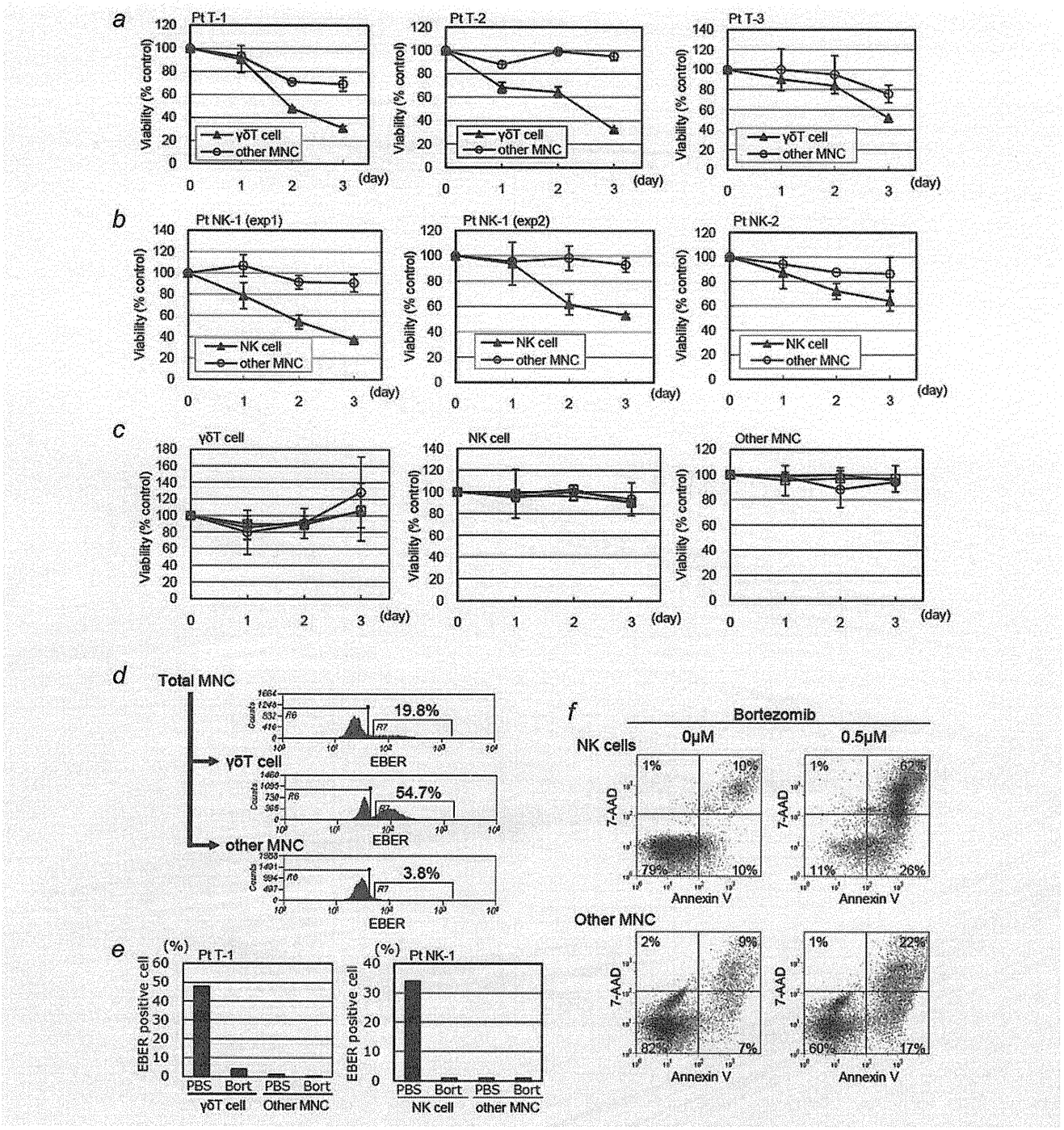

Figure 6. Bortezomib decreases the viability of EBV-infected cells from patients with EBV-associated diseases. (*a, b, c*) Bortezomib (0.5 μM) was administered to each sample of cells, and the viable cells were counted for 3 days. (*a*) γδT cells and other MNCs from three patients (Patients T-1, T-2, and T-3) with hydroa vacciniforme-like lymphoma, (*b*) NK cells and other MNCs from two patients (Patients NK-1, and NK-2) with NK cell-type chronic active EBV infection, and (*c*) γδT cell, NK cell, and non-γδT, non-NK cell (other MNC) from three healthy donors were separated by magnetic sorting. For Patient NK-1, experiments were performed twice on different visits (exp.1 and exp.2). Bars indicate standard errors. (*d*) EBER-positive cells of Patient T-1 with hydroa vacciniforme-like lymphoma were quantified using a FISH assay on unsorted MNCs (total MNCs), the γδT cell fraction (γδT cell), and the non-γδT cell fraction (other MNCs). (*e*) The EBER-positive rate of each sorted cells from Patient T-1 (left) and Patient NK-1 (right) were quantified using a FISH assay after 3 days treatment with bortezomib or PBS. (*f*) NK cells and other MNCs of Patient NK-1, with NK cell-type chronic active EBV infection were separated by magnetic sorting. Cells were treated with 0.5 μM bortezomib for 24 hr and analyzed by flow cytometry. Viable cells were defined as those negative for annexin V-PE and 7-AAD staining, and early apoptotic cells were defined as those positive for annexin V-PE and negative for 7-AAD staining.

The existence of EBV may have little effect on cell death induced by bortezomib. In a previous study, the killing effect of bortezomib was not different between EBV-positive and -negative Burkitt lymphoma cell lines,[8,41] although Zou *et al.*[8] reported that bortezomib had a greater effect in B cell lines with latency type III than those with Type I. The effect of bortezomib may be different in cells with different latency types due to distinct patterns of viral gene expression. The existence of EBV may have some effect on bortezomib because LMP1 is known to activate NF-κB.[42] In the study by Zou *et al.*,[8] however, transfection of LMP1 into an EBV-negative B cell line did not change the sensitivity to bortezomib. The EBV-positive T and NK cell lines used in our study are classified as latency type II[25] and express LMP1. Bortezomib seemed to have little impact on LMP1-mediated NF-κB activation because the presence of EBV did not influence its effects in our study. Comparing cell lines that are naturally EBV positive with derivative cell lines that have lost EBV is necessary to prove that EBV does not affect the outcome of bortezomib treatment. However, establishing such EBV-depleted cell lines is very difficult and, to our knowledge, there are no existing EBV-depleted T- or NK-cell lines. Alternatively, we compared EBV-negative cell lines and those that have been infected with EBV *in vitro*. We admit that this is an artificial system that may have limited relevance.

