Valproate (Sigma, St Louis, MO, USA) was dissolved in distilled water. Bortezomib, a gift from Millennium Pharmaceuticals (Cambridge, MA, USA), was dissolved in PBS.

Cell viability. Cell viability was quantified by Trypan blue exclusion. These experiments were performed in duplicate.

Immunoblotting. Cells were lysed directly in SDS sample buffer. Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies. Antibodies directed against acetyl-Histone 3, caspase-3, cleaved caspase-3, poly(ADP-ribose) polymerase (PARP; Cell Signaling Technology, Beverly, MA, USA), and β-actin (Sigma) were used.

Flow cytometry apoptosis assays. Apoptosis was measured by flow cytometry using an annexin V–phycoerythrin (PE)/7-aminoactinomycin D (7-AAD) apoptosis assay kit (BD Pharmingen Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Cell cycle assay. Cells were treated with 1 mM of VPA for 48 h, fixed with 70% ethanol, and then washed with ice-cold PBS. Fixed cells were treated with 10 μg/mL DNase-free RNase and stained with 5 μg/mL propidium iodide (Sigma).

Real-time RT-PCR. Viral mRNA expression was quantified by RT-PCR, as described previously.[23,24] β2-Microglobulin (β2m) was used as an endogenous control and reference gene for relative quantification.[25] Each experiment was performed in triplicate. The Mann-Whitney *U*-test was used to compare expression levels and $P < 0.05$ were considered significant.

Patients. Mononuclear cells (MNC) were collected from three patients with EBV-associated diseases. Patients T-1 (a 7-year-old boy) and T-2 (a 6-year-old girl) had hydroa vacciniforme-like lymphoma, a newly classified EBV-associated T cell lymphoma.[2] In these patients, approximately 10% of the MNC were infected with EBV and the EBV-infected cells were primarily γδT cells.[26] The third patient, NK-1 (a 14-year-old boy), had chronic active EBV disease, NK cell type.[27–29] Chronic active EBV disease is now considered an EBV-associated T/NK lymphoproliferative disease.[30,31] In this patient, approximately 40% of the MNC were infected with EBV and the EBV-infected cells were NK cells. Mononuclear cells from three healthy donors were used as controls. Informed consent was obtained from all participants or their guardians. The present study was approved by the Institutional Review Board of Nagoya University Hospital.

Flow cytometric *in situ* hybridization (FISH). To quantify EBV-infected cells and to identify the cell type(s) infected by EBV, a FISH assay was performed.[26] Briefly, 5×10^5 MNC were stained with monoclonal antibodies for 1 h at 4°C. Cells were fixed, permeabilized, and hybridized with a fluorescein-labeled EBV-encoded small RNA (EBER)-specific peptide nucleic acid probe (Y5200; Dako, Glostrup, Denmark). Stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA, USA).

Magnetic cell sorting. Primarily infected cell fractions were separated by magnetic sorting using a TCRγ/δ$^+$ T Cell Isolation kit or CD56 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity and recovery rates were 98.3% and 80.0%, respectively, with the TCRγ/δ$^+$ T Cell Isolation kit, and 96.4% and 80.9%, respectively, with the CD56 MicroBeads.

Results

Effects of VPA on HDAC in T and NK cell lines. Acetylated histone 3 levels were determined in T cell lines (SNT16 and Jurkat) and NK cell lines (KAI3 and KHYG1) after 24 h exposure to 0.1-5 mM of VPA. Valproate increased acetylated histone 3 levels in a dose-dependent manner (Fig. 1a), indicating that VPA inhibits HDAC in these cell lines.

Effects of VPA on the viability of T and NK cell lines. To evaluate the effects of VPA on cells viability, EBV-positive T cell lines (SNT13 and SNT16), an EBV-negative T cell line (Jurkat), EBV-positive NK cell lines (KAI3 and SNK6), and an EBV-negative NK cell line (KHYG1) were exposed to 0.1-5 mM of VPA for 24 h. The cell viability of all six cell lines tested was reduced by VPA in a dose-dependent manner (Fig. 1b,d). In another series of experiments, the same six cell lines were exposed to 1 mM VPA for 4 days, with viability evaluated every 24 h. In these experiments, VPA reduced the viability of all six cell lines by between 22% and 52% after 96 h (Fig. 1c,e). There were no obvious differences between the effects of VPA on EBV-positive and -negative cell lines. Furthermore, to directly compare the effects of VPA on EBV-positive and -negative cell lines, we exposed MT2/hyg and MT2/rEBV/9-7 (Fig. 1f,g) and NKL and TL1 (Fig. 1h,i) cells to VPA and found that 0.1-5 mM of VPA had almost identical effects on the EBV-positive and -negative cell lines.

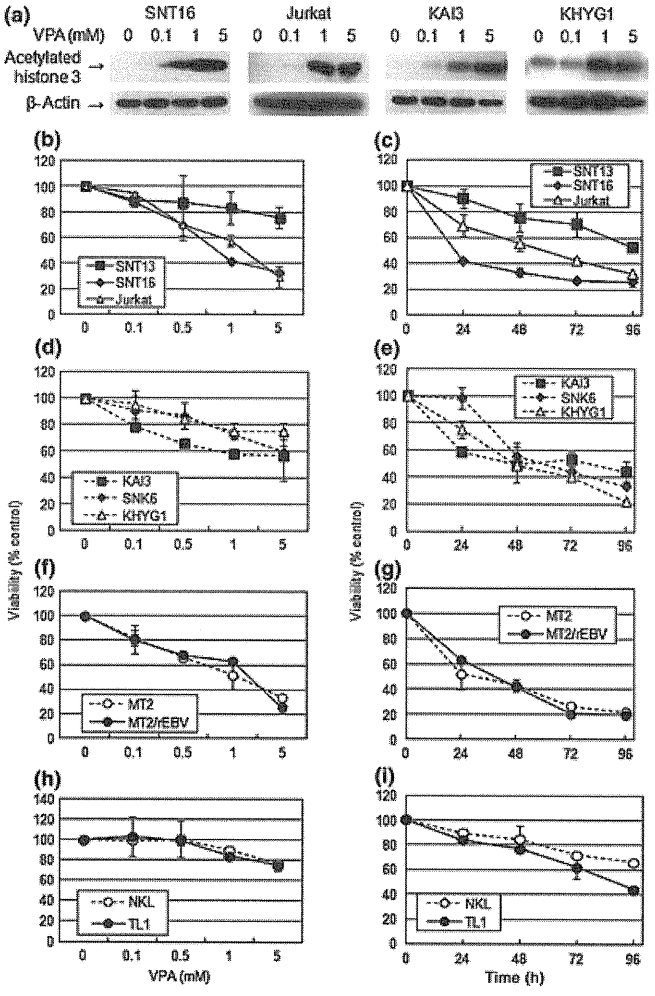

Fig. 1. Valproate (VPA) inhibits histone deacetylase (HDAC) and reduces viability of T and natural killer (NK) cell lines. (a) Acetylated histone 3 was detected by immunoblotting in T and NK cell lines treated with various concentrations of VPA for 24 h. β-Actin was used as a loading control. Viability of (b,c) Epstein–Barr virus (EBV)-positive T cell lines (SNT13 and SNT16) and an EBV-negative T cell line (Jurkat), (d,e) EBV-positive NK cell lines (KAI3 and SNK6) and an EBV-negative NK cell line (KHYG1), (f,g) an EBV-positive T cell line (MT2/rEBV) and its parental cell line (MT2/hyg), and (h,i) an EBV-positive NK cell line (TL1) and its parental line (NKL) that were either treated with VPA at the concentrations indicated for 24 h (b,d,f,h) or with 1 mM VPA for 96 h (c,e,g,i). Viability is shown as the ratio of viable cells in the different treatment groups to distilled water-treated cells, as assessed by Trypan blue exclusion. Data are the mean ± SEM.

doi: 10.1111/j.1349-7006.2011.02127.x

Effects of VPA on the apoptosis of T and NK cell lines. To determine whether VPA induces apoptosis in these cell lines, the cleavage of caspase-3 and PARP was analyzed by immunoblotting. One mM of VPA increased levels of cleaved caspase-3 and PARP in Jurkat and KHYG1, which are EBV-negative T and NK cell lines, respectively (Fig. 2a), suggesting that VPA induces apoptosis in these two cell lines. Analysis of the induction of apoptosis by flow cytometry showed that VPA only increased the number of apoptotic cells in the SNT16 cell line (Fig. 2b). In the other cell lines tested, increases in the number of apoptotic cells were not confirmed, although the number of dead cells increased. A representative result for KHYG1 cells is shown in Figure 2(c).

Effects of VPA on the cell cycle in T and NK cell lines. To investigate the effects of VPA on the cell cycle, cells were treated with 1 mM VPA for 48 h, stained with propidium iodide, and then analyzed by flow cytometry. The population of cells in the G_1 phase was increased following exposure

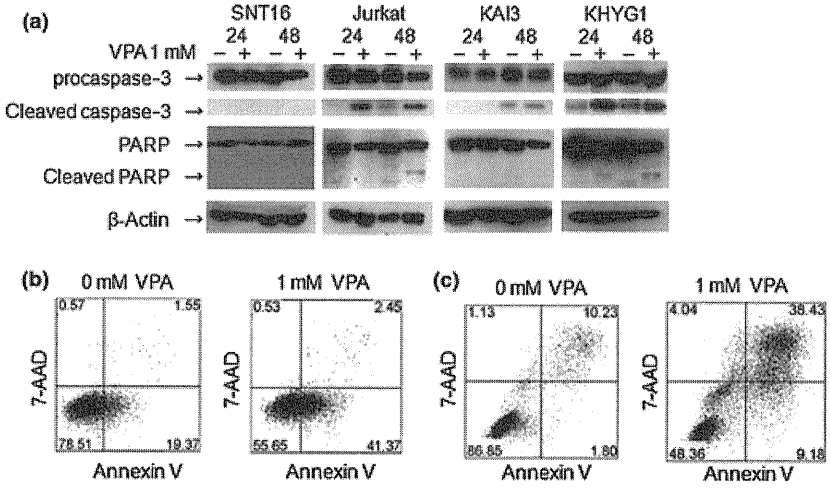

Fig. 2. Effects of valproate (VPA) on apoptosis. (a) T and natural killer (NK) cell lines were treated with 1 mM VPA for 24 or 48 h. β-Actin was used as a loading control. Valproate induced the cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP) in Jurkat and KHYG1 cells. (b,c) T and NK cell lines were treated with 1 mM VPA for 48 h. Viable cells were defined as those negative for annexin V–phycoerythrin (PE) and 7-amino-actinomycin D (7-AAD). (b) The number of early apoptotic SNT16 cells, defined as those positive for annexin V-PE and negative for 7-AAD, was increased, as was (c) the numbers of dead KHYG1 cells, defined as those positive for both annexin V-PE and 7-AAD.

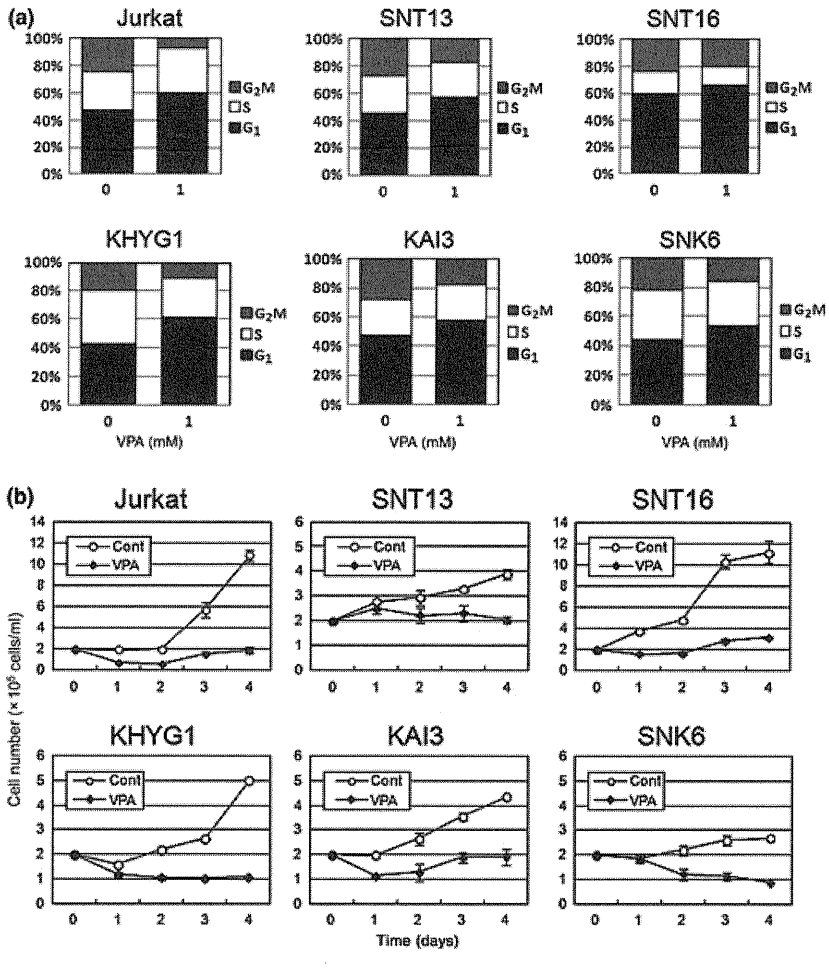

Fig. 3. Effects of valproate (VPA) on the cell cycle. (a) T cell lines (SNT13, SNT16, and Jurkat), and natural killer (NK) cell lines (KAI3, SNK6, and KHYG1) were treated with 1 mM VPA or distilled water for 48 h, fixed, and stained with propidium iodide. Cell cycle profiles were assessed by flow cytometry. (b) Cells were treated with 1 mM VPA or distilled water (control) and viable cells were counted using the Trypan blue exclusion test. Experiments were performed in duplicate. Data are the mean ± SEM.

to VPA and VPA arrested the cell cycle in all T and NK cell lines tested (Fig. 3a). To confirm that VPA arrested the cell cycle, proliferation was compared in the presence and absence of VPA. Proliferation was inhibited in all VPA-treated cells compared with control cells (Fig. 3b).

