytes were normal in number with no appearance of atypical lymphocytes. Platelets were normal, too. LDH (480 IU/L) and conjugated bilirubin (3.1 mg/dL) were elevated (Fig. 1). Both direct and indirect Coombs tests were positive, and haptoglobin was undetectable. Cold agglutinin was negative. From these findings, the patient was diagnosed with a hemolytic crisis of AIHA. Because fever preceded the progression of anemia, we suspected infection as a trigger of this crisis and blood was obtained for bacterial examination and detection of viral DNA. The results showed a significant increase in EBV DNA copies, 2.3×10^5 copies/μg DNA (Table 1). Anti-EBVIgG and anti-EBNA were positive, and anti-EBVIgM was not high titer. We considered these findings revealed reactivation of EBV. Parvo virus B19 DNA was not detected. Bacterial examination, and the antigens and antibodies for the hepatitis B and C virus were negative, too (data not shown).

After RBC transfusion, we immediately increased the PSL to 50 mg/day (1 mg/kg). His LDH and bilirubin, then decreased gradually, suggesting that the hemolysis had resolved. However, it was more than 5 days before the numbers of reticulocytes and RBCs began to increase. ABPC/SBT was discontinued on hospital day 7 when his fever resolved. We started to taper the PSL on hospital day 11, and he was discharged on hospital day 19 receiving 30 mg/day of PSL. Anemia improved and PSL was tapered to 10 mg without recurrence of AIHA. EBV DNA copies decreased to 1.6×10^2 copies/μg DNA. We examined the bone marrow later, and neither dysplasia nor chromosomal abnormality could be detected.

To investigate the cause of reactivation of EBV, we tried to detect EBV-specific CTL activity after achieving remission. First, we performed flow cytometric analysis of PBMCs and detected that they consisted of 5.3% of CD19-positive, 39.8% of CD4-positive, 29.6% of CD8-positive, and 23.5% of CD56-positive cells. CD19-positive cells were markedly decreased. Second, we tried and successfully established LCL from CD19-positive cells in PBMCs. Since these cells proliferated spontaneously in vitro and were positive for EBV as those of the control patient of acute phase IM, it was speculated that the EBV-infected cells of the patient were B-cells and might be activated and immortal in vivo. From these results, we considered that his state was the same as LPD. To investigate CTL activity, we performed ELISPOT assay against these cells. As shown in Fig. 2D, significant suppression of CTL activity was detected and considered the cause of EBV reactivation.

Discussion

The patient had been diagnosed with AIHA and had been administered low dose PSL for about one year. On his admission, he suddenly developed worsening AIHA with fever. ABPC/SBT, which had been started one day before admission, could have been the cause of hemolysis. However, it was resolved despite continuing the administration of ABPC/SBT. On the other hand, the crisis was accompanied by reactivation of EBV. The EBV-DNA titer was closely associated with the course of anemia, which suggested that EBV reactivation might have played a role in the development of anemia.

Erythropoietic failure due to EBV infection can be developed by two major mechanisms; one is hemolysis, the other is suppression of erythropoiesis itself. AIHA is accompanied by a 0.1-3% EBV infection rate (7) but usually by a primary EBV infection. To the best of our knowledge, this is the first case of AIHA accompanied by reactivation of an EBV infection. The mechanism of the development of AIHA due to an EBV infection has not been clarified. Antibodies against EBV may cross-react with antigens expressed on the surface of RBCs and directly attack and destroy

them. Riboldi et al (8) reported that EBV-infected B cells produce anti-i IgM cold agglutinin, which cross-reacts with an RBC surface antigen *in vitro*. Anti-i IgM cold agglutinins are autoantibodies mainly responsible for the development of AIHA (9), suggesting that the RBCs in our patient, who also had EBV-infected B cells, might have been destroyed by this mechanism. Actually we could not detect cold agglutinin in the patient, but we examined it after increasing the PSL up to 1 mg/day. Increasing the dose was effective possibly by suppressing the EBV-infected B cells which produced antibodies, including cold agglutinin.

The other possible mechanism of erythropoietic failure due to EBV is suppression of erythropoiesis in the bone marrow. Pure red cell aplasia (PRCA) can occur with an EBV infection (10) and five such cases have been reported to occur due to reactivation of EBV (11-15). There was no reactive increase in reticulocytes on admission in this patient which suggested that the erythroid cells might be dominantly suppressed. The pathogenesis of EBV-induced PRCA has not been determined. Erythroid cells do not express the CD21 molecule which is known as the target of EBV infection. However EBV can interact with other molecules including HLA-class II (16) which is expressed on the surface of erythroid cells. In addition, EBV infection induces T-cell activation and its cytokine production (17). Produced cytokines may suppress hematopoietic cells including immature erythroid cells (18). Further study is necessary to prove these hypotheses and identify whether EBV infection suppresses erythroid cells directly or indirectly.

The EBV-infected LCL was successfully established from CD19-positive cells in PBMC. Furthermore, an ELISPOT assay proved that the CTL activity was suppressed against the LCL. In addition, as clonality could not be proved due to the lack of cells, it was considered that the cells might become immortal and his status might be EBV-associated B-cell LPD.

The present patient had received low dose PSL for about one year. Although a relationship between low dose administration of PSL and EBV reactivation has not been reported, it may suppress CTL activity and produce reactivation of EBV, just as methotrexate. Cases should be accumulated to examine this possibility. Another possible cause of EBV reactivation is aging. This disorder was summarized by Oyama et al in 2003 (3) and it is now defined as EBV-positive diffuse large B-cell lymphoma of the elderly in the new WHO classification (4). It is an EBV-positive clonal B-cell proliferation that occurs in patients over the age of 50 who are not immunodeficient and who do not have prior lymphoma. It had been speculated that its pathogenesis was due to immunological deterioration by aging. We actually detected CTL suppression against EBV and it was compatible with the hypothesis.

The present patient highlighted the fact that EBV reactivation can cause not only lymphoma but also various diseases including AIHA. Further investigation should be conducted to clarify their clinical features and determine the mechanism of the EBV reactivation.

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Journal of General Virology (2010), **91**, 42–50 DOI 10.1099/vir.0.013482-0

Quantitative analysis of Epstein–Barr virus (EBV)-related gene expression in patients with chronic active EBV infection

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Received 22 May 2009
Accepted 25 September 2009

Chronic active Epstein–Barr virus (CAEBV) infection is a systemic Epstein–Barr virus (EBV)-positive lymphoproliferative disorder characterized by persistent or recurrent infectious mononucleosis-like symptoms in patients with no known immunodeficiency. The detailed pathogenesis of the disease is unknown and no standard treatment regimen has been developed. EBV gene expression was analysed in peripheral blood samples collected from 24 patients with CAEBV infection. The expression levels of six latent and two lytic EBV genes were quantified by real-time RT-PCR. EBV-encoded small RNA 1 and *Bam*HI-A rightward transcripts were abundantly detected in all patients, and latent membrane protein (LMP) 2 was observed in most patients. EBV nuclear antigen (EBNA) 1 and LMP1 were detected less frequently and were expressed at lower levels. EBNA2 and the two lytic genes were not detected in any of the patients. The pattern of latent gene expression was determined to be latency type II. EBNA1 was detected more frequently and at higher levels in the clinically active patients. Quantifying EBV gene expression is useful in clarifying the pathogenesis of CAEBV infection and may provide information regarding a patient's disease prognosis, as well as possible therapeutic interventions.