Bortezomib induced lytic infection only in T cell lines. Inhibition of the NF-κB pathway has been shown to induce the EBV lytic cycle.[43] Although the reason for the difference in lytic induction between T and NK cell lines is unclear, T cell lines seem to express lytic infection genes more often than NK cell lines, consistent with our previous report.[25] Histone deacetylase inhibitors, such as butyric acid and valproic acid, and phorbol 12-myristate 13-acetate are reported to induce an EBV lytic cycle in B cell and epithelial cell lines.[5,26] To our knowledge, however, no agent induces a lytic cycle in EBV-positive NK cell lines. We administered valproic acid, a class I histone deacetylase inhibitor, to NK-cell lymphoma cell lines. Lytic induction was not induced in the NK cell lines (data not shown).

Such lytic induction could contribute to the decreased viability of T cell lines. However, this lytic induction is not a major mechanism of bortezomib-induced cell death because no significant difference was observed in its effect between T and NK cell lines, or between EBV-positive and -negative cell lines. In SNT-16 cells, bortezomib induced a marked decrease in viability, and the recovery in viability following administration of a pan-caspase inhibitor was incomplete (Fig. 3*c*). Thus, the induction of lytic infection by bortezomib could play a partial role in this cell line. Additionally, this result suggests the potential utility of bortezomib as a novel strategy against EBV-positive T cell lymphoma. In EBV-infected T cells, a few latent genes with low antigenicity are expressed.[25] Moreover, the increased expression of lytic proteins, which are generally antigenic, can be recognized in virus-specific cytotoxic T lymphocytes, resulting in lysis of EBV-infected T cells.

Bortezomib killed EBV-infected γδT cells of hydroa vacciniforme-like lymphoma and EBV-infected NK cells of chronic active EBV infection. Taken together with the flow cytometry results, bortezomib induced apoptosis in EBV-infected immortalized cells. These results indicate that bortezomib may be an effective therapy against EBV-associated T and NK lymphoma/lymphoproliferative diseases. We administered 0.5 μM bortezomib to peripheral blood cells in an *ex vivo* study. This concentration is approximately equal to the plasma concentration after administering 1.3 mg/m^2 bortezomib in a recent Phase I and II study of multiple myeloma.[44]

Although recent studies have shown that the effect of bortezomib could be enhanced synergistically in combination with a histone deacetylase inhibitor,[45–47] further studies with appropriate *in vivo* models are essential to confirm this possibility. EBV infects only humans and no good animal models exist, although recently, humanized mouse models with reconstituted human lymphocytes and EBV infection have been reported.[48–50] Despite the complexity of this mouse model, it could be useful for evaluating new therapies, including molecular targeted therapy. The *ex vivo* administration model, coupled with magnetic sorting, is a convenient method to evaluate molecular targeted therapy against EBV-associated lymphoma.