Effects of VPA on lytic infection of EBV-positive T and NK cell lines. The expression of the following eight viral genes were analyzed using real-time RT-PCR: lytic genes encoding BZLF1 and gp350/220; and latent genes encoding EBV nuclear antigen (EBNA) 1, EBNA2, latent membrane protein (LMP) 1, LMP2, EBER1, and *Bam*HI-A rightward transcripts (BARTs). BZLF1, but not gp350/220, was detected in the T cell lines. Conversely, neither BZLF1 nor gp350/220 were detected in the NK cell lines (Fig. 4). The expression of the two lytic genes and six latent genes did not differ significantly between VPA-treated cells and controls. Representative results for two latent genes (those encoding LMP1 and EBER1) are shown in Figure 4.

Effects of the combination of VPA and bortezomib on cell death. Because the antitumor effect of VPA alone was modest (1 mM VPA treatment for 96 h reduced cell viability by between 22% and 56%) (Fig. 1b–e), we evaluated the effects of the combination of VPA (1 mM) and the proteasome inhibitor bortezomib (0.01 μM) in several cell lines. In Jurkat and KAI3 cells, the combination of VPA plus bortezomib enhanced cell death (Fig. 5); however, in SNT16 and KHYG1 cells, the effects of this combination were difficult to assess because 0.01 μM bortezomib alone killed almost all the cells (Fig. 5).

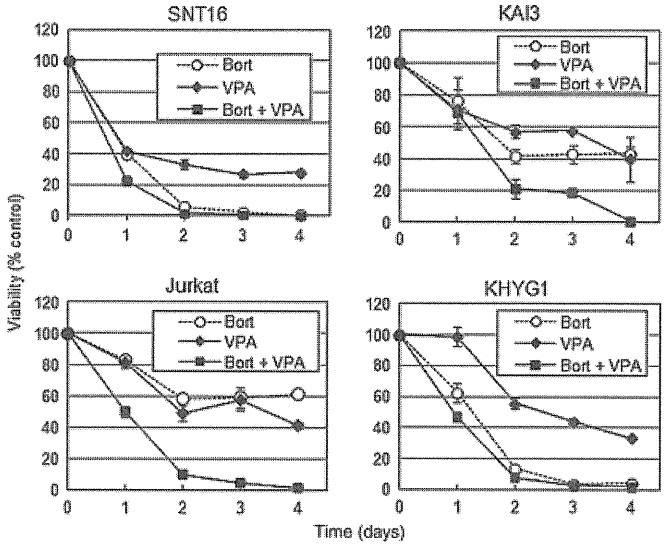

Fig. 5. Combined effects of valproate (VPA) and bortezomib. T cell lines (SNT16 and Jurkat) and natural killer (NK) cell lines (KAI3 and KHYG1) were treated with 1 mM VPA and/or 0.01 μM bortezomib for 96 h and cell viability was assessed. VPA and bortezomib had additive effects in reducing the viability of T and NK cell lines. Data are the mean ± SEM.

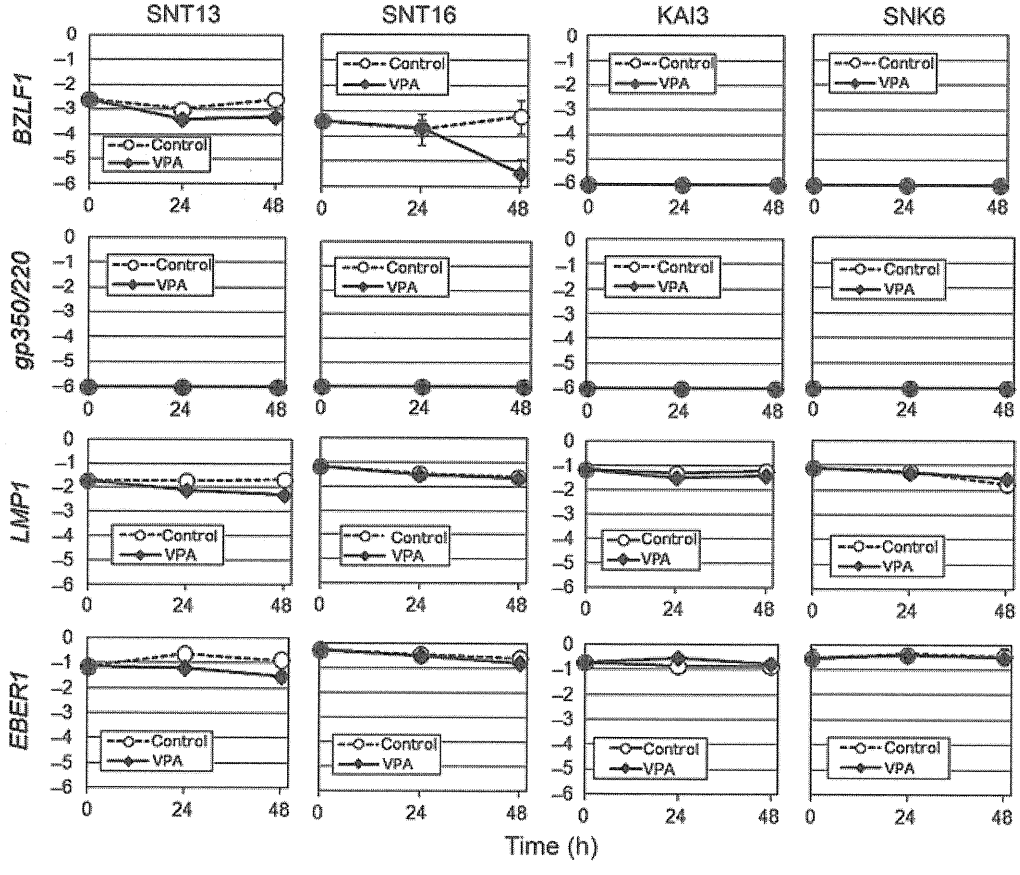

Fig. 4. Effects of valproate (VPA) treatment on the expression of Epstein–Barr virus (EBV)-encoded genes. The EBV-positive T cell lines (SNT13 and SNT16) and EBV-positive natural killer (NK) cell lines (KAI3 and SNK6) were treated with 1 mM VPA and harvested at 0, 24, and 48 h to evaluate gene expression using real-time RT-PCR. *BZLF1* is an immediate early gene and *gp350/220* is a late gene. *LMP1* and *EBER1* are latent genes. β2-Microglobulin was used as an internal control and reference gene for relative quantification and assigned an arbitrary value of 1 (10^0). Data are the mean ± SEM.

doi: 10.1111/j.1349-7006.2011.02127.x

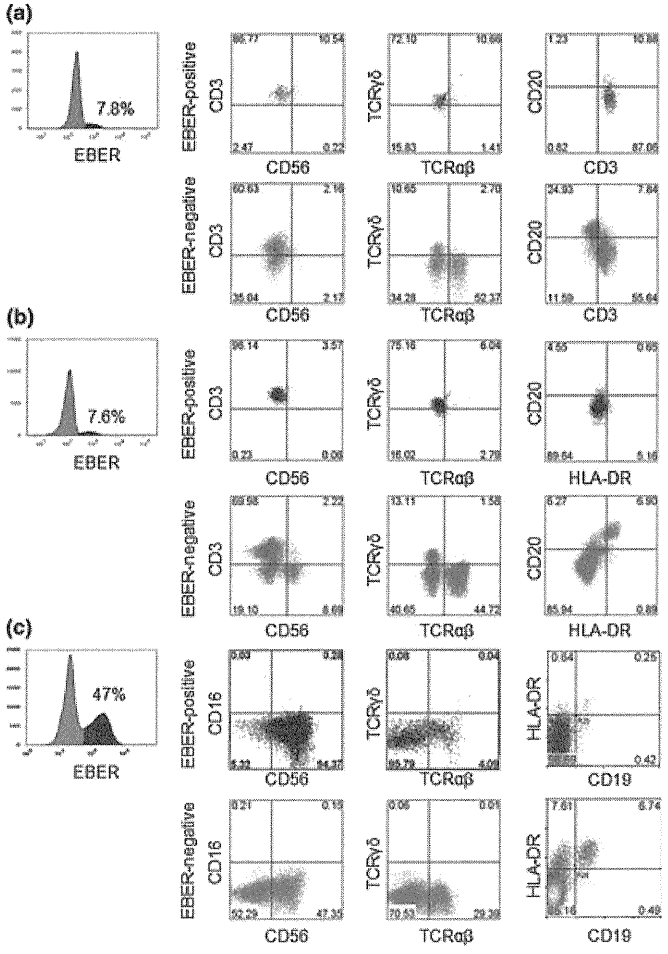

Fig. 6. Identification of Epstein–Barr virus (EBV)-infected cell fractions in patients with EBV-associated T/natural killer (NK) lymphoma. (a, b) Patients T-1 (a) and T-2 (b), who had hydroa vacciniforme-like lymphoma. (c) Patient NK-1, who had chronic active EBV disease, NK cell-type. Mononuclear cells were analyzed in a FISH assay. The EBV-encoded small RNA (EBER)-positive (black) and -negative (gray) lymphocytes were gated and plotted in quadrants.

Effects of VPA on the viability of EBV-infected cells from patients with EBV-associated lymphoma. The *ex vivo* effect of VPA on lymphoma cells from patients with EBV-associated T/NK lymphoma or lymphoproliferative diseases was evaluated. To identify the fractions that contained EBV-infected cells, MNC were stained with surface marker antibodies and then subjected to *in situ* hybridization with EBER in a FISH assay. In patients T-1 and T-2, who had hydroa vacciniforme-like lymphoma, the FISH assay showed that 7.8% and 7.6% of MNC were EBER positive, respectively. Most of the EBER-positive MNC in these patients were CD3+ and TCRγδ+ T cells (Fig. 6a,b). Conversely, in patient NK-1, who had chronic active EBV disease, 47% of MNC were EBER positive. Most of the EBER-positive MNC in this patient were CD56+ NK cells (Fig. 6c). Magnetic sorting was then used to separated γδT cells from other MNC in patients T-1 and T-2, and NK cells from the other MNC in patient NK-1. Bortezomib (0.5 μM) and/or VPA (1 mM) was administered to each fraction and viable cells were counted over a period of 3–4 days. Individually, bortezomib and VPA had greater killing effects on the fractions containing EBV-infected cells compared with the other MNC, whereas the combination of bortezomib plus VPA produced the strongest killing effect (Fig. 7a–c). In the γδT and NK cell fractions, the absolute number of control viable cells was stable or increased slightly, but was reduced by treatment (data not shown). The viability of cells obtained from blood samples from three healthy donors after combined treatment with bortezomib plus VPA for 4 days ranged between 75% and 100%, indicating that bortezomib and VPA do not affect non-tumor cells (Fig. 7d).

Discussion

Several studies have reported that HDAC inhibitors have anticancer activities and some have even been tested in clinical trials.[32–34] Valproate is used to treat epilepsy, is easily accessible, and has a well-established safety profile. Therefore, evaluation of an anticancer effect of VPA may be very useful in the treatment of malignant diseases. In the present study, VPA reduced the viability of T and NK lymphoma/leukemia cell lines independently of the presence of EBV. However, the killing effect of VPA was smaller than that of bortezomib, despite the fact that the concentration of VPA tested (1 mM) was higher than that used in the treatment of epilepsy (0.3–0.6 mM).

The HDAC inhibitors affect tumor cell growth and survival via multiple biological effects. For example, they induce tumor cell death with all the biochemical and morphological characteristics of apoptosis. The HDAC inhibitors induce cell cycle arrest at the G_1/S boundary via upregulation of *CDKN1A*, which encodes $p21^{WAF1/CIP1}$, and/or downregulation of cyclins. They can suppress angiogenesis by reducing the expression of proangiogenic factors and also have immunomodulatory effects, enhancing tumor cell antigenicity and altering the expression of key cytokines, including tumor necrosis factor-α, interleukin-1, and interferon-γ.[9] In the present study, we analyzed the mechanism by which VPA reduces the viability of T and NK cell lines. In some cell lines, VPA induced apoptosis, whereas in most there was evidence of cell cycle arrest. Thus, VPA probably activates other pathways to kill tumor cells than apoptosis and cell cycle arrest.

The proteasome inhibitor bortezomib has strong killing effects on T and NK lymphoma/leukemia cell lines (independent of the presence of EBV) and EBV-infected tumor cells from patients with EBV-associated T/NK lymphoproliferative diseases.[14] Bortezomib is used in the treatment of myeloma and has also been assessed for efficacy against a variety of other malignancies. Recently, bortezomib and an HDAC inhibitor were reported to have synergistic effects in human and mouse models.[35,36] Therefore, in the present study we evaluated the effects of the combination of bortezomib and VPA. Bortezomib and VPA were found to have additive killing effects on T and NK cell lines and EBV-infected MNC from patients. In the two cell lines tested, the effect of bortezomib was too strong to evaluate the killing effect of the combination treatment, despite the low bortezomib concentration used. Conversely, in Jurkat and KAI3, in which a low concentration of bortezomib killed approximately half the cells, the combination treatment killed nearly all cells within 4 days. Furthermore, the combination treatment had a stronger killing effect in EBV-infected MNC from patients than in uninfected cells. These results suggest the potential usefulness of the combination of VPA and bortezomib in the treatment of EBV-associated T/NK lymphoproliferative diseases.