INTRODUCTION

Epstein–Barr virus (EBV) is the causative agent of infectious mononucleosis and is associated with several malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma and post-transplant lymphoproliferative disorders (Cohen, 2000; Rickinson & Kieff, 2007; Williams & Crawford, 2006). Chronic active EBV (CAEBV) infection is a systemic EBV-positive lymphoproliferative disorder characterized by persistent or recurrent infectious mononucleosis-like symptoms in patients with no known immunodeficiency (Kimura, 2006; Okano *et al.*, 2005; Straus, 1988; Tosato *et al.*, 1985). The clonal expansion of EBV-infected T cells or natural killer (NK) cells plays a pathogenic role in patients with CAEBV, particularly among those in east Asia or central America (Kanegane *et al.*, 2002; Kimura, 2006; Quintanilla-Martinez *et al.*, 2000). These patients can be classified into two groups based on the predominantly infected cell type, T cells or NK cells (Kimura *et al.*, 2001, 2003). Nonetheless, the detailed pathogenesis of CAEBV remains elusive and no standard treatment regimen has been developed. Recently, haematopoietic stem cell transplantation (HSCT) was introduced as a curative therapy for CAEBV (Fujii *et al.*, 2000; Okamura *et al.*, 2000; Taketani *et al.*, 2002); however, transplant-related complications are common in such patients (Gotoh *et al.*, 2008; Kimura *et al.*, 2001, 2003). Alternatively, the EBV-related antigens expressed by infected cells are possible targets for treatment with EBV-specific cytotoxic T lymphocytes (CTLs) (Heslop *et al.*, 1996; Rooney *et al.*, 1998).

Viral gene expression in EBV-associated diseases is classified into one of three latency patterns (Cohen, 2000; Kieff & Rickinson, 2007). Latency type I, which is found in Burkitt's lymphoma, is characterized by EBV nuclear antigen (EBNA) 1, EBV-encoded small RNAs (EBERs) and *Bam*HI-A rightward transcripts (BARTs) expression (Tao *et al.*, 1998). In latency type II, which is characteristic

A supplementary table of primer sequences is available with the online version of this paper.

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of Hodgkin's lymphoma and nasopharyngeal carcinoma, EBNA1, latent membrane protein (LMP) 1, LMP2, EBERs and BARTs are expressed (Brooks *et al.*, 1992; Deacon *et al.*, 1993). In latency type III, which is associated with post-transplant lymphoproliferative disorders, all of the above latent genes (EBNA1, EBNA2, EBNA3A, 3B, 3C, EBNA-LP, LMP1, LMP2, EBERs and BARTs) are expressed (Young *et al.*, 1989).

We recently reported that EBV gene expression could be quantitatively assessed by multiplex real-time RT-PCR (Kubota *et al.*, 2008). This method not only helps quantify EBV gene expression but also can be used to clarify the pathogenesis of EBV-associated diseases and to provide information about their prognosis and possible therapeutic interventions. Thus, in this study, we quantified the expression of six latent (EBNA1, EBNA2, LMP1, LMP2, EBER1 and BARTs) and two lytic [BZLF1 and glycoprotein (gp) 350/220] EBV genes in the peripheral blood of patients with CAEBV.

RESULTS

First, we quantified the expression of several EBV genes in B, T and NK cell lines by real-time RT-PCR (Fig. 1a). In the EBV-positive B cell lines (Raji, LCL-1 and LCL-2), all six latent genes (EBNA1, EBNA2, LMP1, LMP2, EBER1 and BARTs) were detected, and the gene expression pattern was consistent with latency type III. Both lytic genes were detected in LCL-1 and -2 cells. However, none of the target genes was detected in BJAB, an EBV-negative cell line. EBNA1, LMP1, LMP2, EBER1 and BARTs, but not EBNA2, were detected in both the T (SNT-8, -13, -15 and -16) and NK cell lines (SNK-1, -6, -10 and KAI-3). The pattern of expression in the T and NK cell lines was latency type II.