In conclusion, bortezomib killed T/NK lymphoma cells by inducing apoptosis. No significant difference in killing was observed between EBV-positive and -negative cell lines, although bortezomib induced lytic infection in EBV-infected T cells. Following *ex vivo* administration, bortezomib had a greater killing effect on EBV-positive cells than other MNCs. These results give rationale for the use of bortezomib on T/NK lymphomas, although existence of EBV may have little effect on cell death induced by bortezomib.

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Antitumor activities of valproic acid on Epstein–Barr virus-associated T and natural killer lymphoma cells

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Epstein–Barr virus (EBV), which infects B cells, T cells, and natural killer (NK) cells, is associated with multiple lymphoid malignancies. Recently, histone deacetylase (HDAC) inhibitors have been reported to have anticancer effects against various tumor cells. In the present study, we evaluated the killing effect of valproic acid (VPA), which acts as an HDAC inhibitor, on EBV-positive and -negative T and NK lymphoma cells. Treatment of multiple T and NK cell lines (SNT13, SNT16, Jurkat, SNK6, KAI3 and KHYG1) with 0.1-5 mM of VPA inhibited HDAC, increased acetylated histone levels and reduced cell viability. No significant differences were seen between EBV-positive and -negative cell lines. Although VPA induced apoptosis in some T and NK cell lines (SNT16, Jurkat and KHYG1) and cell cycle arrest, it did not induce lytic infection in EBV-positive T or NK cell lines. Because the killing effect of VPA was modest (1 mM VPA reduced cell viability by between 22% and 56%), we tested the effects of the combination of 1 mM of VPA and 0.01 μM of the proteasome inhibitor bortezomib. The combined treated of cells with VPA and bortezomib had an additive killing effect. Finally, we administered VPA to peripheral blood mononuclear cells from three patients with EBV-associated T or NK lymphoproliferative diseases. In these studies, VPA had a greater killing effect against EBV-infected cells than uninfected cells, and the effect was increased when VPA was combined with bortezomib. These results indicate that VPA has antitumor effects on T and NK lymphoma cells and that VPA and bortezomib may have synergistic effects, irrespective of the presence of EBV. (*Cancer Sci* 2012; 103: 375–381)

The ubiquitous Epstein–Barr virus (EBV) infects most individuals by early adulthood and typically remains latent throughout life. Not only does EBV infect B cells, T cells, and natural killer (NK) cells, but it is also associated with multiple lymphoid malignancies, including Burkitt lymphoma, diffuse large B cell lymphoma, Hodgkin lymphoma, post-transplant lymphoproliferative disorders, nasal NK/T-cell lymphoma, hydroa vacciniforme-like lymphoma, aggressive NK cell leukemia, and chronic active EBV disease.[1–4] Epstein–Barr virus plays an important role in the pathogenesis of many of these malignancies via its ability to establish latent infection and induce the proliferation of infected cells.[5] Some of these EBV-associated lymphoid malignancies are refractory and resistant to conventional chemotherapies. Rituximab, a humanized monoclonal antibody against CD20, targets B cell-specific surface antigens present on EBV-transformed malignant cells. Currently, rituximab is used for the treatment and prophylaxis of B cell lymphoma and lymphoproliferative disorders.[6,7] However, the need remains for effective treatments for T and NK cell lymphoid malignancies and novel approaches to molecular targeting are desirable.

Sodium valproate (VPA) is a short chain fatty acid that is widely used to treat epilepsy. It is easily accessible and has a well-established safety profile. Recently, VPA was reported to be a potent histone deacetylase (HDAC) inhibitor and inducer of DNA demethylation.[8] It has been found that HDAC inhibitors have potent anticancer activities, with remarkable tumor specificity, and some have even demonstrated therapeutic potential.[9] The HDAC inhibitors can affect tumor cell growth and survival through multiple biological effects. For example, they induce tumor cell death with all of the biochemical and morphological characteristics of apoptosis. Several HDAC inhibitors have been used in the treatment of leukemias and lymphomas, such as cutaneous T cell lymphoma, myelodysplastic syndrome, and diffuse B cell lymphoma.[9] They have been used alone or in combination with DNA demethylating agents or other anticancer chemotherapies. Valproate has been reported to induce cell death in human leukemia cell lines[10] and endometrial tumor cells,[11] and to enhance the efficacy of chemotherapy in EBV-positive tumors.[12] Furthermore, VPA was shown to activate lytic viral gene expression in cells infected with EBV.[12,13]