Valproate has been reported to induce lytic infection by EBV,[12,13] human cytomegalovirus,[37] and Kaposi sarcoma-associated herpes virus.[38] Induction of the lytic cycle is an advantage for the treatment of EBV-associated malignant diseases because of the lysis of EBV-infected tumor cells, the possible availability of antiviral therapy, and the recognition of expressed viral lytic proteins by the host immune system. Furthermore, the combination of VPA and an antiviral drug may

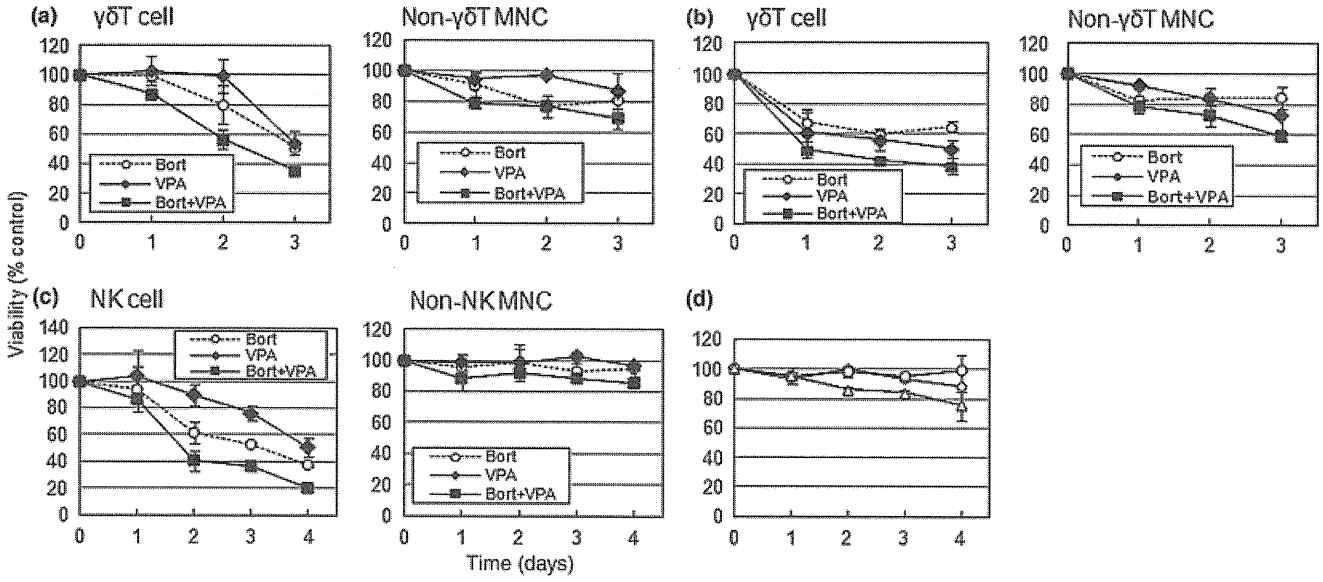

Fig. 7. Effects of the combination of valproate (VPA) and bortezomib on Epstein–Barr virus (EBV)-infected lymphoma cells. Cell populations were separated by magnetic sorting. Each fraction was exposed to VPA (1 mM) and/or bortezomib (0.5 μM) and viable cells were counted over 3 or 4 days. (a,b) Viability of γδT cells and other mononuclear cells (MNC) from patients T-1 (a) and T-2 (b) with hydroa vacciniforme-like lymphoma. (c) Viability of NK cells and other MNC from patient NK-1 with chronic active EBV disease, NK cell-type. (d) Viability of MNC from three healthy donors treated with 1 mM VPA and 0.5 μM bortezomib for 4 days. Data are the mean ± SEM.

increase cell killing because some antiviral drugs inhibit virus DNA polymerase and are more effective in the lytic state than in the latent state.[39] To our knowledge, this is the first report of the effects of VPA on T and NK cell lines. In previous studies showing that VPA induces the EBV lytic cycle, a gastric carcinoma cell line and B cell lines were used.[12,13] In the present study, VPA did not induce the EBV lytic cycle in any of the T or NK cell lines tested. In the two EBV-positive T cell lines tested, expression of only the immediate early gene *BZLF1* was detected (expression of the late gene *gp350/220* was not detected). In the NK cell lines, the expression of neither gene was detected. These results are consistent with our previous report.[23] In addition, bortezomib only induced the EBV lytic cycle in EBV-positive T cell lines.[23] Therefore, it seems that lytic infection can be induced in EBV-positive T cell lines. Nevertheless, VPA treatment did not induce lytic infection in EBV-positive T cell lines in the present study.

In summary, the results of the present study suggest that VPA has potential antitumor activity, regardless of whether EBV is present, although its efficacy may not be sufficient. The combination of VPA plus bortezomib may be a useful treatment because of the potential synergistic effects. Our results indicate that VPA has killing effects on T and NK lymphoma cells. Other HDAC inhibitors, such as suberoylanilide hydroxamic acid and depsipeptide, have potent activity against T cell lymphoma[40] and may produce beneficial effects in EBV-associated malignancies by inducing the lytic cycle or suppressing the expression of EBV-related genes.[13]

Acknowledgments

The authors thank Shigeyoshi Fujiwara (National Research Institute for Child Health and Development, Tokyo, Japan) for the MT2/hyg and MT2/rEBV/9-7 cell lines and Koichi Sugimoto and Yasushi Isobe (Juntendo University, Tokyo, Japan) for the NKL and TL1 cell lines. The KAI3 and KHYG1 cells were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). The authors thank Millennium Pharmaceuticals (Cambridge, MA, USA) for providing the bortezomib. This study was supported, in part, by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (19591247) and by a grant for Research on Measures for Emerging and Reemerging Infections (Intractable Infectious Diseases in Organ Transplant Recipients, H21-Shinko-Ippan-094) from the Ministry of Health, Labor, and Welfare of Japan.

Disclosure Statement

The authors have no conflict of interest.

References

1 Cohen JI. Epstein–Barr virus infection. *N Engl J Med* 2000; **343**: 481–92.
2 Quintanilla-Martinez L, Kimura H, Jaffe ES. EBV+ T-cell lymphoma of childhood. In: Jaffe ES, Harris NL, Stein H, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, 4th edn. Lyon: IARC Press, 2008; 278–80.
3 Rickinson AB, Kieff E. Epstein–Barr virus. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia: Lippincott Williams & Wilkins, 2007; 2655–700.
4 Williams H, Crawford DH. Epstein–Barr virus: the impact of scientific advances on clinical practice. *Blood* 2006; **107**: 862–9.
5 Kieff ED, Rickinson AV. Epstein–Barr virus and its replication. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia: Lippincott Williams & Wilkins, 2007; 2603–54.
6 Cartron G, Watier H, Golay J, Solal-Celigny P. From the bench to the bedside: ways to improve rituximab efficacy. *Blood* 2004; **104**: 2635–42.
7 Heslop HE. How I treat EBV lymphoproliferation. *Blood* 2009; **114**: 4002–8.
8 Detich N, Bovenzi V, Szyf M. Valproate induces replication-independent active DNA demethylation. *J Biol Chem* 2003; **278**: 27586–92.
9 Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discov* 2006; **5**: 769–84.
10 Kawagoe R, Kawagoe H, Sano K. Valproic acid induces apoptosis in human leukemia cells by stimulating both caspase-dependent and -independent apoptotic signaling pathways. *Leuk Res* 2002; **26**: 495–502.
11 Takai N, Desmond JC, Kumagai T *et al.* Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. *Clin Cancer Res* 2004; **10**: 1141–9.

doi: 10.1111/j.1349-7006.2011.02127.x

12 Feng WH, Kenney SC. Valproic acid enhances the efficacy of chemotherapy in EBV-positive tumors by increasing lytic viral gene expression. *Cancer Res* 2006; **66**: 8762–9.
13 Hui KF, Chiang AK. Suberoylanilide hydroxamic acid induces viral lytic cycle in Epstein–Barr virus-positive epithelial malignancies and mediates enhanced cell death. *Int J Cancer* 2010; **126**: 2479–89.
14 Iwata S, Yano S, Ito Y *et al.* Bortezomib induces apoptosis in T lymphoma cells and natural killer lymphoma cells independent of Epstein–Barr virus infection. *Int J Cancer* 2011; **129**: 2263–73.
15 Zhang Y, Nagata H, Ikeuchi T *et al.* Common cytological and cytogenetic features of Epstein–Barr virus (EBV)-positive natural killer (NK) cells and cell lines derived from patients with nasal T/NK-cell lymphomas, chronic active EBV infection and hydroa vacciniforme-like eruptions. *Br J Haematol* 2003; **121**: 805–14.
16 Tsuge I, Morishima T, Morita M, Kimura H, Kuzushima K, Matsuoka H. Characterization of Epstein–Barr virus (EBV)-infected natural killer (NK) cell proliferation in patients with severe mosquito allergy; establishment of an IL-2-dependent NK-like cell line. *Clin Exp Immunol* 1999; **115**: 385–92.
17 Kaplan J, Tilton J, Peterson WD Jr. Identification of T cell lymphoma tumor antigens on human T cell lines. *Am J Hematol* 1976; **1**: 219–23.
18 Yagita M, Huang CL, Umehara H *et al.* A novel natural killer cell line (KHYG-1) from a patient with aggressive natural killer cell leukemia carrying a p53 point mutation. *Leukemia* 2000; **14**: 922–30.
19 Miyoshi I, Kubonishi I, Yoshimoto S *et al.* Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. *Nature* 1981; **294**: 770–1.
20 Fujiwara S, Ono Y. Isolation of Epstein–Barr virus-infected clones of the human T-cell line MT-2: use of recombinant viruses with a positive selection marker. *J Virol* 1995; **69**: 3900–3.
21 Robertson MJ, Cochran KJ, Cameron C, Le JM, Tantravahi R, Ritz J. Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia. *Exp Hematol* 1996; **24**: 406–15.
22 Isobe Y, Sugimoto K, Matsuura I, Takada K, Oshimi K. Epstein–Barr virus renders the infected natural killer cell line, NKL resistant to doxorubicin-induced apoptosis. *Br J Cancer* 2008; **99**: 1816–22.
23 Iwata S, Wada K, Tobita S *et al.* Quantitative analysis of Epstein–Barr virus (EBV)-related gene expression in patients with chronic active EBV infection. *J Gen Virol* 2010; **91**: 42–50.
24 Kubota N, Wada K, Ito Y *et al.* One-step multiplex real-time PCR assay to analyse the latency patterns of Epstein–Barr virus infection. *J Virol Methods* 2008; **147**: 26–36.
25 Patel K, Whelan PJ, Prescott S *et al.* The use of real-time reverse transcription-PCR for prostate-specific antigen mRNA to discriminate between blood samples from healthy volunteers and from patients with metastatic prostate cancer. *Clin Cancer Res* 2004; **10**: 7511–9.
26 Kimura H, Miyake K, Yamauchi Y *et al.* Identification of Epstein–Barr virus (EBV)-infected lymphocyte subtypes by flow cytometric in situ hybridization in EBV-associated lymphoproliferative diseases. *J Infect Dis* 2009; **200**: 1078–87.
27 Kimura H, Hoshino Y, Hara S *et al.* Differences between T cell-type and natural killer cell-type chronic active Epstein–Barr virus infection. *J Infect Dis* 2005; **191**: 531–9.
28 Kimura H, Hoshino Y, Kanegane H *et al.* Clinical and virologic characteristics of chronic active Epstein–Barr virus infection. *Blood* 2001; **98**: 280–6.
29 Kimura H, Morishima T, Kanegane H *et al.* Prognostic factors for chronic active Epstein–Barr virus infection. *J Infect Dis* 2003; **187**: 527–33.
30 Cohen JI, Kimura H, Nakamura S, Ko YH, Jaffe ES. Epstein–Barr virus-associated lymphoproliferative disease in non-immunocompromised hosts: a status report and summary of an international meeting, 8–9 September 2008. *Ann Oncol* 2009; **20**: 1472–82.
31 Kimura H. Pathogenesis of chronic active Epstein–Barr virus infection: is this an infectious disease, lymphoproliferative disorder, or immunodeficiency? *Rev Med Virol* 2006; **16**: 251–61.
32 Fakih MG, Fetterly G, Egorin MJ *et al.* A Phase I, pharmacokinetic, and pharmacodynamic study of two schedules of vorinostat in combination with 5-fluorouracil and leucovorin in patients with refractory solid tumors. *Clin Cancer Res* 2010; **16**: 3786–94.
33 Kirschbaum M, Frankel P, Popplewell L *et al.* Phase II Study of vorinostat for treatment of relapsed or refractory indolent non-Hodgkin's lymphoma and mantle cell lymphoma. *J Clin Oncol* 2011; **29**: 1198–203.
34 Niesvizky R, Ely S, Mark T *et al.* Phase 2 trial of the histone deacetylase inhibitor romidepsin for the treatment of refractory multiple myeloma. *Cancer* 2011; **117**: 336–42.
35 Jagannath S, Dimopoulos MA, Lonial S. Combined proteasome and histone deacetylase inhibition: a promising synergy for patients with relapsed/refractory multiple myeloma. *Leuk Res* 2010; **34**: 1111–8.
36 Kawada J, Zou P, Mazitschek R, Bradner JE, Cohen JI. Tubacin kills Epstein–Barr virus (EBV)–Burkitt lymphoma cells by inducing reactive oxygen species and EBV lymphoblastoid cells by inducing apoptosis. *J Biol Chem* 2009; **284**: 17102–9.
37 Kuntz-Simon G, Obert G. Sodium valproate, an anticonvulsant drug, stimulates human cytomegalovirus replication. *J Gen Virol* 1995; **76**: 1409–15.
38 Klass CM, Krug LT, Pozharskaya VP, Offermann MK. The targeting of primary effusion lymphoma cells for apoptosis by inducing lytic replication of human herpesvirus 8 while blocking virus production. *Blood* 2005; **105**: 4028–34.
39 Bazarbachi A, Suarez F, Fields P, Hermine O. How I treat adult T-cell leukemia/lymphoma. *Blood* 2011; **118**: 1736–45.
40 Zain JM, O'Connor O. Targeted treatment and new agents in peripheral T-cell lymphoma. *Int J Hematol* 2010; **92**: 33–44.

