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Heterogeneous, restricted patterns of Epstein–Barr virus (EBV) latent gene expression in patients with chronic active EBV infection

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Epstein–Barr virus (EBV) has been shown to infect T lymphocytes and to be associated with a chronic active infection (CAEBV), which has been recognized as a mainly non-neoplastic T-cell lymphoproliferative disorder (T-cell LPD). The systemic distribution of EBV genomes was studied, by real-time PCR, in multiple tissues from six patients with CAEBV, including three patients with T-cell LPD, one patient with B-cell LPD and two patients with undetermined cell-type LPD. There were extremely high loads of EBV genomes in all tissues from the patients. This reflects an abundance of circulating and infiltrating EBV-infected cells and a wide variety of clinical symptoms in the affected tissues. We chose one sample from each patient that was shown by real-time PCR to contain a high load of EBV genomes and examined the expression of EBV latent genes by RT–PCR. EBER1 and EBNA1 transcripts were detected in all samples. Only one sample also expressed EBNA2, LMP1 and LMP2A transcripts in addition to EBER1 and EBNA1 transcripts. Two of the remaining five samples expressed LMP1 and LMP2A transcripts. One sample expressed LMP2A but not LMP1 and EBNA2 transcripts. Another sample expressed EBNA2 but not LMP1 and LMP2A transcripts. The other sample did not express transcripts of any of the other EBNAs or LMPs. None of the samples expressed the viral immediate-early gene BZLF1. These results showed that EBV latent gene expression in CAEBV is heterogeneous and that restricted forms of EBV latency might play a pathogenic role in the development of CAEBV.

Introduction

Epstein–Barr virus (EBV) is a human herpesvirus associated with an array of conditions that range from asymptomatic infection and infectious mononucleosis to lethal lymphoproliferative disease (LPD), nasopharyngeal carcinoma (NPC), Burkitt's lymphoma (BL), and B-cell lymphoma in the immunocompromised host (Griffin, 2000). EBV has recently been implicated in the aetiology of an increasing number of diseases, including gastric carcinoma (Imai *et al.*, 1994), Hodgkin's lymphoma (HD) (Pallesen *et al.*, 1993), NK/T-cell lymphoma (Jones *et al.*, 1988; Harabuchi *et al.*, 1990; Su *et al.*, 1991), and non-neoplastic T-cell LPD such as chronic active EBV infection (CAEBV) (Kikuta *et al.*, 1988) and EBV-associated haemophagocytic syndrome (EBV-AHS) (Kawaguchi *et al.*, 1993; Su *et al.*, 1994). CAEBV is characterized by such clinical symptoms as fever, lymphadenopathy, splenomegaly, hepatitis, interstitial pneumonitis, nephritis, coronary artery aneurysms and uveitis, which persist over a period of months to several years. Laboratory findings include anaemia, thrombocytopenia, leukopenia and hypergammaglobulinaemia with extremely high titres of antibodies against EBV lytic-cycle proteins (Rickinson, 1986; Schooley *et al.*, 1986). CAEBV is a life-threatening illness leading to the development of lymphoma, myelodysplastic syndrome, EBV-AHS, opportunistic infection or multiple organ failure. The most striking finding of CAEBV is that EBV usually infects T lymphocytes and only occasionally B lymphocytes (Kikuta *et al.*, 1989; Ohga *et al.*, 1999). Though EBV-infected cells show a clonal proliferation in CAEBV (Kikuta *et al.*, 1989), the EBV-infected cells have the morphology not of lymphoblastoid cells but of small- to medium-sized lymphoid cells in

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the majority of cases. This makes us suspect that EBV-infected cells must represent a different form of EBV latency from that of neoplastic T-cell LPD.