Previously, we reported that the proteasome inhibitor bortezomib induced apoptosis in T and NK lymphoma cells.[14] Bortezomib produced a stronger killing effect in EBV-infected tumor cells compared with uninfected cells from patients with EBV-associated lymphoproliferative diseases, although the killing effect of bortezomib in cell lines was not affected by the presence of EBV. In the present study, we administered VPA to EBV-positive and -negative T cell lines and NK cell lines, and evaluated its antitumor effects by analyzing cell viability, the induction of apoptosis, cell cycle arrest, and expression of EBV-encoded genes. Finally, we evaluated the antitumor effect of the combination of VPA and bortezomib using both *in vitro* cell lines and *ex vivo* primary cultures of EBV-infected T and NK lymphoma cells.

Materials and Methods

Cell lines and reagents. Of the cell lines used in the present study, SNT13 and SNT16 are EBV-positive T cell lines,[15] SNK6[15] and KAI3[16] are EBV-positive NK cell lines, and Jurkat[17] and KHYG1[18] are EBV-negative T and NK cell lines, respectively. The SNT13, SNT16, SNK6, and KAI3 cells were derived from patients with chronic active EBV disease or nasal NK/T-cell lymphoma. The MT2/rEBV/9-7 cell line[19] was established through infection of MT2 cells with the hygromycin-resistant B95-8 strain.[20] The MT2/hyg cell line was transfected with a hygromycin resistance gene. Similarly, the NKL cell line[21] was derived from a patient with NK cell leukemia, and the TL1 cell line[22] was established from NKL cells infected with an Akata-transfected recombinant EBV strain carrying a neomycin resistance gene.

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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.

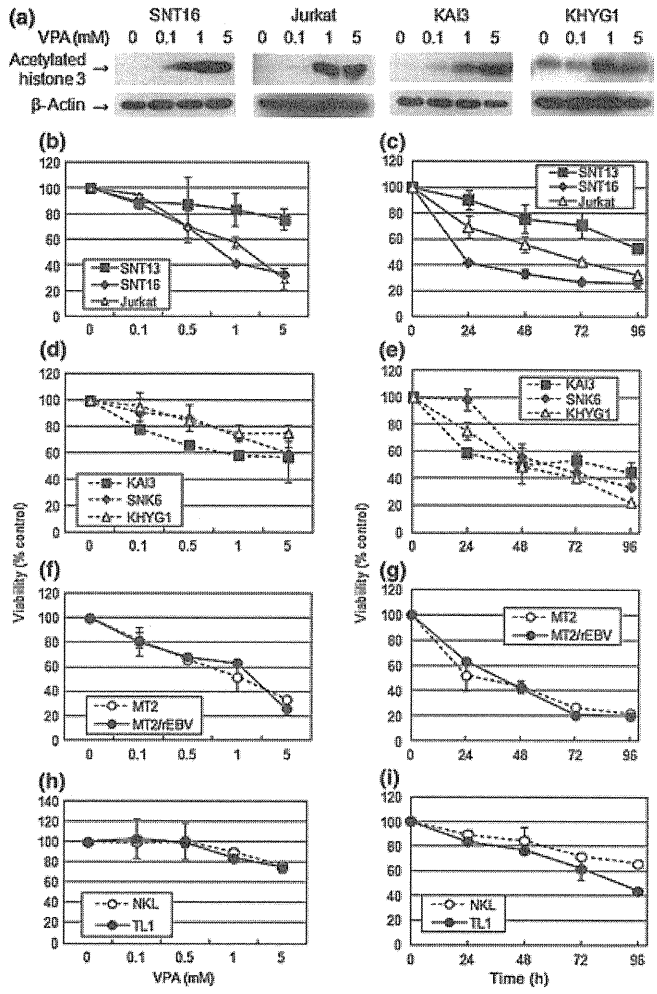

Fig. 1. Valproate (VPA) inhibits histone deacetylase (HDAC) and reduces viability of T and natural killer (NK) cell lines. (a) Acetylated histone 3 was detected by immunoblotting in T and NK cell lines treated with various concentrations of VPA for 24 h. β-Actin was used as a loading control. Viability 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.