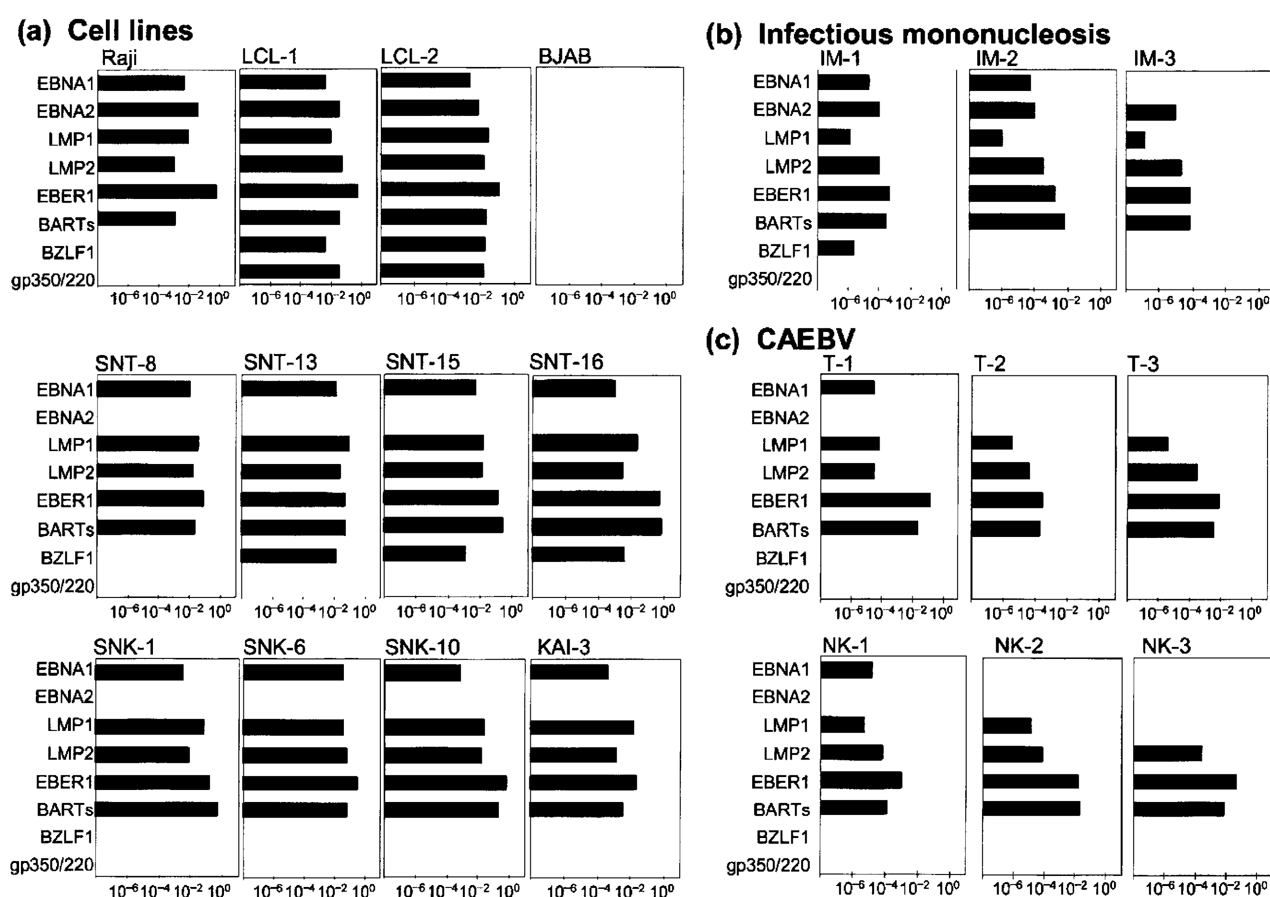


Fig. 1. Analysis of EBV gene expression by real-time RT-PCR. β2-Microglobin (β2 m) was used as an endogenous control and reference gene for relative quantification and was assigned an arbitrary value of 1 (10^0). (a) The quantity of each EBV gene in B, T and NK cells. Raji, LCL-1 and LCL-2 are EBV-positive B cell lines. BJAB is an EBV-negative B cell line. SNT-8, -13, -15 and -16 are EBV-positive T cell lines. SNK-1, -6, -10 and KAI-3 are EBV-positive NK cell lines. (b) Quantitative expression of the EBV genes in patients with infectious mononucleosis. (c) Representative results showing the relative expression of EBV genes in patients with a CAEBV infection. T-1, -2 and -3 are T-cell-type cases (patients 6, 9 and 11 in Table 1), while NK-1, -2 and -3 are NK-cell-type cases (patients 14, 15 and 19 in Table 1).

BZLF1 was detected in three of four T-cell lines, while gp350/220 was not detected in any of the cell lines, indicating an abortive lytic cycle. These results are consistent with those from previous reports (Leenman *et al.*, 2004; Tao *et al.*, 1998; Tsuge *et al.*, 1999; Zhang *et al.*, 2003), indicating the reliability of our system. We evaluated the sensitivity for each latent EBV gene using a cell mixture containing 1×10^6 EBV-negative BJAB cells and 10-fold serial dilutions of LCL-1 with latency III. The detection limits for EBNA1, EBNA2, LMP1, LMP2, EBER1 and BARTs were 0.1, 0.1, 0.01, 0.01, 0.001 and 0.01 % of LCL-1 cells, respectively. To evaluate the sensitivity for lytic genes, cell mixtures containing BJAB and Akata cells with a lytic infection, induced by human immunoglobulin G, were used. The detection limits for BZLF1 and gp350/220 were 0.1 % of Akata cells.

Next, we analysed blood from three patients with acute-phase infectious mononucleosis (Fig. 1b). EBNA2, LMP1, LMP2, EBER1 and BARTs were detected in the PBMCs of the patients, whereas EBNA1 was detected in two patients. The gene expression pattern in each case was latency type III. BZLF1 was detected in one patient, whereas gp350/220 was not detected in any patient. Furthermore, we analysed the PBMCs of 23 healthy carriers. Four healthy carriers were positive for EBV DNA. Real-time RT-PCR detected EBER1 and BARTs in the PBMCs of one carrier, while EBER1 alone was detected in a single additional carrier.

We next quantified the expression level of each gene in 24 patients with CAEBV. PBMCs collected at the time of diagnosis or referral were used in the analysis. The expression profiles of each patient are shown in Table 1, while the positive rates for each EBV gene are summarized in Table 2. EBER1 and BARTs were detected in each patient, while LMP2 was detected in most patients. EBNA1 and LMP1 were detected less frequently compared with EBER1 and BARTs ($P<0.0001$ and $P=0.004$, respectively). EBNA2 and the lytic genes BZLF1 and gp350/220 were undetected in all of the patients. Representative

Table 1. Characteristics and EBV gene expression profiles of 24 patients with chronic active EBV infection

ND, Not done. EBNA2, BZLF1 and gp350/220 were not expressed in any samples. EBER1 and BARTs were expressed in all samples.

Patient	Age (years)	Gender	Cell type infected	Viral load*				Disease type†	HSCT	Outcome	Viral load‡	EBV gene expression		
				PBMC	CD3$^+$	CD19$^+$	CD56$^+$					EBNA1	LMP1	LMP2
1	6	M	T	85925	**157196**	32828	62047	I	−	Alive	241000	−	+	+
2	5	M	T	74915	**119024**	12292	**77651**	I	−	Alive	392203	+	−	+
3	25	M	T	10749	**12106**	2742	5739	I	−	Alive	297	−	−	−
4	10	M	T	18308	**23422**	12665	**27106**	I	−	Alive	19363	−	+	+
5	6	M	T	14162	**22559**	1583	1073	A	+	Alive	14162	−	−	+
6	4	F	T	15776	**17312**	5243	4321	A	+	Alive	15776	+	+	+
7	11	M	T	60097	**143852**	23212	6352	A	+	Alive	60097	−	−	+
8	18	F	T/B	93458	**118026**	**174042**	**267078**	A	+	Alive	392734	+	+	+
9	14	F	T	30633	**32730**	8345	4760	I	+	Alive	30633	−	+	+
10	24	F	T	8589	**43469**	2388	**12555**	A	−	Dead	37148	+	−	+
11	23	F	T	5684	**7990**	4200	250	I	+	Dead	2764	−	+	+
12	13	M	T	3176	**3579**	948	839	I	+	Dead	10681	+	+	+
13	16	F	T	52978	**55431**	37536	**84110**	I	+	Dead	52978	−	+	+
14	11	M	NK	370000	31600	100000	**1800000**	I	−	Alive	339589	+	+	+
15	9	M	NK	77884	7428	17083	**89352**	I	−	Alive	89930	−	+	+
16	4	M	NK	74550	11288	18423	**86361**	A	+	Alive	74550	+	+	+
17	5	F	NK	11200	330	3300	**23400**	A	+	Alive	1108	+	+	+
18	3	M	NK	131957	1591	16450	**917500**	I	+	Alive	131957	−	+	+
19	9	M	NK	263429	92057	206565	**425956**	I	+	Alive	263429	−	−	+
20§	26	F	NK	18889	ND	ND	ND	I	+	Alive	18889	−	+	−
21	14	F	NK	1559	53	105	**4302**	A	−	Dead	1051	−	−	−
22	14	F	NK	20126	3288	1866	**35252**	I	+	Dead	44750	+	+	+
23§	16	M	NK	69121	ND	ND	ND	I	+	Dead	69121	+	+	+
24§	14	F	NK	1041	ND	ND	ND	I	+	Dead	1041	−	−	−

*Bold type indicates that EBV DNA was concentrated by fractionation; copies (μg DNA)$^{-1}$.

†Patients with severe symptoms were defined as having a clinically active disease (A); patients with no symptoms or with only skin symptoms were defined as having an inactive disease (I).

‡Indicates the EBV DNA in the PBMCs used for real-time RT-PCR analysis; copies (μg DNA)$^{-1}$.

§Infection was confirmed by *in situ* hybridization with EBER using fractionated cells.