EBV-infected cells may sustain three distinct forms of virus latency (Rowe *et al.*, 1992; Griffin, 2000). EBV infects B lymphocytes and induces indefinite cell proliferation. The lymphoblastoid cell lines (LCLs) carry episomal viral genomes and are usually non-lytic for virus replication. The LCL expresses the full spectrum of latent proteins or transcripts (latency III), including six EBV-encoded nuclear antigens [EBNAs 1, 2, 3A, 3B, 3C and latent protein (LP)], three latent membrane proteins (LMPs 1, 2A and 2B), two small non-polyadenylated nuclear RNAs (EBERs 1 and 2), and transcripts from the *Bam*HI-A region of the virus genome (BARF0) (Brooks *et al.*, 1993; Chen *et al.*, 1999). This form of latency is also encountered in some post-transplant LPD cases. In EBV-positive cases of NPC (Brooks *et al.*, 1992) and HD (Pallesen *et al.*, 1991), EBERs, BARF0, EBNA1 and the LMPs are expressed (latency II), whereas in BL, only EBERs, BARF0 and EBNA1 have been detected (latency I) (Chen *et al.*, 1995; Niedobitek *et al.*, 1995). The cell phenotype-dependent differences in EBV latent gene expression may reflect the strategy of the virus in relation to latent infection.

An EBV-infected T-cell lymphoma is a clonal expansion of a single EBV-infected cell with a pattern of gene expression that is distinct from that in BL but similar to that in NPC. However, despite the accumulation of clinical evidence in cases of neoplastic T-cell LPD (Pallesen *et al.*, 1993; Suzushima *et al.*, 1995), nothing is known about the form of EBV latency in cases of non-neoplastic T-cell LPD, including CAEBV. To understand the pathogenesis of CAEBV, especially T-cell LPD, it is essential to characterize the EBV latent gene expression in the disorder. Real-time PCR was first applied to the measurement of copy numbers of the EBV genome in various tissues in order to avoid underestimation of EBV latent gene expression by RT–PCR. Then EBV latent gene expression in the samples with high viral loads was examined. To the best of our knowledge, this is the first report describing EBV latent gene expression in patients with CAEBV.

Methods

■ **Tissue samples and cells.** Multiple tissue samples were obtained from six patients with CAEBV, including three patients with T-cell LPD, one patient with B-cell LPD and two patients with undetermined cell-type LPD. Samples were obtained at autopsy except for Pt-3 (at biopsy) after obtaining informed consent from the patients' parents. The tissue samples were immediately snap-frozen at −80 °C. The diagnosis of CAEBV was based on clinical, serological and virological evidence (Rickinson, 1986; Schooley *et al.*, 1986). The results of the virological study are summarized in Table 1. For three of the six patients, we determined the clonality of EBV-infected cells by using terminal repeat analysis (Kikuta *et al.*, 1989). All patients died in spite of treatment with a wide variety of agents. Raji cells were used as a positive control in real-time PCR. B95-8 EBV-immortalized reference cell lines (R-LCL) and BJAB cell lines were used as positive and negative controls, respectively, for EBV latent gene expression in RT–PCR. The B95-8 cell line, with 3% of cells in the lytic cycle, was used as a positive control for lytic replication.

■ **DNA and RNA extraction.** Genomic DNA was extracted from the frozen tissue samples, cells and cell lines by digestion with proteinase K, extraction with phenol–chloroform and precipitation with ethanol. Total RNA was extracted from the frozen tissue samples and cell lines by using the RNAzol B (TEL-TEST, Texas, USA) method according to the manufacturer's protocol.