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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

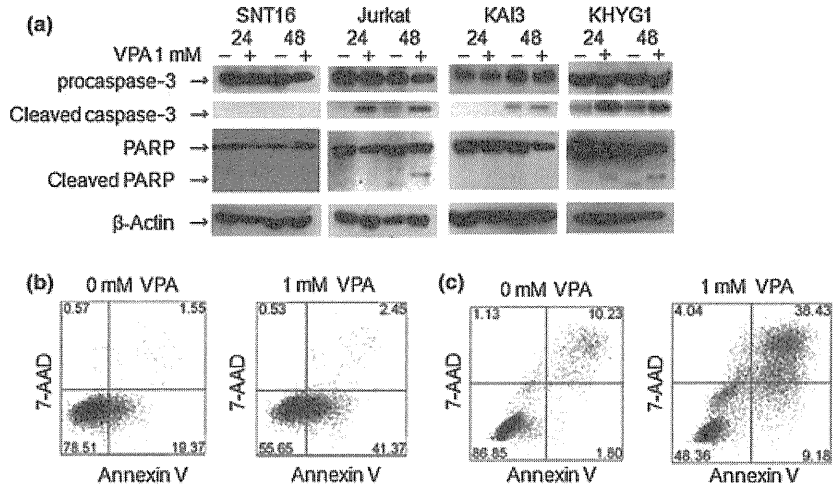

Fig. 2. Effects of valproate (VPA) on apoptosis. (a) T and natural killer (NK) cell lines were treated with 1 mM VPA for 24 or 48 h. β-Actin was used as a loading control. Valproate induced the cleavage of caspase-3 and poly (ADP-ribose) polymerase (PARP) in Jurkat and KHYG1 cells. (b,c) T and NK cell 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.

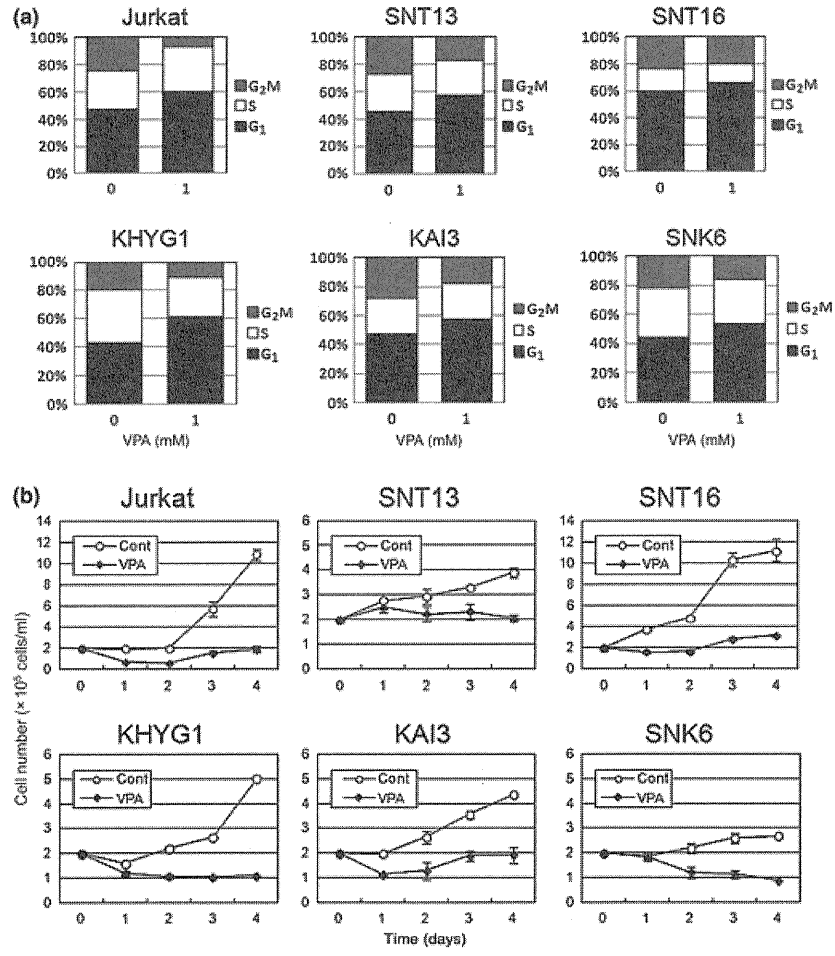

Fig. 3. Effects of valproate (VPA) on the cell cycle. (a) T cell lines (SNT13, SNT16, and Jurkat), and natural killer (NK) cell lines (KAI3, SNK6, and KHYG1) were treated with 1 mM VPA or distilled water for 48 h, fixed, and stained with propidium iodide. Cell cycle profiles were assessed by flow cytometry. (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).