INTERNAL MEDICINE

□ CASE REPORT □

Recurrence of Chronic Active Epstein-Barr Virus Infection from Donor Cells after Achieving Complete Response Through Allogeneic Bone Marrow Transplantation

Ayako Arai[1], Ken-Ichi Imadome[2], Ludan Wang[1], Nan Wu[1], Tetsuya Kurosu[1], Atsushi Wake[3], Hisashi Yamamoto[3], Yasunori Ota[4], Masayoshi Harigai[5], Shigeyoshi Fujiwara[2] and Osamu Miura[1]

Abstract

We report the case of a 35-year-old woman with chronic active Epstein-Barr virus (EBV) infection (CAEBV). She underwent allogeneic bone marrow transplantation (BMT) from an unrelated male donor and achieved a complete response. However, her CAEBV relapsed one year after BMT. EBV-infected cells proliferated clonally and revealed a 46XY karyotype. In addition, the infecting EBV strain differed from that detected before BMT. These findings indicated that her disease had developed from donor cells. This is the first report of donor cell-derived CAEBV that recurred after transplantation, suggesting that host factors may be responsible for the development of this disease.

Key words: chronic active Epstein-Barr virus infection, bone marrow transplantation, systemic lupus erythematosus

(Intern Med 51: 777-782, 2012)
(DOI: 10.2169/internalmedicine.51.6769)

Introduction

Epstein-Barr virus (EBV) can be detected not only in B-cell tumors but also in T- and NK-cell tumors, which are known as EBV-positive T/NK-cell lymphoproliferative diseases (EBV-T/NK-LPDs). EBV-T/NK-LPDs comprise extranodal NK/T-cell lymphoma nasal type (ENKL), aggressive NK-cell leukemia, and chronic active EBV infection (CAEBV). CAEBV is a rare disorder accompanied by the clonal proliferation of EBV-infected cells (1). Its T-cell infecting type is designated as "EBV-positive T-cell lymphoproliferative disease of childhood" in the WHO classification revised in 2008 (2). However, adult-onset cases have been reported (3, 4).

The pathogenesis of CAEBV is assumed to be due to the EBV infection of T or NK cells followed by their immortalization and expansion. However, the mechanisms responsible for the clonal expansion of infected cells remain unclear.

We report here the case of CAEBV in a female patient. In spite of achieving a complete response (CR) after bone marrow transplantation (BMT), CAEBV recurred. At recurrence, the infected cells were clonally proliferating donor cells, and the infecting virus differed from that originally causing the disease. We describe her clinical course and discuss the possible pathological mechanism responsible for the recurrence.

Methods

The detection and isolation of infected cells (5) and sequence analysis for *perforin* (6) were performed as de-

[1]Department of Hematology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Japan, [2]Department of Infectious Diseases, National Research Institute for Child Health and Development, Japan, [3]Department of Hematology, Toranomon Hospital, Japan, [4]Department of Pathology, Toranomon Hospital, Japan and [5]Department of Pharmacovigilance, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Japan
Received for publication October 18, 2011; Accepted for publication December 12, 2011
Correspondence to Dr. Ayako Arai, ara.hema@tmd.ac.jp

scribed previously. For the sequence analysis of the variable region of *lmp1*, the genomic DNA extracted from infected cells was amplified by PCR. The following primers were used: 5-AAGGGAGTGTGTGCCATTAAG-3 (fwd) and 5-A CCCCCACTCTGCTCTCAA-3 (rev); their nucleotide positions in B95.8 (Genbank No.V01555) were 168052-168073 and 168619-168601, respectively. The conditions for PCR reactions were as follows: 94℃ for 5 minutes, 94℃ for 15 seconds, 60℃ for 30 seconds, and 72℃ for 60 seconds; 35 cycles. The amplicon was directly sequenced using the same primers. The ethics committee of Tokyo Medical and Dental University Hospital approved this study, and written informed consent was obtained from the patient.

Case Report

A 35-year-old woman developed fever and cervical lymphadenopathy and was transferred to our hospital. She had systemic lupus erythematosus (SLE) for the previous 13 years and was receiving prednisolone (PSL) at 5 mg/day. Her anti-EBV antibodies on admission were 1:20,480 for anti-VCA-IgG and 1:1,280 for anti-EA-DRIgG, which were extremely elevated. Anti-VCA-IgM was undetectable, and the titer of anti-EBNA was 1:40. EBV DNA copy numbers in peripheral blood (PB) were elevated to 1×10^7 copies/μg DNA.

EBV-positive T-cell lymphoproliferative disease was diagnosed by cervical lymph node biopsy (Fig. 1A). Infiltrating cells were positive for CD8, Granzyme B, and EBER (Fig. 1B-D). In addition, activated $CD8^+$ cells were increased in the PB (Fig. 1E). These cells were EBV-positive; they were clonally similar to those in the lymph node, which involved a *TCRJβ1* gene rearrangement (Fig. 1F-H).

Chemotherapy was administered followed by BMT as described by Koyama et al (7). The donor was a 29-year-old unrelated male. His HLA type was A2 (0207) / A26 (2602), B46 (1501) / B62 (4601), and DR14 (1403) / DR14 (1406). The patient's type was A2 (0207) / A26 (2602), B46 (1501) / B62 (4601), and DR14 (1401) / DR14 (1401). Their serologic HLA types were identical, whereas the DNA types displayed disparities in 2 HLA-DR alleles.

The conditioning regimen for transplantation comprised fludarabine (37.5 mg/m^2 intravenously, once daily from days -6 to -2), melphalan (60 mg/m^2 intravenously, once daily from days -6 to -5), and total body irradiation (4 Gy in 2 fractions on day -1). Cyclosporine (3 mg/kg, from day -1) and short-term methotrexate (5 mg, 10 mg, and 10 mg on days 1, 3, and 6, respectively) were administered for the prophylaxis of acute graft-versus-host disease. Engraftment was confirmed 1 month after BMT, and the EBV genome in PB became undetectable after 2 months and remained so for nearly 12 months.

Although graft-versus-host disease had not developed, administration of low-dose corticosteroid (hydrocortisone, 10 mg/day) was continued to compensate for her endogenous cortisol deficiency due to the long-term administration of PSL. One year later, her EBV DNA level began to increase and reached 1.7×10^4 copies/μg DNA. Three years after BMT, it was 1.0×10^5 copies/μg DNA, and the number of CD8-positive cells had increased among her PB mononuclear cells (PBMC; Fig. 2A).

Infected cells in PB were investigated again; these were identified as CD8-positive T cells. Their clonality was confirmed by detecting a *TCRJβ1* gene rearrangement, which revealed a difference from the original (Fig. 1I). EBV-infected cells (Fig. 2B) and a lymphoblastoid cell line (LCL) established from the patient's PBMC soon after engraftment (Fig. 2C) had XY karyotype, confirming that these were donor cells. Furthermore, sequence analysis of the variable region of *lmp1* showed that the infecting virus differed from that detected in CD8-positive cells before BMT and was identical to that detected in LCL (Fig. 2D). Although we did not examine whether the donor was seropositive for EBV, the virus obtained from LCL might have been of donor origin.

Liver dysfunction developed gradually 4 years after BMT. Liver biopsy was performed, and a significant sinusoidal infiltration of atypical cells (CD8- and EBV-positive) was detected (Fig. 3A-C). Her PBMC retained the 46XY karyotype (Fig. 3D) and mainly comprised activated CD8-positive cells. In addition, CD4-positive cells were detected (Fig. 3E).

The EBV DNA copy numbers, the chimerisms of nucleated cells and lymphocytes, and the percentage of CD4- and CD8-positive cells in peripheral blood are summarized in Table 1. The chimerism maintained the donor type during the clinical course. An abnormal XXYY clone suggesting donor origin appeared 4.5 years after BMT as the disease progressed. From these results, the diagnosis of CAEBV, which developed from donor cells infected with a different virus, was confirmed.

Discussion

The mechanisms responsible for CAEBV development have not been elucidated. Some investigators reported that EBV-infected T or NK cells could be detected during primary infection (8, 9), indicating that EBV could infect these cells under a high level of viral load. However, some factors leading to disease development may exist because CAEBV shows a marked geographic preference for East Asia. Although the strains identified in the present patient before and after BMT were not identical, the relationship between strains and disease development needs to be investigated. In addition, a patient's genetic background may be involved. In our patient, recurrence after BMT underlines the importance of non-hematological factors for disease development.

According to Ohshima et al, following infection with EBV, T, or NK cells can undergo poly-, oligo-, or monoclonal expansion, resulting in CAEBV (10). For the expansion of EBV-infected T or NK cells, suppression of cytotoxic T-cell (CTL) activity may play an important role. Sugaya et al

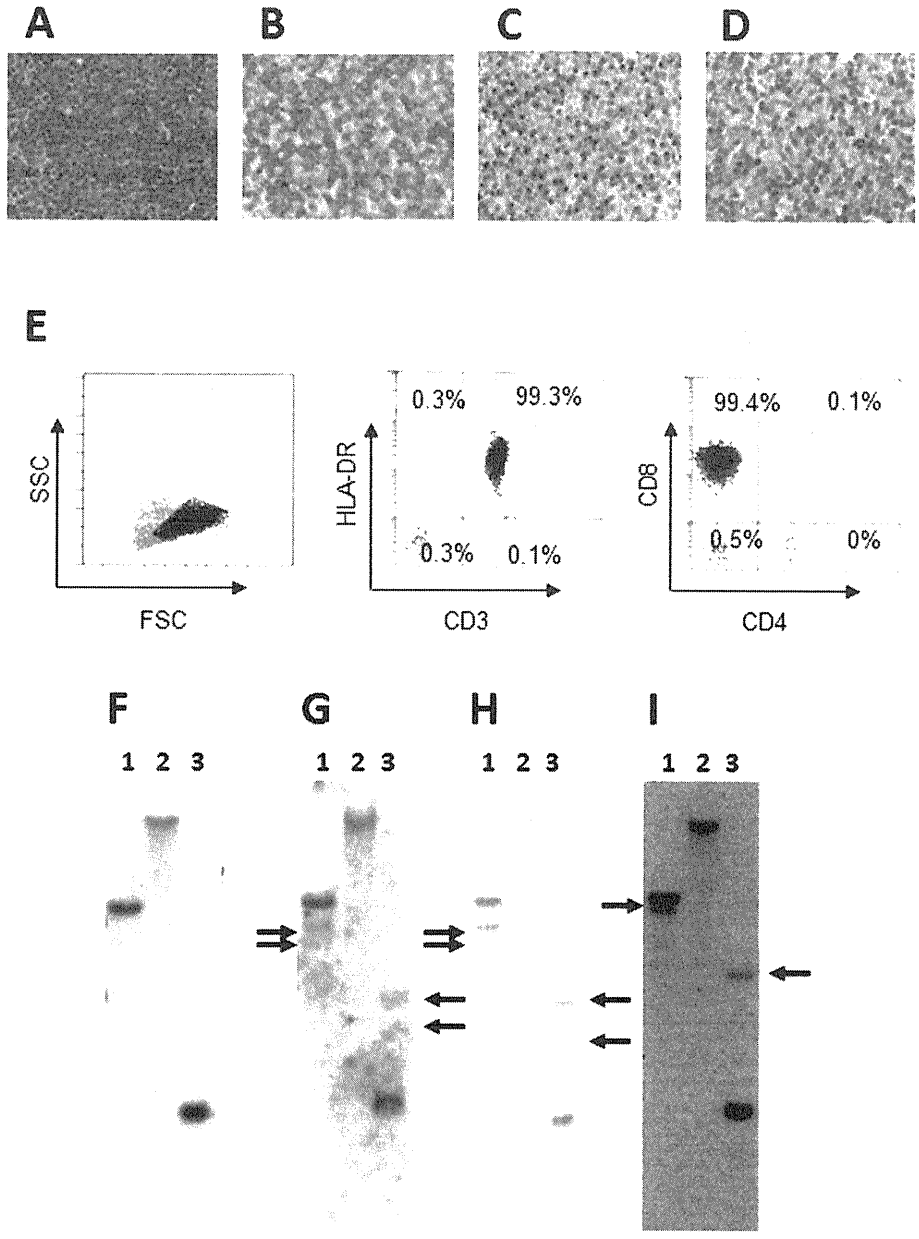

Figure 1. Analysis of Epstein-Barr virus-infected cells at the onset of chronic active EBV infection. A-D: Biopsy specimens of a cervical lymph node (original magnification, ×200). A: Hematoxylin and Eosin staining shows diffuse infiltration of atypical cells. B: Stained with the anti-CD8 antibody. C: Stained with the anti-granzyme B antibody. D: *In situ* hybridization of Epstein-Barr virus-encoded mRNA. Neoplastic cells were positive for CD3 and CD5; these cells were negative for CD4, CD20, and CD56 (data not shown). E: Analysis of peripheral blood mononuclear cells by flow cytometry at disease onset. (F-I) Southern blot analysis for *T-cell receptor Jβ1 gene.* After digestion with *EcoR*I (1), *BamH*I (2), and *Hind*III (3), DNA was analyzed to detect gene rearrangements. Arrows show rearranged bands. F: Negative control. G: DNA extracted from peripheral blood (PB) at disease onset. H: DNA extracted from a cervical lymph node at disease onset. I: DNA extracted from PB at recurrence.

demonstrated suppressed EBV-specific CTL activity in CAEBV patients using human leukocyte antigen (HLA)-A* 2402-restricted tetramers (11). In addition, Katano et al reported that mutations in both alleles of the *perforin* gene, which is indispensable for CTL activity, resulted in its reduced expression and could play a role in CAEBV development (12).