■ **Real-time PCR.** A real-time PCR assay was used to measure copy numbers of the EBV genome in tissues. The PCR primers for this assay were based on the EBNA1-encoding BKRF1 gene; the forward and reverse primer sequences were 5′ GGATGCGATTAAGGACCTTGTT 3′ (nucleotide position 109677–109698) and 5′AAAGCTGCACAC-AGTCACCCT 3′ (109749–109729) (Baer *et al.*, 1984), respectively. The real-time PCR assay was carried out using a *Taq*Man PCR regent kit (PE Applied Biosystems) according to the manufacturer's instructions. A fluorogenic probe [5′-(6-FAM)-TGACAAAGCCCGCTCCTACCTGC-AAT (nucleotide position 109700–109725)-(TAMRA)-3′] located between the PCR primers was synthesized by Sawady Technology Co. (Tokyo, Japan). The PCR reaction mixture consisted of 200 μmol each of dATP, dCTP and dGTP, 400 μmol dUTP, 1·25 U Ampli*Taq* Gold (PE Applied Biosystems), 50 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8·3), 5 mmol/l of $MgCl_2$, 10 mmol/l EDTA, 0·5 U AmpErase uracil *N*-glycosylase (UNG), 25 pmol of each primer, 5 pmol *Taq*Man probe and 1 μg DNA in a volume of 50 μl. After activation of the UNG (2 min, 50 °C) and activation of the Ampli*Taq* Gold for 10 min at 95 °C, 50 cycles (15 s at 95 °C and 1 min at 60 °C) were carried out with an ABI PRISM 7700 Sequence Detection system (PE Applied Biosystems). To set up the assay, a standard curve of the threshold cycle (C_T) values was obtained from serial 10-fold dilutions of 1 μg of Raji cell DNA on the basis that 1 μg of Raji cell DNA was extracted from $2{\cdot}5 \times 10^5$ cells and one Raji cell contains 50 copies of the EBV genome. The C_T value was determined by the first cycle number at which fluorescence was greater than or equal to 10 times that of the background. The C_T values from clinical samples were plotted on the standard curve, and the copy number was calculated automatically using Sequence Detector software version 1.6 (PE Applied Biosystems). The lower limit of sensitivity was $2{\cdot}5 \times 10$ EBV DNA copies/ μg DNA.

■ **RT–PCR.** In the present study, one tissue sample that was shown to contain high viral loads of EBV genomes by real-time PCR was selected and used as a representative tissue in each patient for RT–PCR. The tissue samples included those from five spleens and one lymph node, and they were examined for the presence of viral RNA transcripts using RT–PCR. A 3·2 μg sample of each RNA was incubated in a solution containing 100 ng of random hexadeoxynucleotides and 200 U of Moloney murine leukaemia virus reverse transcriptase (First-Strand cDNA Synthesis Kit; Amersham Pharmacia) in a final volume of 15 μl at 37 °C for 1 h to synthesise cDNA. A 0·2 μl sample of the cDNA was subjected to PCR analysis using different primer combinations to determine expression of EBV latent genes and the *β*-actin gene. To evaluate the sensitivity of RT–PCR for detection of latent genes in tissues, the R-LCL was used as an EBV-positive control. Serial 10-fold dilutions of cDNA from the R-LCL were subjected to PCR. The primer sequences and PCR conditions used in this study, the expected sizes of PCR products and the sensitivities are summarized in Tables 2 and 3. The PCR reaction mixture consisted of 100 μmol of each deoxyribonucleotide, 1·0 U Ampli*Taq* Gold, 50 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8·3), 1·5 mmol/l $MgCl_2$, 0·01% (w/v) gelatin, 10 pmol of each primer and cDNA in a volume of 25 μl.

■ **Southern blot hybridization.** Aliquots (10 μl) of the PCR-

Table 1. EBV virology in patients with chronic active EBV infection

Abbreviations: CAEBV, chronic active EBV infection; VCA, viral capsid antigen; EA, early antigen; EBNA, EBV-determined nuclear antigen; ND, not determined.

Patient	Age (yr)/sex	Diagnosis	EBV serology				Clonality*/EBV-infected cells	Reference
			VCA-IgG	VCA-IgM	EA-IgG	EBNA		
Pt-1	5/F	CAEBV	320	< 10	40	< 10	ND/ND	
Pt-2	22/M	CAEBV	560	40	160	640	+/B	Kikuta *et al.* (1989)
Pt-3	16/F	CAEBV	1280	< 10	160	20	ND/CD45RO	Sugiyama *et al.* (1997)
Pt-4	2/M	CAEBV	10240	< 10	2560	160	+/CD4	Kikuta *et al.* (1989)
Pt-5	5/F	CAEBV	5120	< 10	5120	10	ND/CD2	Kikuta *et al.* (1993)
Pt-6	6/F	CAEBV	20480	< 10	5120	40	+/ND	

* Clonality of EBV-infected cells was determined by terminal repeat analysis.