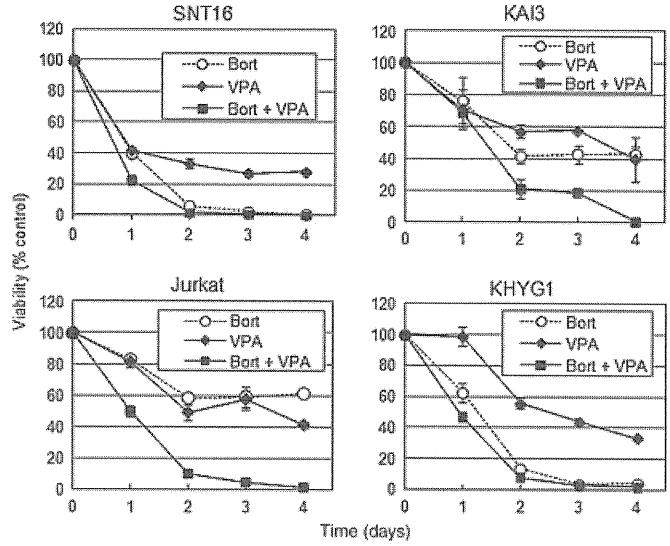

Fig. 5. Combined effects of valproate (VPA) and bortezomib. T cell lines (SNT16 and Jurkat) and natural killer (NK) cell lines (KAI3 and KHYG1) were treated with 1 mM VPA and/or 0.01 μM bortezomib for 96 h and cell viability was assessed. VPA and bortezomib had additive effects in reducing the viability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

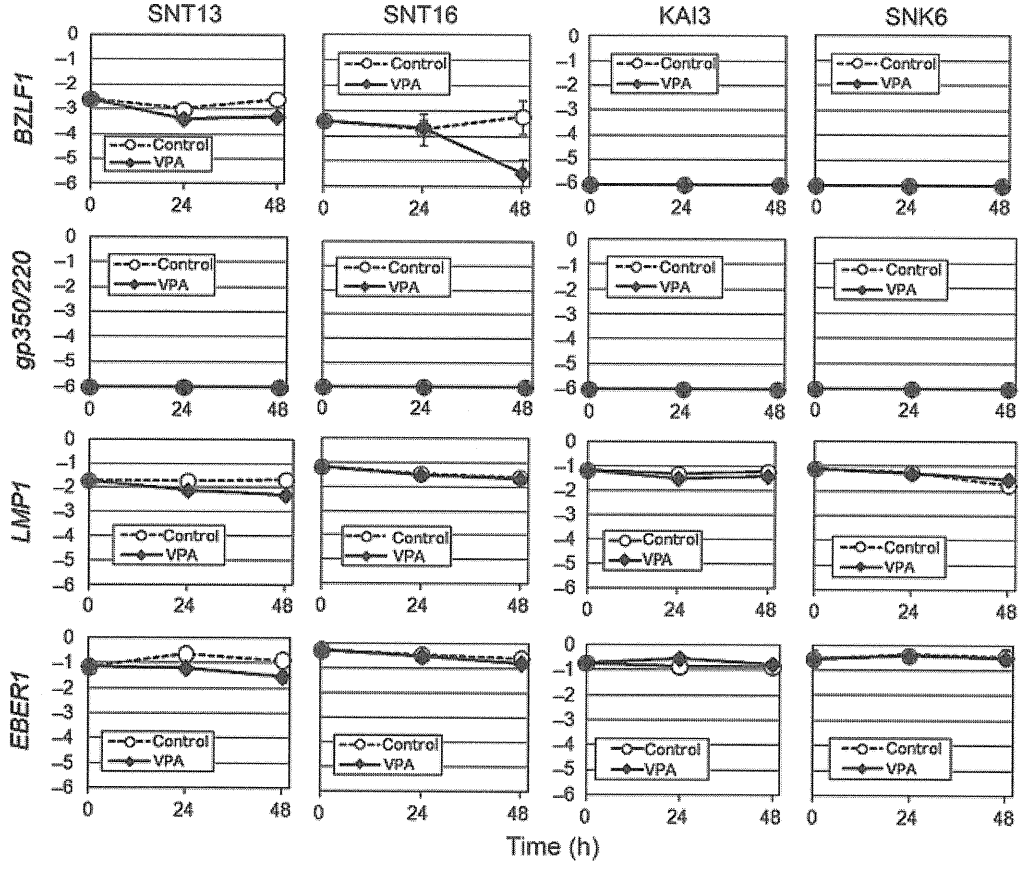

Fig. 4. Effects of valproate (VPA) treatment on the expression of Epstein–Barr virus (EBV)-encoded genes. The EBV-positive T cell lines (SNT13 and SNT16) and EBV-positive natural killer (NK) cell lines (KAI3 and SNK6) were treated with 1 mM VPA and harvested at 0, 24, and 48 h to evaluate gene expression 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.