However, we were unable to detect *perforin* gene mutations in CAEBV cells from the present patient (data not shown). We previously reported suppressed CTL activity against EBV-infected B cells in an EBV-B-LPD patient who had been administered low-dose PSL for more than 7 years (13). The present patient and one in another report who had SLE developed CAEBV during PSL administration (14). Thus, PSL, even at low doses, may suppress CTL activity and trigger disease development.

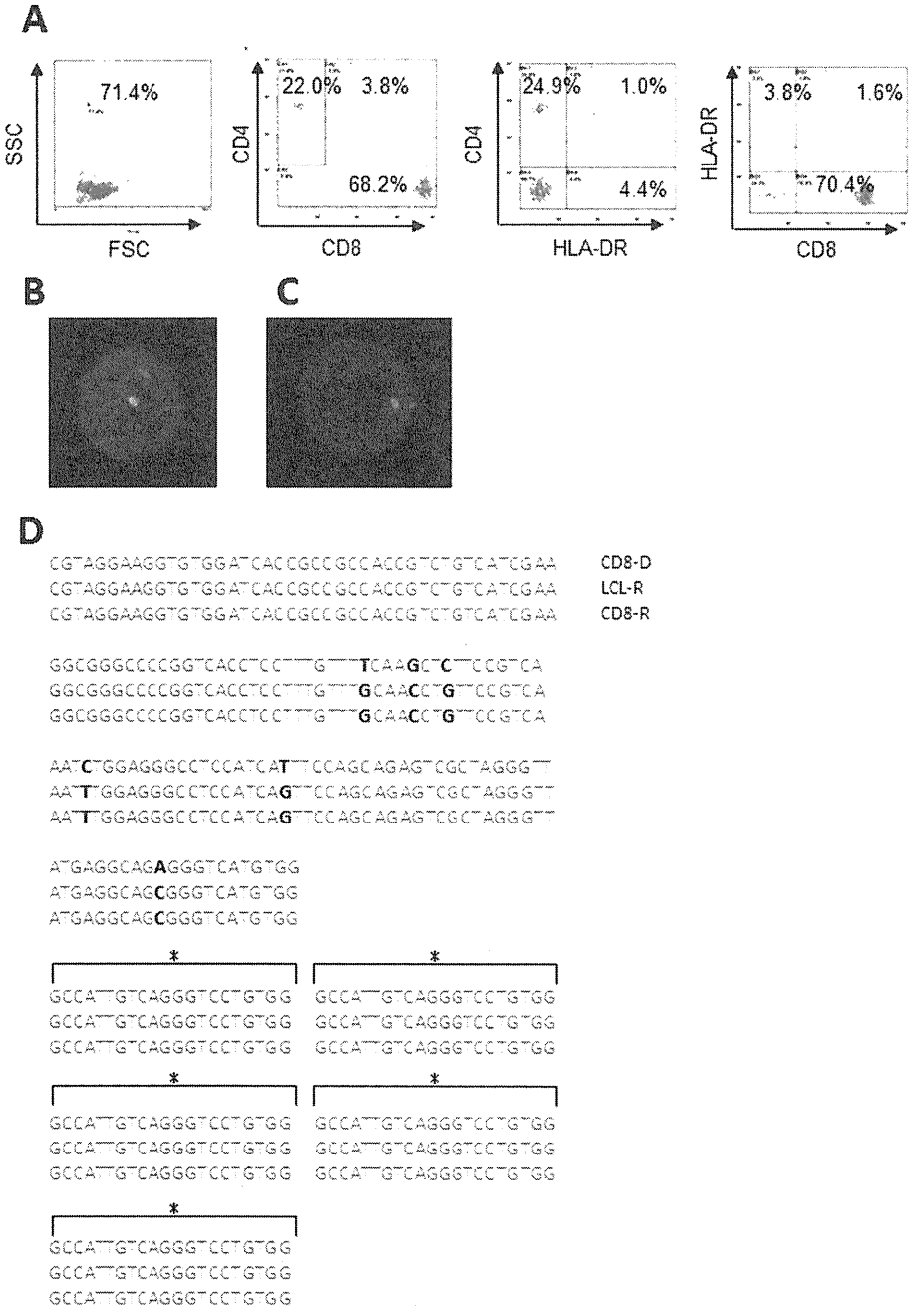

Figure 2. Analysis of Epstein-Barr virus-infected T cells 3 years after BMT at recurrence of chronic active EBV infection. A: Analysis of peripheral blood mononuclear cells by flow cytometry at the diagnosis of recurrence. B, C: Fluorescence *in situ* hybridization (FISH) analysis. Red and green signals indicate X and Y chromosomes, respectively. B: Lymphoblastoid cell line (LCL) established from patient's PBMC soon after engraftment. The XY signal was positive in 96.8% of cells and was considered to be of donor origin. EBV-DNA titer, 1.4×10^6 copies/μg DNA. C: CD8-positive cells from PB at recurrence. The XY signal was positive in 98.4% of CD8-positive cells. EBV-DNA titer, 2.4×10^6 copies/μg DNA. D: *Lmp1* sequence analysis of CD8-positive T cells at diagnosis (CD8-D, upper lane) of LCL, established from patient's PBMC soon after engraftment (LCL-R, middle lane), and of CD8-positive T cells at recurrence (CD8-R, lower lane). The first nucleotide corresponds to nucleotide No. 168238 of B95.8 (Genbank No.V01555). Asterisks indicate repeat regions; black letters indicate distinctive nucleotides.

EBV itself can contribute to the clonal proliferation of infected T or NK cells. NF-kB was constitutively activated in EBV-infected T or NK cells derived from CAEBV patients and protected them from VP-16-induced apoptosis, suggesting that EBV infection of T or NK cells could directly contribute to their immortalization (15). However, EBV-induced immortalization of infected cells may be insufficient for CAEBV development.

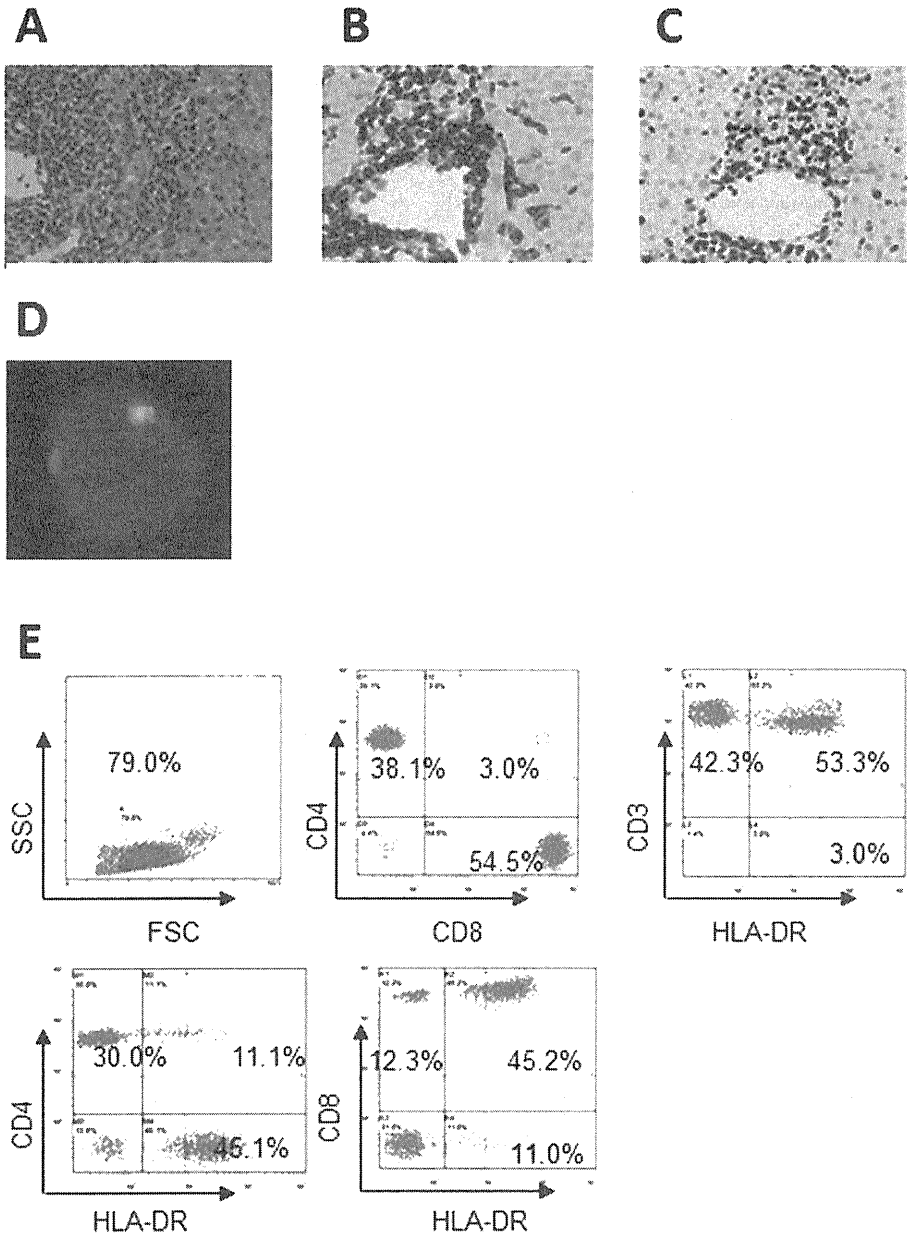

Figure 3. Analysis of Epstein-Barr virus-infected T cells at liver dysfunction development. A-C: Biopsy liver specimens at recurrence (original magnification, ×200), showing severe sinusoidal infiltration of atypical cells. A: Hematoxylin and Eosin staining. B: Stained with the anti-CD8 antibody. C: *In situ* hybridization of Epstein-Barr virus-encoded mRNA. D: FISH analysis of peripheral nucleated cells. Red and green signals indicate X and Y chromosomes, respectively. E: Analysis of peripheral blood mononuclear cells by flow cytometry at the time of liver biopsy.

We recently generated a xenograft model of CAEBV by transplanting a patient's PBMC to NOD/Shi-*scid*/IL-2Rγ-null strain mice (16). In this model, neither EBV-infected T and NK cell engraftment nor CAEBV development occurred without CD4-positive T cells. This indicates that both infected cells and CD4-positive T cell-associated mechanisms (e.g., interactions with CD4-positive T cells, CD4- positive T cell-related cytokines, and so on) may be necessary for CAEBV development. At recurrence, the present patient had activated CD4-positive cells that may have originated from the donor's PBMC (Fig. 3E and Table 1). Three other cases of CAEBV have been reported in patients with autoimmune diseases (14, 17, 18). Hyperactivated, uninfected T cells, including CD4-positive T cells, may facilitate the expansion of EBV-infected T or NK cells, as in our murine model.

In conclusion, the present case indicates that certain background host factors may predispose a patient to CAEBV development. Further studies should be conducted in order to determine these factors.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

This work was originated from Department of Hematology,

Table 1. Chemerism and Lymphocyte Subsets of Peripheral Blood after Bone Marrow Transplantation

	Years after Bone Marrow Transplantation				
	0	1	3	4	4.5
Chimerism of nucleated cells (%)	XX 0.6% XY 99.4%	NE	NE	XX 0% XY 100%	XX 0% XY 100%
Chimerism of T cells		NE	XX 0.5%	NE	XX 0%
		NE	XY 99.5%	NE	XY 78.5%, XXYY 21.5%
The Percentage of CD4-positive cells in CD3-positive cells (in MC)		NE	25 % (24%)	NE	43% (41%)
The Percentage of CD8-positive cells in CD3-positive cells (in MC)		NE	75% (71%)	NE	57% (57%)
Epstein-Barr virus-DNA (copies/μgDNA)	ND	1.7×10^4	1×10^5	1×10^5	5.6×10^6

ND: not detected
NE: not examined
MC: monocuclear cells

Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University.

This work was supported by a grant from Ministry of Health, Labor and Welfare of Japan (H22-Nanchi-080), and a grant from Ministry of Education, Culture, Sports, Science, and Technology of Japan (23591375).