Table 2. Primer sequences used for RT–PCR

Transcript	Primer	Sequence (5′ → 3′)	Coordinates*	Amplified product (bp)	Reference
EBNA1	5′ primer	GATGAGCGTTTGGGAGAGCTGATTCTGCA	67510–67539	184	Imai *et al.* (1996)
	3′ primer	CATTTCCAGGTCCTGTACCT	107986–107967		Brooks *et al.* (1992)
EBNA2	5′ primer	GCTGCTACGCATTAGAGACC	47892–47911	339	Imai *et al.* (1996)
	3′ primer	TCCTGGTAGGGATTCGAGGG	48616–48597		Imai *et al.* (1996)
LMP1	5′ primer	TCCTCCTCTTGGCGCTACTG	169383–169364	490	Imai *et al.* (1996)
	3′ primer	TCATCACTGTGTCGTTGTCC	169740168759		Imai *et al.* (1996)
LMP2A	5′ primer	ATGACTCATCTCAACACATA	166874–166893	280	Brooks *et al.* (1992)
	3′ primer	CATGTTAGGCAAATTGCAAA	380–361		Brooks *et al.* (1992)
EBER1	5′ primer	AAAACATGCGGACCACCAGC	6776–6795	167	Tierney *et al.* (1994)
	3′ primer	AGGACCTACGCTGCCCTAGA	6648–6629		Tierney *et al.* (1994)
BZLF1	5′ primer	TTCCACAGCCTGCACCAGTG	102719–102700	182	Tierney *et al.* (1994)
	3′ primer	GGCAGCAGCCACCTCACGGT	102330–102341/102426–102433		Tierney *et al.* (1994)
β-Actin	5′ primer	CCTTCCTGGGCATGGAGTCCT		202	Busson *et al.* (1992)
	3′ primer	CCTCGTTACTAGAACTAGAAG			Busson *et al.* (1992)

* The coordinates are given with reference to the B95-8 genomic sequence (Baer *et al.*, 1984).

amplified product were subjected to electrophoresis through a 1·0% agarose gel and transferred onto Hybond ECL nitrocellulose membranes (Amersham). Hybridization and washing were then done according to the manufacturer's protocol. The cloned fragments of the RT–PCR-amplified products from the positive controls were labelled with the ECL random prime labelling and detection systems (Amersham) and used as probes.

Results

Real-time PCR

The EBV DNA load ranged from $6{\cdot}5 \times 10^2$ to $3{\cdot}7 \times 10^6$ copies/μg DNA in all tissue samples with a mean load of $4{\cdot}0 \times 10^5$ copies/μg DNA (Fig. 1). Elevated EBV DNA loads in all tissues as well as in peripheral blood mononuclear cells (PBMC) have been reported in patients with CAEBV (Kimura *et al.*, 1999; Maeda *et al.*, 1999). The viral loads in B-cell LPD (Pt-2) appeared to be lower than in T-cell LPD. EBV-infected resting B cells have been shown to carry an episomal viral genome at a low copy number (2–5 copies per cell) (Thorley-Lawson *et al.*, 1996). As we were unable to determine the copy numbers of the EBV genome in the EBV-infected cells from CAEBV patients, we estimated the copy number to be 5 at maximum. One μg of genomic DNA from the tissue samples