Table 2. Detection of eight EBV-related genes in 24 patients with a CAEBV infection

Gene	No. positive patients (%)	P-value*
EBNA1	10 (42)	<0.001
EBNA2	0 (0)	<0.001
LMP1	16 (67)	0.004
LMP2	20 (83)	0.11
EBER1	24 (100)	–
BARTs	24 (100)	–
BZLF1	0 (0)	<0.001
gp350/220	0 (0)	<0.001

*Comparison with EBER1 and BARTs. All *P*-values were obtained using Fisher's exact test.

quantitative results for each EBV gene are shown in Fig. 1(c).

The negative results obtained for EBNA1 and LMP1 raise the possibility that the test was not sensitive enough to detect low levels of expression. Therefore, we examined the correlation between the relative expression level for each gene and the EBV DNA load in the PBMCs (Fig. 2). For all of the EBV genes examined, the expression level correlated with the EBV DNA load. However, the samples with a low EBV DNA load were not always negative for EBNA1; similar findings were seen for LMP1.

To confirm the EBV gene expression profiles in various cell populations, we separated $CD3^+$, $CD19^+$ and $CD56^+$ cells from the PBMCs by immunomagnetic sorting and quantified the gene expression in each population by real-time RT-PCR using selected patients and healthy carriers. In one patient with T-cell-type CAEBV (patient 2 in Table 1; $CD3^+$ $CD56^+$ T cells harboured EBV), type II latent genes, such as EBNA1, LMP2, EBER1 and BARTs, were detected in both the $CD3^+$ and $CD56^+$ cell populations (Fig. 3a). In a patient with NK-cell-type CAEBV (patient 14 in Table 1), type II latent genes were detected primarily in the $CD56^+$ population (Fig. 3b). On the other hand, in a healthy carrier, EBER1 and BARTs were detected in the $CD19^+$ population (presumed to be the B-cell fraction; Fig. 3c). Importantly, the gene expression profiles in the mainly infected cells largely corresponded to those in the unsorted PBMCs in all three cases, suggesting that our PBMC data could be applied to the cells in the mainly infected population.

We next estimated the mean expression level for each EBV gene in 24 patients with CAEBV (Fig. 4a). EBER1 had the highest relative expression level, followed by BARTs, LMP2 and EBNA1, whereas LMP1 had the lowest. Next, compared the expression level for each EBV gene between the T- and NK-cell types of CAEBV (Fig. 4b). No significant difference was found, although LMP2 expression tended to be higher in the T-cell type ($P=0.09$). We also compared the expression levels between the clinically active patients, who presented with severe symptoms at the time of sample collection, and clinically inactive patients (Fig. 4c). EBNA1 expression was 8.3 times higher in the active patients than in the inactive patients ($P=0.02$). Additionally, the rate of EBNA1-positive patients in the active group was significantly higher (75 versus 25 %; $P=0.03$). On the other hand, there was no difference in EBV DNA load in the peripheral blood between the active and inactive groups [$10^{4.4}$ versus $10^{4.5}$ copies (μg DNA)$^{-1}$; $P=0.85$]. We also investigated whether EBV gene expression at the time of diagnosis or referral to our hospital was associated with the subsequent disease outcome. We divided the patients into three groups: survivors without HSCT, survivors with HSCT and non-survivors. No significant difference was observed in the gene expression profiles of the three groups (Fig. 4d).

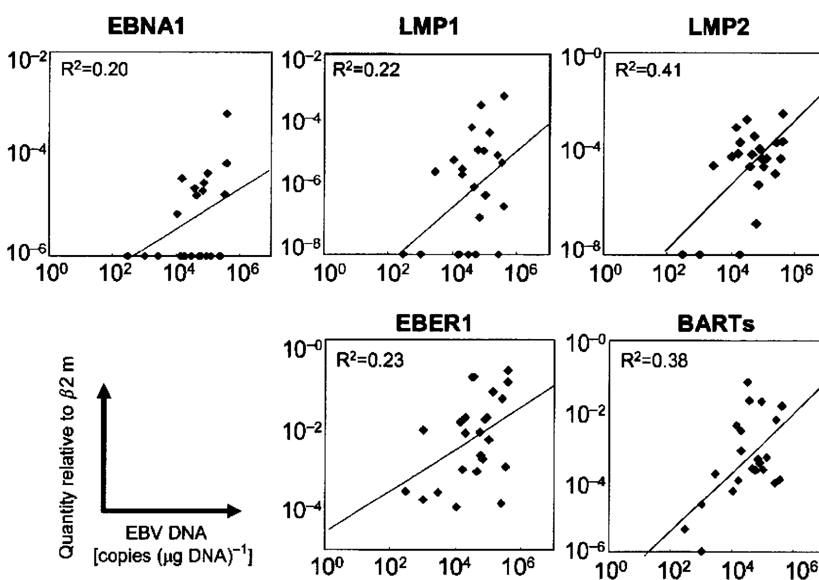
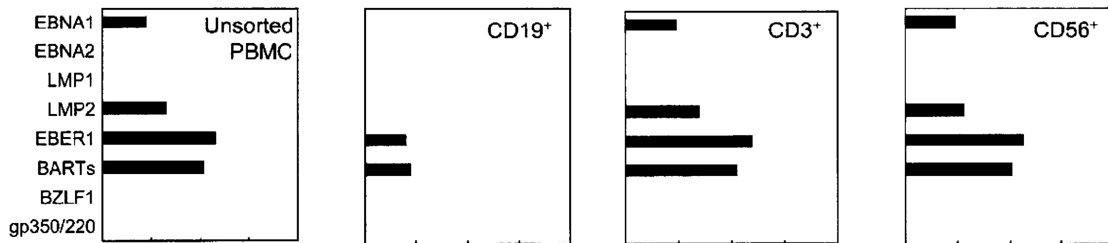
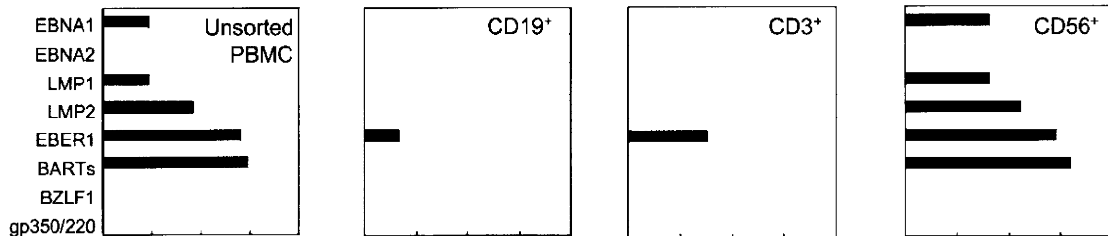


Fig. 2. Relationship between the quantity of each EBV gene and the EBV DNA load in PBMCs from patients with CAEBV. The correlation in all of these was statistically significant.