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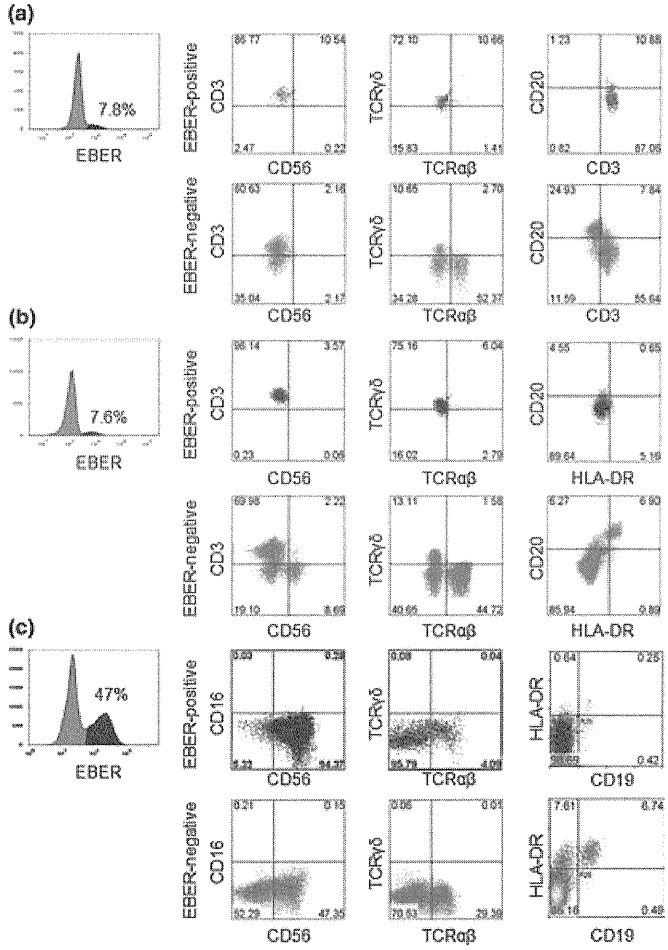

Fig. 6. Identification of Epstein–Barr virus (EBV)-infected cell fractions in patients with EBV-associated T/natural killer (NK) lymphoma. (a, b) Patients T-1 (a) and T-2 (b), who had hydroa vacciniforme-like lymphoma. (c) Patient NK-1, who had chronic active EBV disease, NK cell-type. Mononuclear cells 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 *CDKNIA*, 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

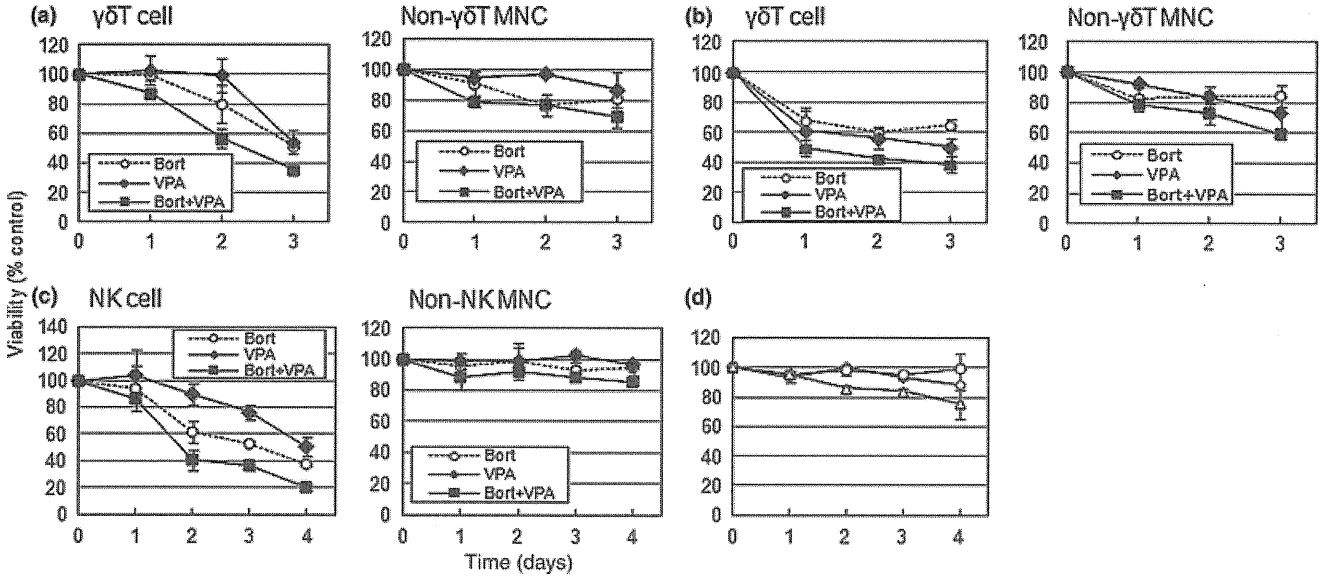

Fig. 7. Effects of the combination of valproate (VPA) and bortezomib on Epstein–Barr virus (EBV)-infected lymphoma cells. Cell populations were separated by magnetic sorting. Each fraction was exposed to VPA (1 mM) and/or bortezomib (0.5 μM) and viable cells were counted over 3 or 4 days. (a,b) Viability 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

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Disclosure Statement

The authors have no conflict of interest.