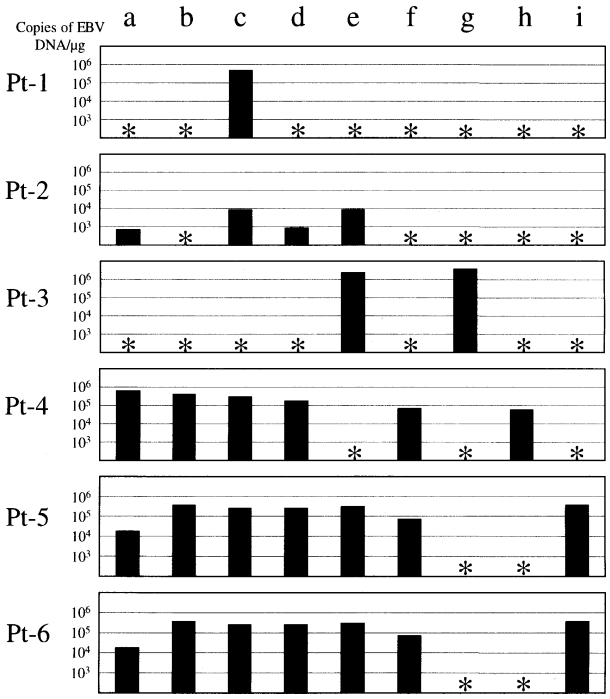

Fig. 1. EBV DNA load in tissue samples of six patients with chronic active EBV infection. a, Liver; b, lung; c, spleen; d, kidney; e, lymph node; f, heart; g, submandibular gland; h, PBMC; i, mononuclear cells from bone marrow; *, not tested.

corresponds to $2{\cdot}5 \times 10^5$ cells. At least five cells ($6{\cdot}5 \times 10^2/5/2{\cdot}5 \times 10^4 = 5{\cdot}2$) in 10^4 cells of the tissues examined would have been infected with EBV in the absence of lytic infection.

RT–PCR

All EBV latent gene transcripts and the BZLF1 transcript could be detected (Fig. 2) down to at least 10^{-4} dilutions of cDNA from the positive controls, as shown in Table 3. This indicated that if there is more than one EBV-infected cell in 10^4 tissue cells, the RT–PCR assay can detect the resulting EBV latent gene expression. Several distinct patterns of gene expressions were identified in the patients with CAEBV, as summarized in Table 4. EBER1 and EBNA1 transcripts were consistently detected by RT–PCR in representative samples from the six patients. Three of the six representative samples, including those from two patients with T-cell LPD (Pt-3, Pt-5) and one patient with undetermined cell-type LPD (Pt-6), expressed LMP1 and LMP2A transcripts. One (Pt-3) of the three samples also expressed EBNA2 transcripts. A sample from the other patient with undetermined cell-type LPD (Pt-1) expressed LMP2A but not LMP1 and EBNA2 transcripts. A sample from the other patient with T-cell LPD (Pt-4) expressed EBNA2 but not LMP1 and LMP2A transcripts. The sample from the patient with B-cell LPD (Pt-2) did not express any of the other EBNAs or LMPs transcripts. None of the samples expressed BZLF1 transcripts.