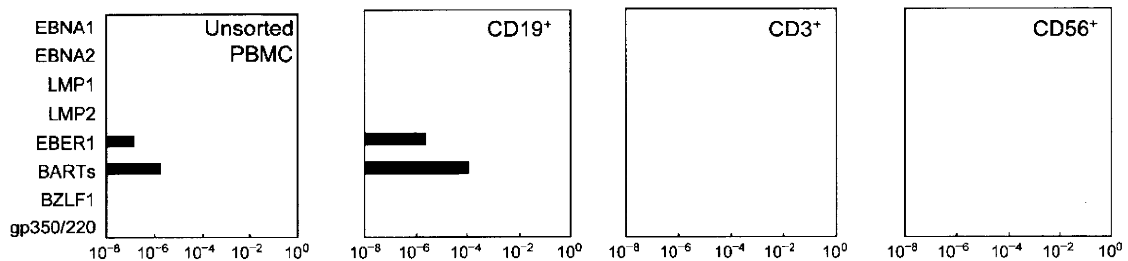


Fig. 3. EBV gene expression in sorted cell populations. CD19$^+$, CD3$^+$ and CD56$^+$ cells were separated by immunomagnetic sorting and analysed by real-time RT-PCR; unsorted PBMCs were analysed. (a) A T-cell-type CAEBV patient (patient 2 in Table 1; CD3$^+$ CD56$^+$ T cells were the main type of infected cells). (b) An NK-cell-type CAEBV patient (patient 14 in Table 1). (c) A healthy carrier whose PBMCs were positive for EBV DNA.

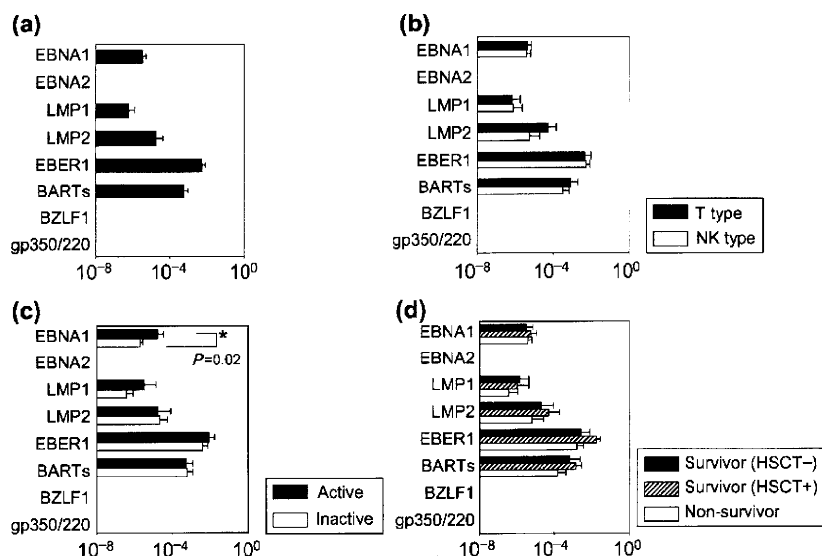


Fig. 4. EBV gene expression profile for patients with CAEBV. The quantity of each EBV gene was analysed by real-time RT-PCR and compared with the $\beta 2$ m level; the mean ± SE (boxes and bars) was calculated for each gene. (a) Average expression of EBV genes in 24 patients with CAEBV. (b) Comparison between T- (13 cases) and NK- (11 cases) cell-types. (c) Comparison between clinically active (8 cases) and inactive (16 cases) patients. (d) Comparisons of surviving patients without HSCT (6 cases), surviving patients with HSCT (10 cases) and non-surviving patients (8 cases). The Mann–Whitney *U*-test was used to compare the expression values between the groups, while the analysis of variance was used to compare the groups of three.

Finally, to eliminate any potential influence of therapeutic interventions, we excluded six patients who had received therapy before entering our hospital and re-evaluated the expression of each gene in the remaining 18 patients. The level of EBNA1 expression in the active patients was 8.2 times higher than that in the inactive patients ($P=0.03$) and the rate of EBNA1-positive patients was significantly higher in the active group (83 versus 25 %; $P=0.04$). We also re-evaluated the disease outcome in these 18 patients. No significant difference was observed in the gene expression profiles between the three groups according to outcome (data not shown).

DISCUSSION

Analysing the expression profile of EBV-related genes is essential to clarify the pathogenesis of EBV-associated diseases and to uncover information regarding the prognosis of individual patients and potential therapeutic interventions. In recent years, a quantitative method for the analysis of EBV gene expression has been applied to infectious mononucleosis (Weinberger *et al.*, 2004), Burkitt's lymphoma and nasopharyngeal carcinoma (Bell *et al.*, 2006). In the present study, we quantified the expression of six latent genes and two lytic genes in 24 patients with CAEBV using one-step multiplex real-time RT-PCR. To our knowledge, this is the first study to quantify EBV gene expression in CAEBV patients. EBNA1, LMP1, LMP2, EBER1 and BARTs were detected in the patient samples, whereas EBNA2 and the two lytic genes were not detected. The gene expression pattern was latency type II, consistent with previous qualitative RT-PCR results (Kimura *et al.*, 2005). Because the lytic genes BZLF1 and gp350/220 were undetected, a lytic infection is unlikely in the peripheral blood of the CAEBV patients. EBER1 and BARTs were detected in abundance in all patients, while LMP2 was found in most patients. EBNA1 and LMP1 were less frequently detected and had lower expression levels than EBER1 and BARTs. These results are in contrast with similar analyses using T or NK cell lines, in which EBNA1, LMP1, LMP2, EBER1 and BARTs were abundantly and comparably expressed. EBNA1, EBNA2 and EBNA3C are the dominant targets of $CD4^{+}$ T-cell responses, while EBNA3A, EBNA3B and EBNA3C are the dominant targets of $CD8^{+}$ T-cell responses (Hislop *et al.*, 2007). In those patients with CAEBV, most or all of these antigens were not expressed, contributing to the evasion of cellular immunity. The decreased frequency and low expression level of EBNA1 may also contribute to the immunological escape mechanism of CAEBV.

The expression profile identified in this study may be useful for obtaining information regarding potential immunotherapies. The EBV-related antigens expressed by infected cells are possible targets for treatment with EBV-specific CTLs. Several studies have reported the use of such therapies for CAEBV, but most have shown only limited effectiveness (Hagihara *et al.*, 2003; Kuzushima *et al.*, 1996; Savoldo *et al.*, 2002). These studies used EBV-specific CTLs that were generated from LCL and targeted latency type III antigens. Our results indicate that EBER1 and BARTs were the most frequently and abundantly expressed EBV genes, followed by LMP2. Because very little EBER1 and BARTs mRNA is translated into protein (Arrand & Rymo, 1982; Kieff & Rickinson, 2007), LMP2 would be the most favourable target for CTL therapy against CAEBV. Recently, EBV-specific CTLs targeted against LMP2 were used to treat Hodgkin's lymphoma and nasopharyngeal carcinoma, both of which are latency type II infections (Bollard *et al.*, 2004, 2007; Straathof *et al.*, 2005). Furthermore, patients with CAEBV generally lack LMP2-specific CTLs (Sugaya *et al.*, 2004). However, to develop effective and useful forms of immunotherapy, additional studies focusing on the nature of the infected cells and the underlying pathology of CAEBV are necessary.

In this study, we quantified the relative expression of EBV latent and lytic genes by real-time RT-PCR. There are a few drawbacks to our system. Firstly, we used β2-microglobulin (β2 m) as a reference for relative quantification; however, comparisons of the levels of expression between different genes may be compromised by variations in the efficiency of the primers used. Another option for such quantification is preparing a standard curve for each cDNA by diluting the plasmid and estimating the number of RNA copies to quantify the expression of each gene more accurately. Secondly, we determined the type of latency based on the patterns of viral gene expression. Promoter usage for EBNA1 is different between latency types I/II and III (Qp versus Cp) (Kieff & Rickinson, 2007). Primers capable of distinguishing between the two EBNA1 promoters would enable us to confirm the type of latency more accurately. Bell *et al.* (2006) used such a system to distinguish latency types and quantify gene expression using different EBNA1 primers.