References

1. Kawa K. Epstein-Barr virus—associated diseases in humans. Int J Hematol **71**: 108-117, 2000.
2. Quintanilla-Martinez L, Kimura H, Jaffe ES. EBV-positive T-cell lymphoproliferative disorders of childhood. In: World Health Organization Classification of Tumors. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. Jaffe ES, Harris NL, Stein H, et al, Eds. IARC Press, Lyon, 2008: 527-533.
3. Kimura H, Morishima T, Kanegane H, et al. Prognostic factors for chronic active Epstein-Barr virus infection. J Infect Dis **187**: 527-533, 2003.
4. Arai A, Imadome K, Watanabe Y, et al. Clinical features of adult-onset chronic active Epstein-Barr virus infection: a retrospective analysis. Int J Hematol **93**: 602-609, 2011.
5. Imadome K, Shimizu N, Arai A, et al. Coexpression of CD40 and CD40 ligand in Epstein-Barr virus-infected T and NK cells and their role in cell survival. J Infect Dis **192**: 1340-1348, 2005.
6. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science **286**: 1957-1959, 1999.
7. Koyama M, Takeshita Y, Sakata A, et al. Cytotoxic chemotherapy successfully induces durable complete remission in 2 patients with mosquito allergy resulting from Epstein-Barr virus-associated T-/natural killer cell lymphoproliferative disease. Int J Hematol **82**: 437-440, 2005.
8. Anagnostopoulos I, Hummel M, Kreschel C, Stein H. Morphology, immunophenotype, and distribution of latently and/or productively Epstein-Barr virus-infected cells in acute infectious mononucleosis: implications for the interindividual infection route of Epstein-Barr virus. Blood **85**: 744-750, 1995.
9. Kasahara Y, Yachie A, Takei K, et al. Differential cellular targets of Epstein-Barr virus (EBV) infection between acute EBV-associated hemophagocytic lymphohistiocytosis and chronic active EBV infection. Blood **98**: 1882-1888, 2001.
10. Ohshima K, Kimura H, Yoshino T, et al. Proposed categorization of pathological states of EBV-associated T/natural killer-cell lymphoproliferative disorder (LPD) in children and young adults: overlap with chronic active EBV infection and infantile fulminant EBV T-LPD. Pathol Int **58**: 209-217, 2008.
11. Sugaya N, Kimura H, Hara S, et al. Quantitative analysis of Epstein-Barr virus (EBV)-specific CD8+ T cells in patients with chronic active EBV infection. J Infect Dis **190**: 985-988, 2004.
12. Katano H, Ali MA, Patera AC, et al. Chronic active Epstein-Barr virus infection associated with mutations in perforin that impair its maturation. Blood **103**: 1244-1252, 2004.
13. Arai A, Imadome K, Fujiwara S, Miura O. Autoimmune hemolytic anemia accompanied by reactivation of an Epstein-Barr virus infection with suppressed CTL response to EBV-infected cells in an elderly man. Intern Med **49**: 325-329, 2010.
14. Ogawa J, Koike R, Sugihara T, et al. An autopsied case of chronic active Epstein-Barr virus infection complicated in systemic lupus erythematosus and antiphospholipid antibody syndrome. Nihon Rinsho Meneki Gakkai Kaishi **25**: 458-465, 2002 (in Japanese, Abstract in English).
15. Arai A, Takahashi M, Imadome K, et al. NF-kB is constitutively activated in EBV-infected T or NK cells and can be a molecular target for EBV-positive T/NK-cell lymphoproliferative disease treatment. Blood **116**: 1998, 2010 (Abstract).
16. Imadome K, Yajima M, Arai A, et al. Novel mouse xenograft models reveal a critical role of CD4+ T cells in the proliferation of EBV-Infected T and NK cells. PLoS Pathog **7**: e1002326, 2011.
17. Young KH, Zhang D, Malik JT, Williams E. Fulminant EBV-driven CD8 T-cell lymphoproliferative disorder following primary acute EBV infection: a unique spectrum of T-cell malignancy. Int J Clin Exp Pathol **1**: 185-197, 2008.
18. Tokuhira M, Hanzawa K, Watanabe R, et al. Co-existence of acute myeloid leukemia with multilineage dysplasia and Epstein-Barr virus-associated T-cell lymphoproliferative disorder in a patient with rheumatoid arthritis: a case report. J Hematol Oncol **2**: 27, 2009.

Int J Hematol
DOI 10.1007/s12185-011-0831-x

ORIGINAL ARTICLE

Clinical features of adult-onset chronic active Epstein–Barr virus infection: a retrospective analysis

Ayako Arai · Ken-Ichi Imadome · Yuko Watanabe · Mayumi Yoshimori · Takatoshi Koyama · Takeharu Kawaguchi · Chiaki Nakaseko · Shigeyoshi Fujiwara · Osamu Miura

Received: 6 June 2010 / Revised: 7 March 2011 / Accepted: 22 March 2011

Abstract We performed a retrospective analysis of patients with adult-onset chronic active Epstein–Barr virus infection (CAEBV). First, we analyzed five patients (aged 28–72) diagnosed at our hospitals with EBV-infected clonally proliferating T cells. Four patients were administered cyclophosphamide/doxorubicin/vincristine/prednisone (CHOP) chemotherapy, but no remarkable decrease of viral load was observed in three of the patients. The other patient died 19 days after initiation of CHOP treatment due to disease progression. Addition of high-dose cytarabine to the regimens of two of the patients was discontinued shortly after administration, due to the development of grade 4 pericardial effusion. Together, these regimens may be insufficient for treating adult-onset CAEBV. We next reviewed 23 adult-onset CAEBV patients, adding 18 previously reported patients to the five patients described in the present study. T cells were frequently infected (87%), whereas NK- and T-cell types are known to be almost equally prevalent in childhood-onset cases. The time duration from the onset of disease to initiation of treatment averaged 20 months. Reports showed that 12 patients died; seven patients died at an average of 8 months after initiation of treatment. Patients' disease courses seemed to be rapidly progressive and more aggressive than those of childhood-onset cases. More cases must be studied to clarify clinical features and establish an optimal treatment strategy.

Keywords Chronic active Epstein–Barr virus infection · Adult-onset · EBV-positive T-cell lymphoproliferative disorders of childhood · Chemotherapy · Clinical features

1 Introduction

Chronic active Epstein–Barr virus infection (CAEBV) is a rare disease characterized by an infectious mononucleosis (IM)-like syndrome persisting for at least 6 months, and is associated with high titers of antibodies against EBV [1]. It shows a marked geographic preference for East Asia, and most reports are from Japan, Korea, and Taiwan. In these patients, T or NK cells are EBV-infected. Clonal expansion of EBV-infected cells has been reported in severe forms of CAEBV and is accompanied by high fever, hepatosplenomegaly, and pancytopenia. Because most of these patients were children and young adults, these severe cases of CAEBV with clonally proliferating T cells were termed EBV-positive T-cell lymphoproliferative disorders of childhood in the WHO classification revised in 2008 [2]. However, as reviewed here, some cases of adult-onset CAEBV have been reported. The placement of adult-onset CAEBV in the WHO classification, its clinical features and differences from pediatric cases, and a recommended treatment have not been determined.

A. Arai (✉) · Y. Watanabe · O. Miura
Department of Hematology,
Tokyo Medical and Dental University,
1-5-45 Yushima, Bunkyoku, Tokyo 113-8519, Japan
e-mail: ara.hema@tmd.ac.jp

K.-I. Imadome · S. Fujiwara
Department of Infectious Diseases,
National Research Institute for Child Health and Development,
Tokyo, Japan

M. Yoshimori · T. Koyama
Department of Laboratory Molecular Genetics of Hematology,
Tokyo Medical and Dental University, Tokyo, Japan

T. Kawaguchi · C. Nakaseko
Department of Hematology,
Chiba University Hospital, Chiba, Japan

Published online: 14 April 2011

CAEBV prognosis is poor and CAEBV pathophysiology is considered to be an EBV infection of T or NK cells resulting in their activation and immortalization. Ohshima et al. [3] indicated that infected cells develop from polyclones and oligoclones, and finally become expanded monoclones to develop into aggressive diseases such as lymphomas or hemophagocytic syndrome. Although stem cell transplantation might be curative for CAEBV [4], no chemotherapeutic regimen has been identified with a confirmed effect on CAEBV. No prospective or retrospective analysis has been performed to evaluate the effects of treatment regimens for CAEBV, especially for adult cases.

In this study, we report 5 adult-onset patients with CAEBV. We will outline their clinical courses and the effects of chemotherapy as well as review the reported adult-onset cases in the literature and analyze their clinical features. The aims of this study are to investigate their clinical features in comparison with those of childhood-onset CAEBV and to discuss an optimal treatment strategy.

2 Materials and methods

2.1 Diagnostic criteria

CAEBV was diagnosed according to the criteria proposed by Okano et al. [1]. Briefly, these criteria are as follows: (1) EBV-related symptoms for more than 6 months, including fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, hypersensitivity to mosquito bites (HMB), etc.; (2) increased quantity of EBV in either affected tissues or peripheral blood (PB) defined as EBV-DNA detected in tissue or PB samples by Southern blot hybridization, EBV encoded small RNA1 (EBER)-positive cells detected in tissue or PB samples, or an EBV-DNA level of $10^{2.5}$ copies/g of DNA detected in peripheral blood mononuclear cells (PBMCs); (3) EBV-infected cells confirmed as T or NK cells; and (4) no evidence of any prior immunologic abnormalities or other recent infection that might explain the condition. Criterion #3 was added to exclude EBV-positive B cell lymphoproliferative disorders. The time of diagnosis was defined as the time when the patient was found to meet the above criteria.

2.2 Detection of infected cells

Detection and isolation of infected cells was performed as described previously [5]. Briefly, PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. Then, CD19-, CD4-, CD8-, and CD56-positive cells were separated using antibody-conjugated magnetic beads (IMag Human Particles-DM; BD Biosciences, Sparks, MD, USA). The EBV-DNA of each fraction and of whole blood was quantified using a real-time quantitative polymerase chain reaction assay based on the TaqMan system (Applied Biosystems, Foster City, CA, USA). The fractions of blood with same or higher EBV-DNA titers than that of whole blood were designated as containing EBV-infected cells. The clonality of EBV was determined by Southern blotting using a terminal repeat probe.

2.3 Treatment protocol

Koyama and colleagues recently reported 2 pediatric patients with EBV-positive T-/NK-cell lymphoproliferative disorders and HMB who were successfully treated with sequential chemotherapy consisting of cyclosporine A and prednisolone (CP), followed by CHOP, Capizzi, and HDCA regimens [6], as described below:

CP: cyclosporine A (3 mg/kg/day), (prednisolone 1–2 mg/kg/day).

CHOP: cyclophosphamide (750 mg/m^2 on day 1), doxorubicin hydrochloride (50 mg/m^2 on day 1), vincristine (1.4 mg/m^2 on day 1), and prednisolone (100 mg on days 1–5).

Capizzi regimen: cytosine arabinoside (cytarabine) (3 g/m^2 every 12 h on days 1 and 2), L-asparaginase (6,000 U/m^2 on day 2), prednisolone (30 mg/m^2 on days 1 and 2).

We treated our patients according to this protocol.

The study complied with the principles of the Declaration of Helsinki and was approved by the Ethical Committee of the Tokyo Medical and Dental University. Written informed consent was obtained from each patient.

3 Results

3.1 Case reports

3.1.1 Case 1

A 48-year-old male was admitted to our hospital due to sustained fever which lasted for 1 year, liver dysfunction, and lymphadenopathy. Histology of his cervical lymph node revealed infiltration of EBER-positive cells without atypia; therefore, a diagnosis of lymphoma could not be made. As shown in Table 1, the EBV-DNA level in PB was significantly elevated in mononuclear cells. EBV-infected cells were identified as clonally proliferating CD4-positive T cells. As shown in Table 2, the anti-EBV antibody titer in PB was significantly elevated. From these findings, a diagnosis of CAEBV was made.

CP and CHOP treatment produced a transient resolution of clinical symptoms, but achieved only one log reduction of viral load (Table 1). Grade 4 neutropenia was sustained

Table 1 Effects and adverse events of chemotherapy of chronic active Epstein–Barr virus infection

Case	Age (years)	Sex	Cell type	EBV-DNA (copies/μg DNA)			Adverse events		
							CHOP	HDCA	
				On admission	After CHOP	After HDCA	Grade 4	Grade 3	Grade 4
1	48	M	CD4	2.3×10^5	1.8×10^4	8.5×10^2	Neutropenia (4 days)		Fever, pericardial effusion
2	28	M	CD8	4.2×10^4	5.6×10^4	2.4×10^4[a]	Neutropenia (8 days)	Fever	Pericardial effusion
3	37	F	CD8	1.0×10^7	2.8×10^6	[b]	Neutropenia (9 days)		[b]
4	72	F	CD4	9.5×10^4	Dead	[b]	Neutropenia (5 days)		[b]

F female, *M* male, *EBV* Epstein–Barr virus, *HDCA* high-dose cytarabine

[a] The titer was examined after adding L-asparaginase

[b] High-dose cytarabine was not performed for Cases 3 and 4

for 4 days (Table 1). We judged that the effect of CHOP was insufficient, and 4.8 g of cytarabine was administered according to the Capizzi regimen. However, we discontinued the 3rd and the 4th cytarabine doses because of grade 4 high fever and grade 4 pericardial effusion. L-Asparaginase was administered after resolution of these events. Although the EBV-DNA level in PB decreased drastically, a Guillain-Barré-like neuropathy developed. The EBV-DNA level was elevated in the cerebrospinal fluid (CSF), and CD4-positive cells were detected in the fluid. Magnetic resonance imaging (MRI) revealed no abnormal lesion in the brain. However, we concluded that EBV-infected CD4-positive cells had infiltrated the CSF, and an intrathecal MTX injection was administered until cells were no longer detectable. BMT was performed from a HLA-matched unrelated donor with reduced intensity conditioning therapy (fludarabine 25 mg/m^2/day, 4 days; melphalan 80 mg/m^2/day, 1 day; total body irradiation, 2 Gy × 2), and engraftment was confirmed 1 month later. EBV-DNA became undetectable in both PB and CSF 1 month after BMT. However, the EBV-DNA level rose to 1.2×10^3 copies/g DNA 1 year after BMT. The patient showed no symptoms and the EBV-DNA level decreased without treatment within 2 months. He has remained in remission for 25 months.