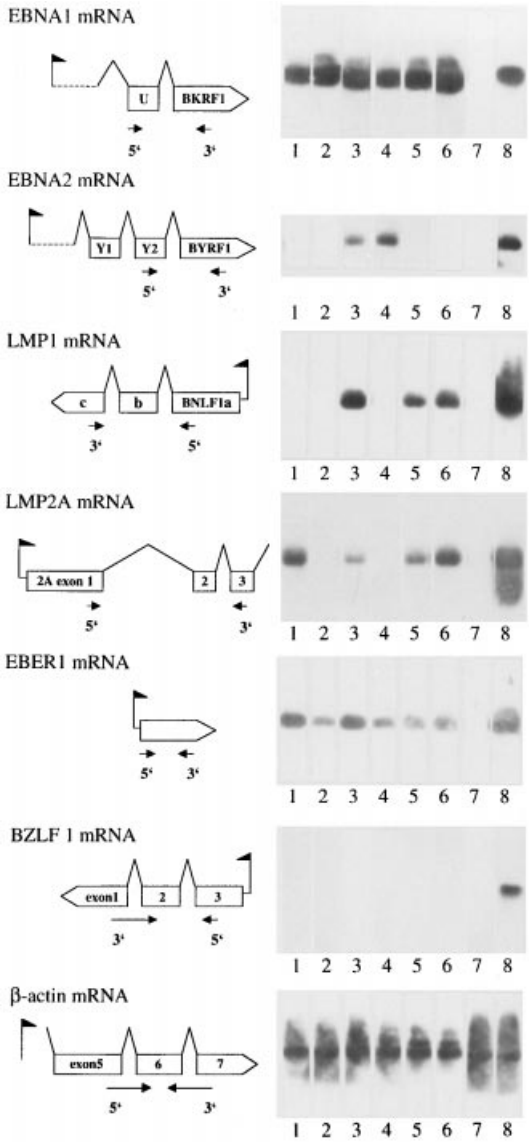

Fig. 2. RT–PCR analysis for mRNA expression of EBV transcripts. Southern blot hybridization is shown together with the exons (boxes), primers (arrows) and sites of transcriptional promoter (flags) for each mRNA. Lane 1, Pt-1; lane 2, Pt-2; lane 3, Pt-3; lane 4, Pt-4; lane 5, Pt-5; lane 6, Pt-6; lane 7, negative control; lane 8, positive control.

Discussion

EBV establishes a life-long infection in humans with distinct virus latency programmes during acute and chronic phases of infection (Thorley-Lawson *et al.*, 1996; Thorley-Lawson & Babcock, 1999; Faulkner *et al.*, 2000). During primary infection, EBV-infected B lymphocytes express the full spectrum of EBNAs and LMPs in addition to BARF0 and the EBERs. EBV-immortalized lymphoblasts are detected during the acute phase of infectious mononucleosis but are eventually eliminated by a strong cellular immune response to highly immunogenic EBNA2 and EBNA3 family proteins (Klein, 1994). This expression pattern, which has been termed latency

Table 3. Amplification conditions and sensitivities of RT–PCR

Name	Cycles	Denaturation	Annealing	Polymerization	Sensitivity*
EBNA1	35	1 min at 94 °C	2 min at 55 °C	2 min at 72 °C	10^{-4}
EBNA2	35	1 min at 94 °C	2 min at 55 °C	2 min at 72 °C	10^{-4}
LMP1	35	1 min at 94 °C	2 min at 55 °C	2 min at 72 °C	10^{-4}
LMP2A	35	1 min at 94 °C	2 min at 55 °C	2 min at 72 °C	10^{-4}
EBER1	35	1 min at 94 °C	1 min at 58 °C	1 min at 72 °C	10^{-5}
BZLF1	35	1 min at 94 °C	2 min at 55 °C	2 min at 72 °C	10^{-4}
β-Actin	35	1 min at 94 °C	2 min at 55 °C	2 min at 72 °C	Not determined

* Maximum dilutions of cDNA in which a positive signal could be detected for each RT–PCR.

Table 4. Summary of EBV transcripts in chronic active EBV infection as determined by RT–PCR

Abbreviations: PC, positive control; NC, negative control; R-LCL, B95-8 EBV-immortalized reference cell line; EBNA, EBV-determined nuclear antigen; LMP, latent membrane protein; EBER, EBV-encoded small RNA.

Patient	Tissue	Copies of EBV DNA (1 μg)	EBNA1	EBNA2	LMP1	LMP2A	EBER1	BZLF1	*β*-Actin
Pt-1	Spleen	$4{\cdot}7 \times 10^5$	+	−	−	+	+	−	+
Pt-2	Spleen	$8{\cdot}7 \times 10^3$	+	−	−	−	+	−	+
Pt-3	Lymph node	$2{\cdot}3 \times 10^6$	+	+	+	+	+	−	+
Pt-4	Spleen	$2{\cdot}8 \times 10^5$	+	+	−	−	+	−	+
Pt-5	Spleen	$2{\cdot}5 \times 10^5$	+	−	+	+	+	−	+
Pt-6	Spleen	$6{\cdot}8 \times 10^4$	+	−	+	+	+	−	+
PC	R-LCL	$3{\cdot}3 \times 10^6$	+	+	+	+	+	+*	+
NC	BJAB	< 25	−	−	−	−	−	−	+

* The B95-8 cell line with 3% of cells in lytic cycle was used as a positive control.