There are several possible reasons why EBNA1 and LMP1 were detected less frequently in our analysis. First, EBV-infected T or NK cells in some patients with CAEBV may indeed express very little LMP1 or EBNA1. A previous experiment performed using nested RT-PCR, which is sensitive but not quantitative, showed that these genes were expressed in less than half of CAEBV patients (Kimura *et al.*, 2005). Second, the sensitivity of the test may be too low to detect these genes. However, those samples with a low EBV DNA load in this study were not always negative for EBNA1 or LMP1, indicating that low sensitivity was not the only reason that the expression of these genes was not detected. Moreover, EBV polymorphisms may have affected our results. Indeed, the primers used for LMP1 are specific for polymorphic regions (Kubota *et al.*, 2008). However, we used mixed primers for LMP1 to account for sequence variations, and the EBNA1 primers were designed to recognize fairly conserved regions. Furthermore, we also examined EBNA1- or LMP1-negative samples by nested RT-PCR using alternate primer sets (Kimura *et al.*, 2005).

Neither EBNA1 nor LMP1 was detected in any of the samples by nested PCR (data not shown).

EBNA1 was detected more frequently and abundantly in the clinically active patients. EBNA1 is the only EBV protein consistently expressed in all proliferating cells, and it plays central roles in the maintenance and replication of the episomal EBV genome. EBNA1 also has a role in cell growth and survival (Kieff & Rickinson, 2007; Thorley-Lawson & Gross, 2004). Recently, Saridakis *et al.* (2005) demonstrated that EBNA1 inhibits apoptosis by binding to USP7, which destabilizes p53. Together with our results, these findings suggest that EBNA1 plays an important part in the pathogenesis and symptoms of CAEBV.

EBV gene expression has been shown to be related to the prognosis of EBV-associated diseases. Kwon *et al.* (2006) evaluated EBER and LMP1 expression in patients with Hodgkin's lymphoma, while Tsang *et al.* (2003) reported a relationship between the recurrence and detection of LMP1 in patients with nasopharyngeal carcinoma. Similarly, we evaluated the relationship between EBV gene expression and the prognosis of CAEBV, but were unable to identify a significant link. Other factors that may have influenced the results of this study include the small sample size, short observation period and therapeutic interventions such as HSCT. Additional studies with a greater number of cases and a longer observation period are necessary to reach conclusions about the prognostic value of EBV gene quantification for CAEBV. In conclusion, we applied a real-time RT-PCR system to PBMCs from patients with CAEBV and identified the expression profiles of several EBV genes. Quantifying EBV gene expression may be useful in clarifying CAEBV pathogenesis and provide further information about therapeutic interventions, such as CTL therapy.

METHODS

Cell lines. The EBV-positive B cell lines used in this study were Raji, Akata, lymphoblastoid cell line (LCL)-1 and LCL-2. BJAB, an EBV-negative B cell line, was used as a negative control. The EBV-positive T cell lines used were SNT-8, -13, -15 and -16 (Zhang *et al.*, 2003). The EBV-positive NK cell lines used were SNK-1, -6 and -10 (Zhang *et al.*, 2003) and KAI-3 (Tsuge *et al.*, 1999). The T/NK cell lines were derived from patients with CAEBV or nasal NK-/T-cell lymphomas.

Patients. Twenty-four patients (13 males and 11 females) with CAEBV, ranging in age from 3 to 26 years (median age 13 years), were enrolled in this study (Table 1). Each patient met the following diagnostic criteria: EBV-related symptoms for at least 6 months (e.g. fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme or hypersensitivity to mosquito bites), an increased EBV load in either the affected tissue or peripheral blood, and no evidence of previous immunological abnormalities or other recent infections that could explain the condition (Kimura, 2006; Kimura *et al.*, 2001). Based on the infected cell type, 13 patients were identified as having T-cell-type CAEBV, while 11 were identified as having NK-cell-type CAEBV. To determine which cells harboured the most EBV, peripheral blood mononuclear cells (PBMCs) were fractionated into $CD3^+$, $CD19^+$ and $CD56^+$ cells and analysed by either quantitative PCR or *in situ* hybridization, using EBER1 as a probe, as described previously (Kimura *et al.*, 2001, 2005). The patients were defined as having a T-cell-type infection if their $CD3^+$ cells contained larger amounts of EBV DNA than their PBMCs, or if their $CD3^+$ cells gave a positive hybridization signal with EBER1. The patients were defined as having an NK-cell-type infection if their $CD56^+$ cells, rather than their $CD3^+$ cells, were the major cells harbouring EBV. The EBV DNA copy numbers in each cell population are shown in Table 1.

Peripheral blood was collected at the time of diagnosis or referral to our hospital. Six of 24 patients had already received steroid therapy or chemotherapy. PBMCs were isolated using Ficoll–Paque density gradients (Pharmacia Biotech) and stored at −80 °C until further use. Eight patients with severe symptoms such as high fever, distinct hepatosplenomegaly, and/or elevated hepatic transaminase levels at the time of sample collection were defined as having clinically active disease, while 16 patients with no symptoms or with only skin symptoms, including hydroa vacciniforme, were defined as having inactive disease. Eight of the patients died after 1–49 months of observation (median 14 months). Sixteen of the patients, 10 of whom received HSCT, were alive after 9–115 months of observation (median 28 months). Twenty-three healthy carrier volunteers who were seropositive for EBV and three patients with infectious mononucleosis (aged 5, 11 and 29 years) were enrolled as controls.

Informed consent was obtained from all patients or their guardians. The institutional review board of Nagoya University Hospital approved the use of the specimens that were examined in this study.

Real-time PCR assay. DNA was extracted from 1×10^6 PBMCs using a QIAmp blood mini kit (Qiagen). EBV DNA was quantified by real-time PCR as described previously. The viral load is expressed as the number of copies (μg DNA)$^{-1}$ (Kimura *et al.*, 1999).

RNA was extracted from 1×10^6 cells using a QIAmp RNeasy mini kit (Qiagen). Contaminating DNA was removed by on-column DNase digestion using the RNase-free DNase set (Qiagen) (Kubota *et al.*, 2008). Viral mRNA expression was quantified by one-step multiplex real-time RT-PCR using an Mx3000P real-time PCR system (Stratagene) as described previously (Kubota *et al.*, 2008). All of the primer/probe combinations, except those for EBER1 lacking an intron, were designed to span introns to avoid amplifying residual genomic DNA. The primer and probe sequences are listed in Supplementary Table S1 (available in JGV Online). The primers used for EBNA1, EBNA2, LMP1 and BZLF1, which were described previously (Kubota *et al.*, 2008), were modified according to sequence variations amongst the strains. The stably expressed housekeeping gene $\beta 2$ m was used as an endogenous control and reference gene for relative quantification (Patel *et al.*, 2004).