3.1.2 Case 2

Two years prior to admission, a 28-year-old male developed fever, mediastinal lymphadenopathy, and polyneuropathy. Biopsies of lymph node lesions were performed at a previous hospital. CD3-positive lymphocyte infiltrations were observed in the specimens, and they were EBER-positive; however, a histological diagnosis of lymphoma was not made due to lack of atypia. However, the anti-EBV antibody titer (Table 2) and the EBV-DNA level in PB were significantly elevated in mononuclear cells. In addition, EBV-infected cells were detected and identified as clonally proliferating CD8-positive T cells (Table 1). From these findings, a diagnosis of CAEBV was made. To evaluate the cause of neuropathy, a sural nerve biopsy was performed and an infiltration of CD8-positive cells was observed in the vascular wall. From these results, we concluded that his neuropathy was due to CAEBV. On examination of his CSF, CD8-positive cells and elevated EBV-DNA levels were detected in the fluid. Although MRI revealed no abnormal lesion in the brain, we concluded that EBV-infected CD8-positive cells had infiltrated the fluid.

Chemotherapy, including an intrathecal MTX injection, was performed. CP and CHOP treatments were first administered. Vincristine was discontinued due to neuropathy. He had transient resolution of clinical symptoms, but without a remarkable decrease in viral load (Table 1). Grade 4 neutropenia was sustained for 8 days (Table 1). We judged that the effect was insufficient, and 5.2 g of cytarabine was administered as part of the Capizzi regimen. However, we discontinued the 3rd and 4th cytarabine doses due to a grade 3 high fever and grade 4 pericardial effusion. We performed a BMT from an unrelated donor with 1 allele-mismatched HLA in DR locus, with reduced intensity of the conditioning therapy (fludarabine 25 mg/m^2/day, 4 days; melphalan 80 mg/m^2/day, 1 day; total body irradiation, 2 Gy × 2), and engraftment was confirmed 1 month later. EBV-DNA became undetectable in PB and CSF 1 month after BMT. However, the EBV-DNA level in PB rose to 1.6×10^4 copies/g DNA 1 year after BMT. He had no symptoms and we detected that the EBV-infected cells were B cells without clonal expansion. The EBV-DNA level decreased without any treatment and has been changing between negative and 4×10^2 copies/g DNA up to the present. We believe that he has remained in remission for 22 months.

3.1.3 Case 3

A 37-year-old female was diagnosed with systemic lupus erythematosus at the age of 24, and 13 years later developed recurrent IM-like symptoms with fever and cervical lymphadenopathy and was transferred to our hospital. Her

Table 2 Reported cases of adult-onset chronic active Epstein–Barr virus infection: clinical features

No.	Sex	Age (years)	Age of onset (years)	Infected cell	Anti-EBV antibodies and EBV-DNA in PB					Clonality	Symptoms and clinical findings on onset	References
					VCA-IgG	EA-IgG	EBNA	VCA-IgM	EBV-DNA			
1	M	24	22	NK cell	5120	5120	40	ND	ND	mono	Fever, LD, hepatosplenomegely, papules	[7]
2	M	36	35	CD4−CD8−T	2560	2560	10	2	ND	mono	Fever, fatigue, facial erythema, parotitis, dry eyes, dry oral cavity	[8]
3	F	25	23	CD4+	>10240	2560	10	ND	103.9 copies/μg	mono	ND	[9]
4	M	29	27	T cell	2560	2560	20	ND	103.6 copies/μg	ND	ND	[9]
5	M	28	26	CD3+	2300 AU/mL	1800 AU/mL	(−)	ND	Positive	ND	Uveitis, iridocyclitis, cardiomyopathy, perimyocarditis, esthesia, paresis	[10]
6	F	35	35	CD3+	18.4[a]	7.8[a]	11.9[a]	0.1[a]	ND	ND	Fever, arthralgia, jaundice, dyspnea	[11]
7	F	57	56	CD4+	5120	1280	10	ND	ND	mono	Fever, eyelid swelling, papules	[12]
8	F	27	24	γδT	2560	320	ND	ND	ND	mono	Fever, LD	[13]
9	F	69	68	CD56+	ND	ND	ND	ND	1.5×10^4 copies/mL	ND	Fever, LD	[14]
10	M	26	24	T cell	1280	ND	40	<10	ND	mono	Fever, weight loss, LD	[15]
11	F	71	68–69	CD3+/CD4−/8−T	2560	1280	20	<10	(+)	oligo	Fever, weight loss, proximal extremities weakness	[16]
12	M	56	54	CD3+/CD4−/8−T	320	1280	10	<10	(+)	oligo	Fever, dysarthria, dysphagia, the proximal extremities weakness	[16]
13	M	27	27	CD3+	ND	ND	ND	ND	140×10^2 copies/mL	ND	LA, motor paralysis, sensory disturbance, involuntary movements	[17]
14	M	52	52	CD8+	Positive	ND	Negative	Positive	9.0×10^4 copies/mL	poly	Fever, night sweats, general malaise	[18]
15	M	59	58	NK cell	(+)	ND	(−)	(−)	2.7×10^3 copies/mL	ND	Fever	[18]
16	F	26	26	T cell	ND	ND	ND	ND	ND	ND	ND	[19]
17	M	45	40	T cell	10240	640	10	ND	ND	ND	Fever, dyspnea, general malaise, hepatosplenomegaly	[20]
18	M	35	33	CD3+	10240	2560	80	<10	7.6×10^3 copies/μg	mono	Fatigue, HMB	[21]
19	M	48	47	CD4+	1280	640	40	<10	2.3×10^5 copies/μg	mono	Fever, LD, LA	OC 1
20	M	28	26	CD8+	2560	2560	<10	<10	4.2×10^4 copies/μg	mono	Fever, polyneuropathy, LA	OC 2
21	F	37	36	CD8+	10240	10240	40	<10	1.0×10^7 copies/μg	mono	Fever, LD, LA	OC 3
22	F	72	72	CD4+	11.2[a]	6.9[a]	3.3[a]	0[a]	9.5×10^4 copies/μg	mono	Fever, diarrhea, LD, body weight loss	OC 4
23	F	62	61	CD4+	2560	2560	40	<10	3.2×10^4 copies/μg	mono	Fever, LD, LA, body weight loss	OC 5

EBV Epstein–Barr virus, *PB* peripheral blood, *M* male, *F* female, *ND* not described, *LD* liver dysfunction, *LA* lymphadenopathy, *HMB* hypersensitivity to mosquito bites, *OC* our case, *mono* monoclonal, *poly* polyclonal, *oligo* oligoclonal

[a] It was measured by enzyme immuno assay. Cut off value was 0.5

clinical course has been reported previously [5]. As shown in Table 1, the EBV-DNA level in PB was elevated in mononuclear cells. Her infected cells were clonally proliferating CD8-positive T cells. As shown in Table 2, the anti-EBV antibody titer in PB was significantly elevated. CP and CHOP treatments produced a transient resolution of clinical symptoms, but without a remarkable decrease in viral load (Table 1). Grade 4 neutropenia was sustained for 9 days (Table 1). BMT was performed from a HLA-matched unrelated donor with reduced intensity conditioning therapy (fludarabine 37.5 mg/m^2/day, 5 days; melphalan 60 mg/m^2/day, 2 days; total body irradiation 2 Gy $\times$ 2). Engraftment was confirmed 1 month after BMT and EBV-DNA in PB became undetectable 2 months after BMT. EBV-DNA remained undetectable for almost 1 year; however, her disease relapsed and chemotherapy was initiated 4 years after BMT.

3.1.4 Case 4

A 72-year-old female was admitted to our hospital due to persistent diarrhea which lasted for 6 months, liver dysfunction, and body weight loss. Biopsies of liver and small intestine were performed and a diagnosis of EBV-positive T-cell lymphoproliferative disorder was made. As shown in Table 1, the EBV-DNA level in PB was elevated in mononuclear cells. The infected cells in the PB were clonally proliferating CD4-positive cells. As shown in Table 2, the anti-EBV antibody titer in PB was significantly elevated. Treatment with CP followed by CHOP was initiated. Grade 4 neutropenia appeared and was sustained for 5 days (Table 1). Unfortunately, her disease progressed and she died 19 days after CHOP initiation.

3.1.5 Case 5

A 62-year-old female, who had been suffering from fever, liver dysfunction, and body weight loss for 8 months, admitted to our hospital. A BM biopsy revealed hemophagocytosis with infiltration of EBV-positive cells. Hepatosplenomegaly was detected by computerized tomography. As shown in Table 2, the anti-EBV antibody titer and EBV-DNA level in PB were elevated in mononuclear cells. EBV-infected cells were clonally proliferating CD4-positive T cells. The diagnosis of CAEBV was made from these results. Both CSF and brain MRI revealed normal findings. She is now in preparation for chemotherapy.

3.2 Analysis of reported cases

To investigate clinical features of adult-onset CAEBV, we retrospectively analyzed the reported cases. We selected patients who met the criteria as described in Sect. 2, and whose onsets were clear after 20 years of age. Eighteen reported patients were selected as shown in Table 2 [7–21]. Although the anti-EBV antibody titer and EBV-DNA level were not described in the report, Case 16 was diagnosed according to the guidelines. After addition of our 5 cases, we analyzed the clinical features of these 23 patients. The age of onset ranged from 22 to 72 years (median 36), in 13 male and 10 female patients. The clinical findings of the onset were described as including fever: 17 cases, liver lesion (dysfunction or hepatomegaly): 10 cases, lymphadenopathy: 5 cases, neurological symptoms: 4 cases, and cardiopulmonary symptoms: 3 cases. HMB was detected in 1 patient. These features were similar to those of childhood-onset cases. The infected cells were NK cells (including CD56-positive cells) in 3 patients, CD4-positive cells in 5 patients, and CD8-positive cells in 3 patients. Three patients had infected T cells that were negative for CD4 and CD8. One patient's infected cells were $\gamma\delta$-T cells. The other 8 patients were CD3-positive, or "T cell" type, and all together, 20 (87%) cases were T-cell type. VCA-IgG was significantly elevated in all described cases. VCA-IgM was undetectable or low except in Case 14, whereas EA-IgG was elevated in most cases. These findings suggested that their infections were not primary, but developed through reactivation of EBV. The elevation of EBV-DNA titer in PB was confirmed in 12 patients. We also confirmed EBV-DNA in PBMC in our 5 patients as in child-onset cases. Clonality was examined in 15 patients. Twelve cases were monoclonal. Their outcomes are summarized in Table 3. The duration from onset to treatment initiation ranged from 6 to 60 months (mean 20 months). Twenty-one patients had their clinical courses documented and 12 (57%) of these patients died. Most patients died from disease progression. The major causes of death were multiple organ failure (4 patients) and hemorrhage (3 patients). Among 8 elderly patients (aged $>$ 50 years; Table 3), 6 patients died form disease progression. Their mortality was 75% besides the younger patients' mortality (aged $<$ 50 years) was 30%. The duration from the onset or treatment initiation to death ranged from 7 to 63 months (mean 27 months) or 4 days to 38 months (mean 8 months), respectively.

4 Discussion

Only a small number of adult CAEBV cases have been reported in the literature; hence, their clinical features are still unclear and treatment strategies have not been evaluated. However, after the diagnostic criteria were suggested in 2005 [1], the diagnosis of CAEBV, especially in adults, has been increasing.