III, is also seen in LCL in culture. During chronic asymptomatic infection in healthy individuals, EBV resides in resting memory $CD23^-$ and $CD80^-$ ($B7^-$) B cells. This population of cells expresses LMP2A, but EBNA1 was not consistently detected (Tierney *et al.*, 1994; Miyashita *et al.*, 1995, 1997; Decker *et al.*, 1996). EBV-associated tumours demonstrate a restricted pattern of latent gene expression (latency I/II) in which only EBNA1 is expressed (latency I) or in which LMP1, LMP2A and LMP2B in addition to EBNA1 are variably expressed (latency II) (Pallesen *et al.*, 1991; Brooks *et al.*, 1992; Chen *et al.*, 1995; Niedobitek *et al.*, 1995). BARF0 and EBERs are commonly expressed in all of the latent forms mentioned above (Brooks *et al.*, 1993; Chen *et al.*, 1999).

EBV is known to be a B-lymphotropic virus. However, recent evidence indicates that EBV can also infect T lymphocytes and may play a role in the development of T-cell LPD. A wide spectrum of non-neoplastic to neoplastic T-cell LPDs has been demonstrated to be frequently associated with EBV infection, including CAEBV (Kikuta *et al.*, 1988; Ohga *et al.*, 1999), EBV-AHS (Kawaguchi *et al.*, 1993; Su *et al.*, 1994), nasal or nasal-type lymphoma (Harabuchi *et al.*, 1990), peripheral T-cell lymphoma (Su *et al.*, 1991) and T-cell lymphoma (Suzushima *et al.*, 1995). Although T-cell LPDs are very rarely seen in Western countries, they are relatively common in Asia (Hsu & Glaser, 2000). CAEBV is a life-threatening illness, which should be considered in the recently recognized spectrum of non-neoplastic T-cell LPDs and occasionally B-cell LPDs. While EBV latent gene expression of neoplastic T-cell LPD and EBV-infected T-cells *in vitro* has been reported, nothing is known about expression in non-neoplastic T-cell LPDs, such as CAEBV and EBV-AHS.

In EBV-positive T-cell lymphoma as well as in NPC and HD, viral transcripts consisting of EBNA1, LMP1 and LMP2A/2B have been detected by RT–PCR (Suzushima *et al.*, 1995). Kelleher *et al.* (1995) and Paterson *et al.* (1995) showed that EBV could infect immature human thymocytes and induce

expression of EBNA1, EBNA2, and BZLF-1 but not LMP2A and EBER1, suggesting that the mode of EBV infection in thymocytes differed from that in B lymphocytes. Two research groups (Koizumi *et al*., 1992; Fujiwara & Ono, 1995) using an intact EBV or a recombinant EBV have shown that the MT-2 cell line can be infected by EBV and that the EBV-infected MT-2 cells expressed only EBNA1 and LMP1 genes, a characteristic of latency II EBV infection. However, the MT-2 cell line is already transformed by human T-cell leukaemia virus type I, and thus EBV-infected cells could not reflect EBV latent gene expression in T cells *in vivo*. Recently, Imai *et al*. (1996) established four EBV-infected IL-2-dependent T-cell lines from patients with CAEBV. These four T-cell lines expressed EBER1, EBNA1, LMP1, LMP2A and LMP2B but not EBNA2, 3A, 3B, 3C or LP. This form of latency was similar to that seen in the EBV-infected MT-2 cells. EBV gene expression in BL appears to be restricted to expression of EBNA1 only. However, most established BL cell lines drift from this restricted viral gene expression to the full spectrum of gene expression seen in LCL. Therefore, it is important to examine gene expression in primary samples. In fact, one of the T-cell lines established by Imai *et al*. (1996) was derived from Pt-4 in this study, while the sample from the patient expressed EBNA2 but not LMP1 and LMP2A transcripts in addition to EBER1 and EBNA1 expression as substantiated in the present study.