Cell sorting and gene expression analysis. $CD3^+$, $CD19^+$ and $CD56^+$ cells were separated from 1×10^7 PBMCs by immunomagnetic sorting using anti-CD3, -CD19 and -CD56 MACS Microbeads, respectively (Miltenyi Biotec). After two rounds of sorting, the purity of the populations exceeded 95 %. RNA was extracted from each cell population for real-time RT-PCR analysis. For comparison, RNA was also extracted from unsorted PBMCs.

Statistical analyses. All statistical analyses were performed using StatView (version 5.0; SAS Institute). Geometric (logarithmic) means were calculated for the expression of each EBV gene. For the negative samples, the default value, which was defined as the lowest level of expression for a particular gene, was used for the calculation. The default values for the undetected genes EBNA1, LMP1 and LMP2 were 10^{-6}, 10^{-8} and 10^{-8}, respectively. The Mann–Whitney *U*-test was used to compare the expression levels between groups, while analysis of variance was used to compare three groups. Fisher's exact

test was used to compare positive rates of gene expression. A regression analysis was used to compare the expression of each gene and the EBV DNA load. P-values <0.05 were deemed to be statistically significant.

ACKNOWLEDGEMENTS

We thank the following people for their contributions to this study: Kayoko Matsunaga (Fujita Health University); Masaki Ito, Atsushi Kikuta, and Mitsuaki Hosoya (Fukushima Medical University); Tomohiro Kinoshita (Nagoya University); Kazuhiko Shirota (National Hospital Organization Shizuoka Medical Center); Mariko Seishima (Ogaki Municipal Hispital); Masashi Shiomi (Osaka City General Hospital); Hajime Katsumata and Yasuo Horikoshi (Shizuoka Children's Hospital); Tsuyoshi Ito (Toyohashi City Hospital); and Sachiyo Kamimura (University of Miyazaki). This study was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (19591247).

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

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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

Received 16 July 2009; accepted 10 February 2010; electronically published 18 June 2010.

Potential conflicts of interest: none reported.

Financial support: Japan Society for the Promotion of Science (Grants in Aid for Scientific Research [C] [20591276] to Y.I.); Ministry of Health, Labor, and Welfare of Japan (grant for research on measures for emerging and reemerging infections [Intractable Infectious Diseases in Organ Transplant Recipients, H21-Shinko-Ippan-009] to H.K.).

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The Journal of Infectious Diseases 2010;202(3):461–469
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0022-1899/2010/20203-0018$15.00
DOI: 10.1086/653737

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 μL 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/μg 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 $\pm$ standard deviation (SD) were 1.6 $\pm$ 1.2 years after the onset of high EBV load status (3.0 $\pm$ 1.8 years after liver transplant) among chronic high EBV load carriers and 4.3 $\pm$ 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 $\pm$ SD were 3.0 $\pm$ 1.8 years after the onset of a high EBV load state (1.6 $\pm$ 1.2 years after liver transplant) among chronic high EBV load carriers and 4.3 $\pm$ 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 2×10^6 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	1.0×10^5	12[d]
2 (M)	1.0	2.8	R^-/D^+	1.5	...	1.6×10^5	12
3 (F)	1.7	2.3	R^-/D^+	1.2	Elevated liver enzyme	1.2×10^5	29
4 (F)	0.6	3.5	R^-/D^+	0.8	...	2.6×10^5	24
5 (M)	0.6	4.3	R^-/D^+	4.5	...	1.4×10^5	8
6 (F)	1.4	3.8	R^-/D^+	10.4	Fever, diarrhea	1.1×10^5	36[d]
7 (M)	5.3	1.2	R^-/D^+	1.0	...	6.8×10^4	15[d]
8 (F)	1.6	5.0	R^-/D^+	1.5	...	5.8×10^4	14
9 (M)	1.2	6.0	R^-/D^+	2.4	Cervical lymphadenopathy	3.7×10^4	23
10 (M)	1.3	6.5	R^-/D^+	2.9	Fever, diarrhea	1.1×10^6	73[d]
11 (F)	1.8	7.4	R^+/D^+	53.2	Atypical lymphocytosis	1.6×10^5	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 β_2-microgloblin (*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

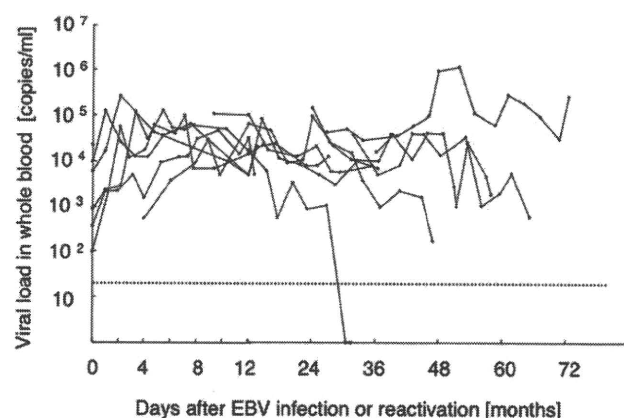

Figure 1. Time courses of Epstein-Barr virus (EBV) loads in whole blood from chronic high EBV load carriers. EBV load was serially measured by real-time polymerase chain reaction. Each line indicates the viral load of an individual patient. The dotted line indicates the detection limit.

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 2.5×10^4 copies/mL (range, 1.9×10^3–3.1×10^6 copies/mL) of whole blood for 15 patients with IM and 4.0×10^5 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, 0–2.7×10^2 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

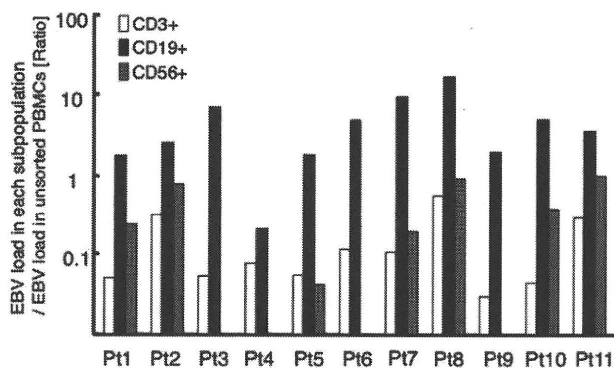

Figure 2. Epstein-Barr virus (EBV) load in each subpopulation of peripheral blood mononuclear cells (PBMCs) from chronic high EBV load carriers. Each subpopulation was positively selected with antibody-coated magnetic beads from PBMCs obtained from chronic high EBV load carriers. Viral load in each compartment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