Adult cases consist of 2 groups, adult-onset and childhood-onset, and according to our analysis, their clinical

Table 3 Reported cases of adult-onset chronic active Epstein–Barr virus infection: clinical courses

No.	Sex	Age at diagnosis (years)	Age at onset (years)	Duration from onset to treatment initiation	Treatment	Outcome (duration from treatment initiation to death)	Duration from onset to death	Cause of death	References
1	M	24	22	24 MO	CHOP/HDCA/allo PBSCT (Sib)	Dead (6 M)	30 M	Pulmonary hemorrhage	[7]
2	M	36	35	12 MO	ND	ND			[8]
3	F	25	23	24 MO	VP16/PSL/CPA	Alive[a]			[9]
4	M	29	27	24 MO	VP16/PSL/CPA	Alive[a]			[9]
5	M	28	26	24 MO	IVIG, PSL + CY	Dead		Heart failure due to infiltration	[10]
6	F	35	35	6 MO	mPSL	Dead (1 MO)	7 M	Respiratory failure	[11]
7	F	57	56	12 MO	PSL, CHOP, CY + VP16	Dead (38 MO)	50 M	MOF due to disease progression	[12]
8	F	27	24	31 MO	allo RIST (Sib)	Alive (180 D)			[13]
9	F	69	68	21 MO	Chemotherapy (ND)	Alive (12 MO)			[14]
10	M	26	24		Observation	Dead	36 M	Respiratory failure	15
11	F	71	68–69	24–36 MO	PSL + CY	Dead		DIC	[16]
12	M	56	54	24 MO	CPA + VCR + CY	Dead		Septic shock	[16]
13	M	33	27	36 MO	Steroid pulse therapy	Dead		Hemorrhagic shock due to HPS	[17]
14	M	52	52	6 MO	Steroid	Dead (4 D)	6 M	MOF due to disease progression	[18]
15	M	59	58	12 MO	CPA + VP16 + DEX/allo RIST (Sib)	Dead (3 M)	15 M	Gastric bleeding	[18]
16	F	26	26	ND	allo RIST (UR)	Alive[a]			[19]
17	M	43	40	60 MO	VP16/PSL/CPA	Dead (3 M)	63 M	MOF, DIC	[20]
18	M	35	33	24 MO	ND	ND			[21]
19	M	48	47	12 MO	CHOP/HDCA/allo RIST (UR BM)	Alive in CR (25 MO)			OC 1
20	M	28	26	24 MO	CHOP/HDCA/allo RIST (UR BM)	Alive in CR (21 MO)			OC 2
21	F	37	36	6 MO	CHOP/allo RIST (UR BM)	Alive in relapse (60 MO)			OC 3
22	F	72	72	6 MO	CHOP	Dead (19 D)	7 M	MOF due to disease progression	OC 4
23	F	62	61	8 MO	In preparation				OC 5

PB peripheral blood, *M* male, *MO* month, *ND* not described, *HDCA* high-dose cytarabine, *allo*, allogeneic, *PBSCT* peripheral blood stem cell transplantation, *Sib* sibling, *F* female, *PSL* prednisolone, *CPA* cyclosporin A, *IVIG* intravenous immunoglobulin, *CY* cyclophosphamide, *mPSL* methyl-prednisolone, *RIST* reduced intensity stem cell transplantation, *MOF* multiple organ failure, *VCR* vincristine, *D* day, *DIC* disseminated intravascular coagulation, *HPS* hemophagocytic syndrome, *DEX* dexamethasone, *UR* unrelated, *BM* bone marrow, *CR* complete remission, *OC* our case

[a] Observation period was not described

symptoms, EBV-DNA titers in PB, and EBV antibodies were similar. EBV-DNA was detected in PBMC in our 5 patients as in child-onset cases. However, their clinical courses were different. In adult-onset cases, the duration from onset to treatment initiation averaged 20 months. Death occurred in 12 cases (57%) and the duration from the onset of the disease or initiation of treatment to death averaged 27 or 8 months, respectively, in the patients whose clinical courses were documented. In contrast, Kimura et al. [9] reviewed 30 Japanese patients consisting of children and young adults and reported that young patients could be observed for 12–336 months (mean 71 months) without treatment. They also reported that the probability of survival at 5 years was 0.68 ± 0.06 for young patients. These data indicate that adult-onset cases may progress more rapidly and their prognosis may be poorer than those of younger patients.

Kimura et al. also analyzed 82 CAEBV patients (aged 9–53 years, median 11.3 years) and reported that the patients aged >8 years or with the T-cell type disease had a poor prognosis [22]. As shown in Table 2, 87% of the adult-onset patients were of the T-cell-infected type, whereas Kimura [9] reported that NK- and T-cell types were almost equal in prevalence (T cell 16/30, NK cell 12/30) in childhood-onset cases. Among adult-onset cases, 4 cases were considered to be $\gamma\delta$-T-cell type (Cases 2, 8, 11, and 12). Because the cause of T-cell dominancy is unknown, it may be one reason for the high mortality in adult cases. In addition, the elderly patients (aged > 50 years; Table 3) may have demonstrated aggressive disease courses and poor prognosis. The mortality of the elderly was significantly higher than the younger's. Analysis of CAEBV patients by age or infected cell types is needed to clarify the relation between these factors and pathophysiology of the disease.

No reports have reviewed the effects of chemotherapy in adult-onset CAEBV. As shown in Table 3, 2 patients (Cases 6 and 14) were treated with steroids alone. The treatment had no effect and the patients died within a month after initiation of treatment. Regarding our 4 patients, CP followed by CHOP treatment improved the clinical profiles of the patients. However, CHOP treatment was insufficient to eradicate EBV-infected cells in 3 patients and could not suppress disease progression in 1 patient. Grade 4 neutropenia was detected in all of our patients. Because the rate of developing grade 4 neutropenia after CHOP treatment is reported as 35% [23], the rate in our CAEBV patients might be high. The effects of CHOP treatment were also considered to be insufficient to eradicate EBV-infected cells in Cases 1 and 7 (Table 3). This chemo-resistant property of CAEBV may be an important factor distinguishing CAEBV from other lymphomas. One of the reasons for the ineffectiveness of CHOP treatment may be an upregulated p-glycoprotein (p-gp) function in EBV-infected T or NK cells (unpublished data). Because doxorubicin and vincristine, included in the CHOP regimen, are removed by p-gp, they may not exercise a sufficiently potent effect on CAEBV cells.

Despite pre-administration of prednisolone, high-dose cytarabine (HDCA), included in the Capizzi regimen, produced a severe grade 4 advanced effect in our 2 cases (Cases 1 and 2). In the original report, the Capizzi regimen, consisting of HDCA and L-asparaginase, produced a grade 4 fever in 34% of patients; however, grade 4 cardiopulmonary disease was demonstrated in <1% of patients [24]. Ek et al. [25] reported that administration of 2 g/m^2 of cytarabine every 12 h to children with leukemia and lymphoma resulted in 13 of 16 patients developing a temperature of >38°C. They also reported that inflammatory cytokines such as tumor necrosis factor-α, interleukin-6, and interferon-γ were elevated; however, no serious disease occurred in these patients [25]. These cytokines can be produced and secreted by T cells, which are significantly activated in CAEBV, indicating that severe acute onset reactions resulting from administration of cytarabine may be peculiar to CAEBV patients, especially patients of the T-cell-infected type. More investigation is needed to evaluate the effect and safety of cytarabine in CAEBV treatment and whether the development of severe adverse events depends on patients' age or infected cell type.

In conclusion, adult-onset CAEBV seems to be more aggressive and has different clinical features from those of childhood-onset cases. Treatment with CHOP may be insufficient, and HDCA may produce severe adverse events. Further study is needed to clarify clinical features and to establish optimal treatments for CAEBV in children and adults.

Acknowledgments This work was supported by a grant from the Ministry of Health, Labor and Welfare of Japan.

Conflict of interest The authors declare no competing financial interests.

References

1. Okano M, Kawa K, Kimura H, Yachie A, Wakiguchi H, Maeda A, et al. Proposed guidelines for diagnosing chronic active Epstein–Barr virus infection. Am J Hematol. 2005;80:64–9.
2. Quintanilla-Martinez L, Kimura H, Jaffe ES. EBV-positive T-cell lymphoproliferative disorders of childhood. In: Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. World Health Organization Classification of Tumors. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: IARC Press; 2008. p. 278–80.
3. Ohshima K, Kimura H, Yoshino T, Kim CW, Ko YH, Lee SS, et al. Proposed categorization of pathological states of EBV-associated T/natural killer-cell lymphoproliferative disorder

(LPD) in children and young adults: overlap with chronic active EBV infection and infantile fulminant EBV T-LPD. Pathol Int. 2008;58:209–17.
4. Sato E, Ohga S, Kuroda H, Yoshiba F, Nishimura M, Nagasawa M, et al. Allogeneic hematopoietic stem cell transplantation for Epstein–Barr virus-associated T/natural killer-cell lymphoproliferative disease in Japan. Am J Hematol. 2008;83:721–7.
5. Imadome K, Shimizu N, Arai A, Miura O, Watanabe K, Nakamura H, et al. Coexpression of CD40 and CD40 ligand in Epstein–Barr virus-infected T and NK cells and their role in cell survival. J Infect Dis. 2005;192:1340–8.
6. Koyama M, Takeshita Y, Sakata A, Sawada A, Yasui M, Okamura T, et al. Cytotoxic chemotherapy successfully induces durable complete remission in 2 patients with mosquito allergy resulting from Epstein–Barr virus-associated T-/natural killer cell lymphoproliferative disease. Int J Hematol. 2005;82:437–40.
7. Fujii N, Takenaka K, Hiraki A, Maeda Y, Ikeda K, Shinagawa K, et al. Allogeneic peripheral blood stem cell transplantation for the treatment of chronic active Epstein–Barr virus infection. Bone Marrow Transplant. 2000;26:805–8.
8. Sato-Matsumura KC, Matsumura T, Kobayashi H, Fujimoto K, Itoh T, Shimizu M, et al. Marked swollen erythema of the face together with sicca syndrome as a sign for chronic active Epstein–Barr virus infection. Br J Dermatol. 2000;143:1351–3.
9. Kimura H, Hoshino Y, Kanegane H, Tsuge I, Okamura T, Kawa K, et al. Clinical and virologic characteristics of chronic active Epstein–Barr virus infection. Blood. 2001;98:280–6.
10. Hauptmann S, Meru N, Schewe C, Jung A, Hiepe F, Burmester G, et al. Fatal atypical T-cell proliferation associated with Epstein–Barr virus infection. Br J Haematol. 2001;112:377–80.
11. Ogawa J, Koike R, Sugihara T, Hagiyama H, Nishio J, Kohsaka H, et al. An autopsied case of chronic active Epstein–Barr virus infection complicated in systemic lupus erythematosus and antiphospholipid antibody syndrome. Nihon Rinsho Meneki Gakkai Kaishi. 2002;25:458–65.
12. Shirasaki F, Taniuchi K, Matsushita T, Hamaguhi Y, Takata M, Takehara K. Epstein–Barr virus-associated T-cell lymphoma: a case of eyelid swelling and intramuscular infiltration mimicking dermatomyositis. Br J Dermatol. 2002;147:1244–8.
13. Yoshiba F, Hagihara M, Tazume K, Ogawa Y, Kishi K, Higuchi A, et al. Complete resolution of severe chronic active Epstein–Barr virus infection by cultured, activated donor T lymphocyte infusion after nonmyeloablative stem cells allografting. Bone Marrow Transplant. 2003;32:107–10.
14. Ogata M, Imamura T, Mizunoe S, Ohtsuka E, Kikuchi H, Nasu M. Natural killer cell-type body cavity lymphoma following chronic active Epstein–Barr virus infection. Am J Hematol. 2003;73:126–30.
15. Shibuya A, Tsuchihashi T, Watanabe M, Nakazawa T, Takeuchi A, Sakurai K, et al. Severe chronic active Epstein–Barr virus infection associated with multiple necrotic lesions in the liver. Hepatol Res. 2003;25:447–54.
16. Uchiyama T, Arai K, Yamamoto-Tabata T, Hirai K, Kishimoto K, Nakamura Y, et al. Generalized myositis mimicking polymyositis associated with chronic active Epstein–Barr virus infection. J Neurol. 2005;252:519–25.
17. Kobayashi Z, Tsuchiya K, Takahashi M, Yokota O, Sasaki A, Bhunchet E, et al. An autopsy case of chronic active Epstein–Barr virus infection (CAEBV): distribution of central nervous system (CNS) lesions. J Neurol Sci. 2008;275:170–7.
18. Sonke GS, Ludwig I, van Oosten H, Baars JW, Meijer E, Kater AP, et al. Poor outcomes of chronic active Epstein–Barr virus infection and hemophagocytic lymphohistiocytosis in non-Japanese adult patients. Clin Infect Dis. 2008;47:105–8.
19. Gotoh K, Ito Y, Shibata-Watanabe Y, Kawada J, Takahashi Y, Yagasaki H, et al. Clinical and virological characteristics of 15 patients with chronic active Epstein–Barr virus infection treated with hematopoietic stem cell transplantation. Clin Infect Dis. 2008;46:1525–34.
20. Takano H, Nakagawa K, Ishio N, Daimon M, Kobayashi Y, Hiroshima K, et al. Active myocarditis in a patient with chronic active Epstein–Barr virus infection. Int J Cardiol. 2008;130:e11–3.
21. Imashuku S, Azuma N, Kanegane H, Kasahara Y. M-protein-positive chronic active Epstein–Barr virus infection: features mimicking HIV-1 infection. Int J Hematol. 2009;90:235–8.
22. Kimura H, Morishima T, Kanegane H, Ohga S, Hoshino Y, Maeda A, et al. Prognostic factors for chronic active Epstein–Barr virus infection. J Infect Dis. 2003;187:527–33.
23. Miller TP, Dahlberg S, Cassady JR, Adelstein DJ, Spier CM, Grogan TM, et al. Chemotherapy alone compared with chemotherapy plus radiotherapy for localized intermediate- and high-grade non-Hodgkin's lymphoma. N Engl J Med. 1998;339:21–6.
24. Capizzi RL, Davis R, Powell B, Cuttner J, Ellison RR, Cooper MR, et al. Synergy between high-dose cytarabine and asparaginase in the treatment of adults with refractory and relapsed acute myelogenous leukemia—a Cancer and Leukemia Group B Study. J Clin Oncol. 1988;6:499–508.
25. Ek T, Jarfelt M, Mellander L, Abrahamsson J. Proinflammatory cytokines mediate the systemic inflammatory response associated with high-dose cytarabine treatment in children. Med Pediatr Oncol. 2001;37:459–64.