In this study, we examined the viral loads in tissue samples and EBV latent gene expression in CAEBV. To determine whether the RT–PCR technique is sensitive enough to definitively analyse EBV latent gene expression, we examined viral loads in a wide variety of primary tissues by real-time PCR. All tissues obtained from patients with CAEBV harboured larger amounts of EBV genomes than PBMC from healthy seropositive controls (Kimura *et al*., 1999). The abundant EBV genomes in the tissues suggested a variety of serious symptoms and subsequent multiple organ failure in the patients. Though CAEBV is characterized by high antibody titres against lytic antigens, BZLF1 transcripts expressed during the lytic programme could not be detected in the tissue samples, indicating that high viral loads in the tissue samples did not seem to reflect virus replication (Takada *et al*., 1986). Furthermore, we could not detect the linear molecules of EBV DNAs found during lytic infection in three patients (Pt-2, -4 and -6) by using terminal repeat analysis (Kikuta *et al*., 1989). Therefore, we decided that the sensitivity of the RT–PCR for EBV latent gene expression was high enough to exclude the possibility of underestimation. All samples consistently expressed EBER1 and EBNA1 transcripts. Expression of EBNA1 would not necessarily be required in EBV-infected resting B cells of healthy carriers. However, EBNA1 may be essential for replication and maintenance of the EBV episomes in proliferating T cells as well as in B cells (Thorley-Lawson & Babcock, 1999). Only one sample expressed all the latent genes including EBER1, EBNA1, EBNA2, LMP1 and LMP2A. This form of latency, termed latency III, is also encountered in some post-transplant LPDs and infectious mononucleosis (Thorley-Lawson & Babcock, 1999). Two of the remaining five samples expressed LMP1 and LMP2A transcripts, showing the latency II form. This form of latency is similar to that seen in a subset of NPC and HD. One sample expressed LMP2A but not LMP1 and EBNA2 transcripts, similar to the latent form in resting B cells of healthy carriers. The transcript of the LMP2A gene is the only gene product consistently detected in latently infected B cells of healthy carriers (Tierney *et al*., 1994; Miyashita *et al*., 1995, 1997; Decker *et al*., 1996). Another sample expressed EBNA2 but not LMP1 and LMP2A transcripts in addition to EBER1 and EBNA1. This latency form has been described in endemic BL (Niedobitek *et al*., 1995). LMP1 and EBNA2 are thought to be responsible for the proliferative phenotype of infected B cells (Thorley-Lawson & Babcock, 1999). Detection of LMP1 and EBNA2 transcripts may, therefore, be indicative of an EBV-driven proliferative state in CAEBV. The other sample did not express any of the other EBNA or LMP transcripts. This expression form resembled that of the phenotypically representative latency I. These results showed that EBV latent gene expression in CAEBV is heterogeneous and restricted, regardless of the cell type of LPD.

As tissue samples could not be examined at the cellular level, the latency form determined by RT–PCR may represent only the predominant form of EBV infection in CAEBV. A tissue sample may comprise different cell populations expressing different latency forms. However, as EBV-infected cells show clonal proliferation in CAEBV as well as in neoplastic T-cell LPD (Kikuta *et al*., 1989), the present results lead us to propose that EBV latent gene expression is heterogeneous and that different states of virus–host interaction exist among cases of CAEBV. Except in one case the EBV-infected cells in CAEBV are distinguishable from those in infectious mononucleosis by the form of EBV latent gene expression. Furthermore, the severity of CAEBV may be due to the restricted expression of latent proteins, resulting in escape from the EBV-specific cytotoxic T lymphocytes and secondary functional impairment of the same T lymphocytes involved in host immunity against the virus.

Expression of different panels of latent gene products (latencies I, II and III) is controlled by use of three different EBNA promoters. While the C/W promoters permit expression of all EBNAs, the Q promoter only gives rise to EBNA1 (Schaefer *et al*., 1991; Zetterberg *et al*., 1999). Studies of EBNA promoters used in CAEBV and methylation patterns of the promoter regions are currently in progress.

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