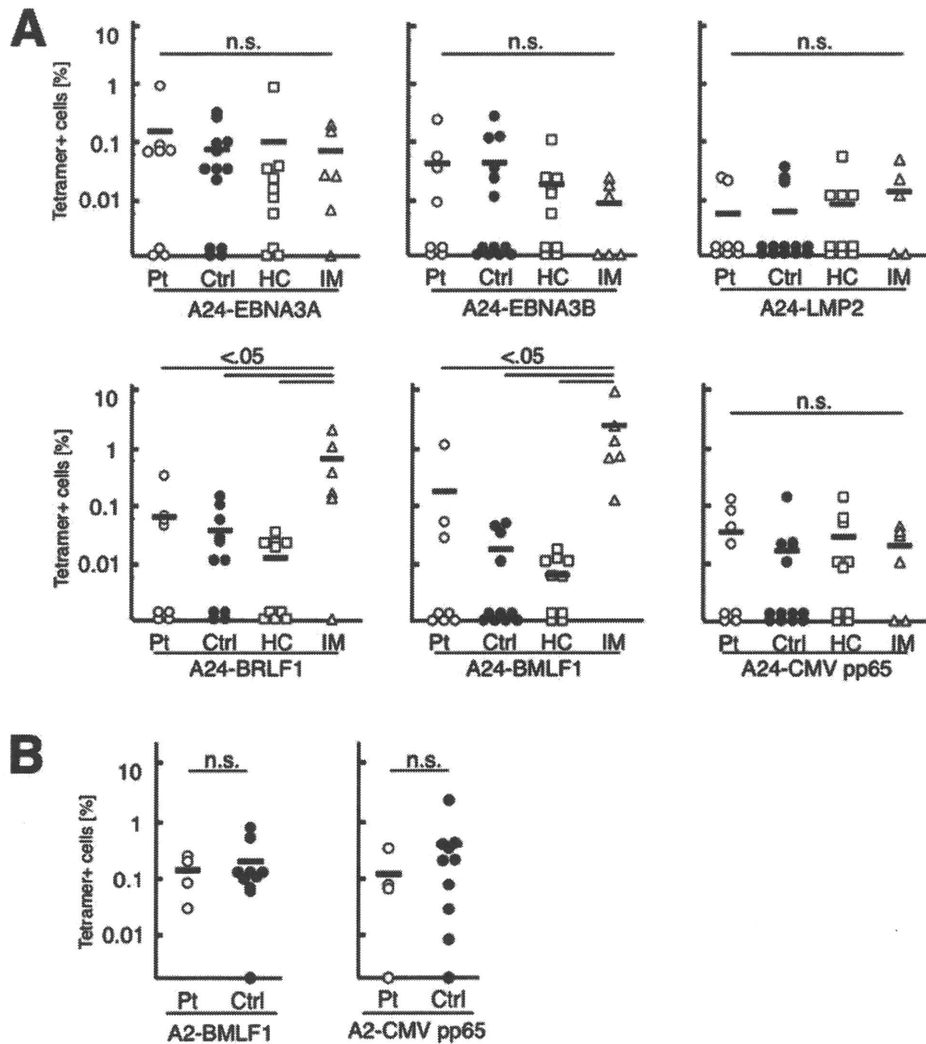

Figure 3. Comparison of the frequency of Epstein-Barr virus (EBV)–specific CD8$^+$ T cells among peripheral blood mononuclear cells (PBMCs). The precursor frequency of EBV-specific CD8$^+$ T cells in each study group was measured using major histocompatibility complex (MHC) peptide tetramers. EBV tetramer–positive 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/μg of DNA (82.4% [14/17] of asymptomatic patients with a positive EBV load had <$10^{2.5}$ copies/μg of DNA), 83% (5/6) of the patients with clinical symptoms had between $10^{2.5}$ and 10^4 copies/μg of DNA, and 100% (5/5) of patients who developed PTLD had >10^4 copies/μg 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/μg 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

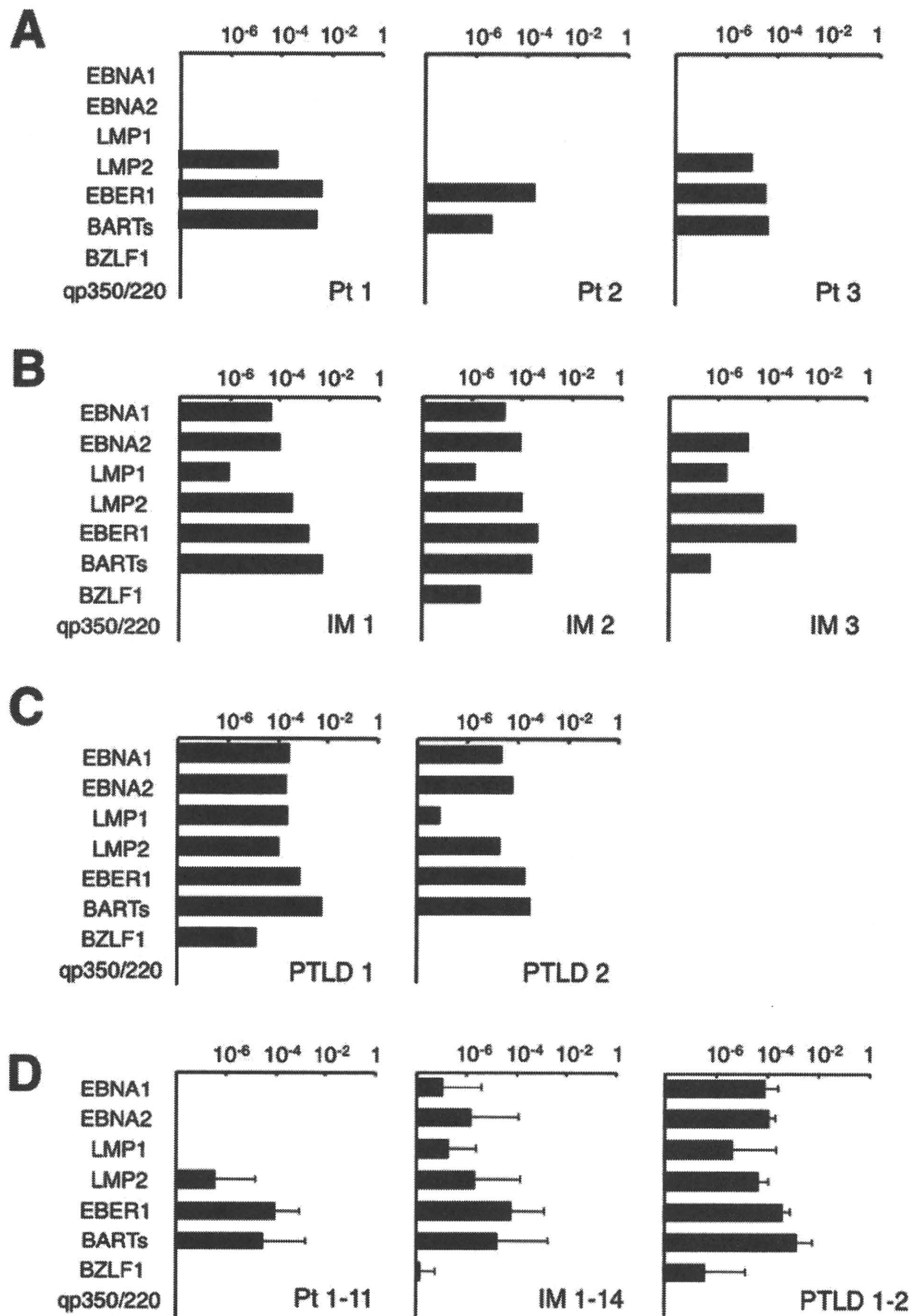

Figure 4. Comparison of the expression patterns of Epstein-Barr virus (EBV)–related genes in peripheral blood mononuclear cells (PBMCs). Relative quantification of EBV genes was performed in each study group by multiplex real-time reverse-transcription polymerase chain reaction. *A*, Representative data 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