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INTERNAL MEDICINE

□ CASE REPORT □

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

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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)
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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-

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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

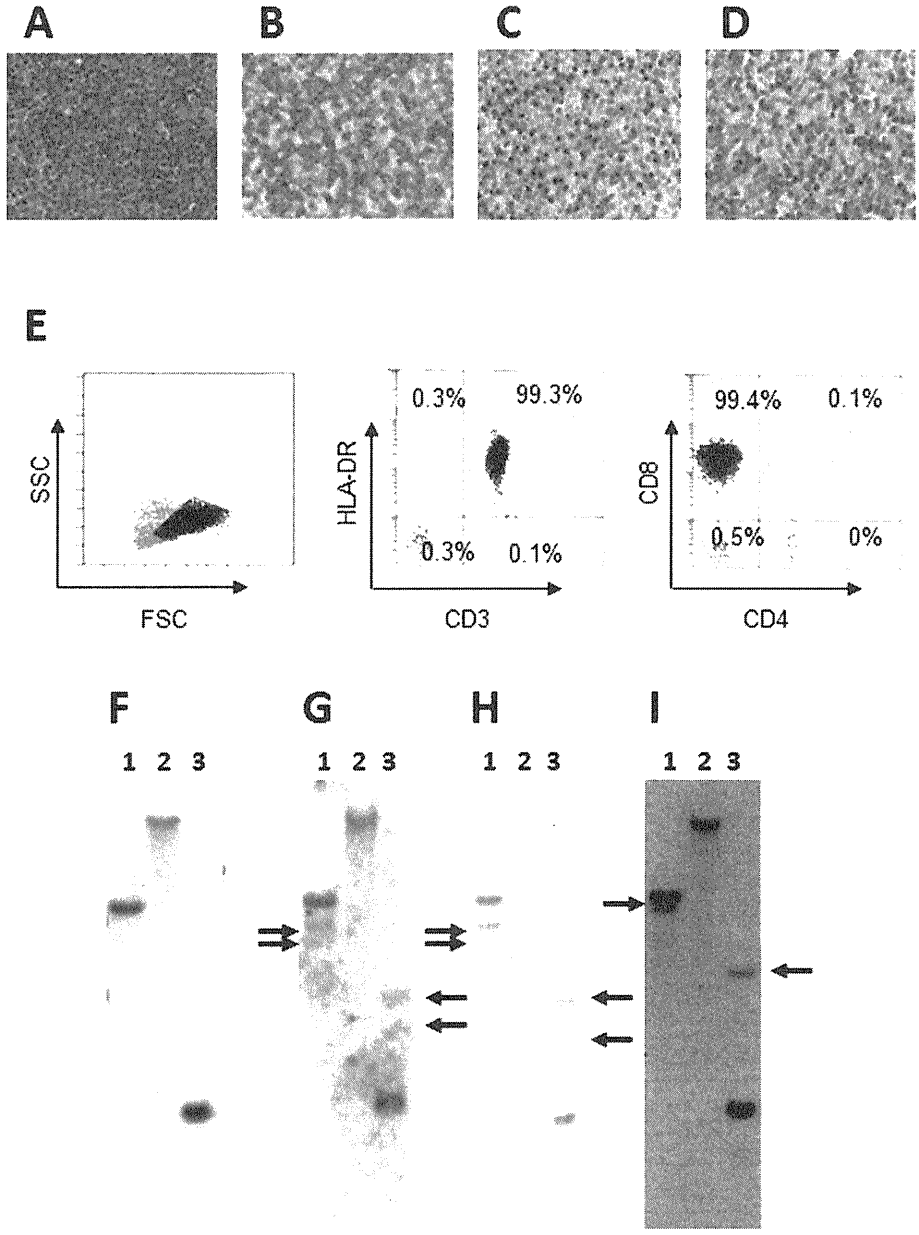

Figure 1. Analysis of Epstein-Barr virus-infected cells at the onset of chronic active EBV infection. A-D: Biopsy specimens of a cervical lymph node (original magnification, ×200). A: Hematoxylin and Eosin staining shows diffuse infiltration of atypical cells. B: Stained with the anti-CD8 antibody. C: 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.

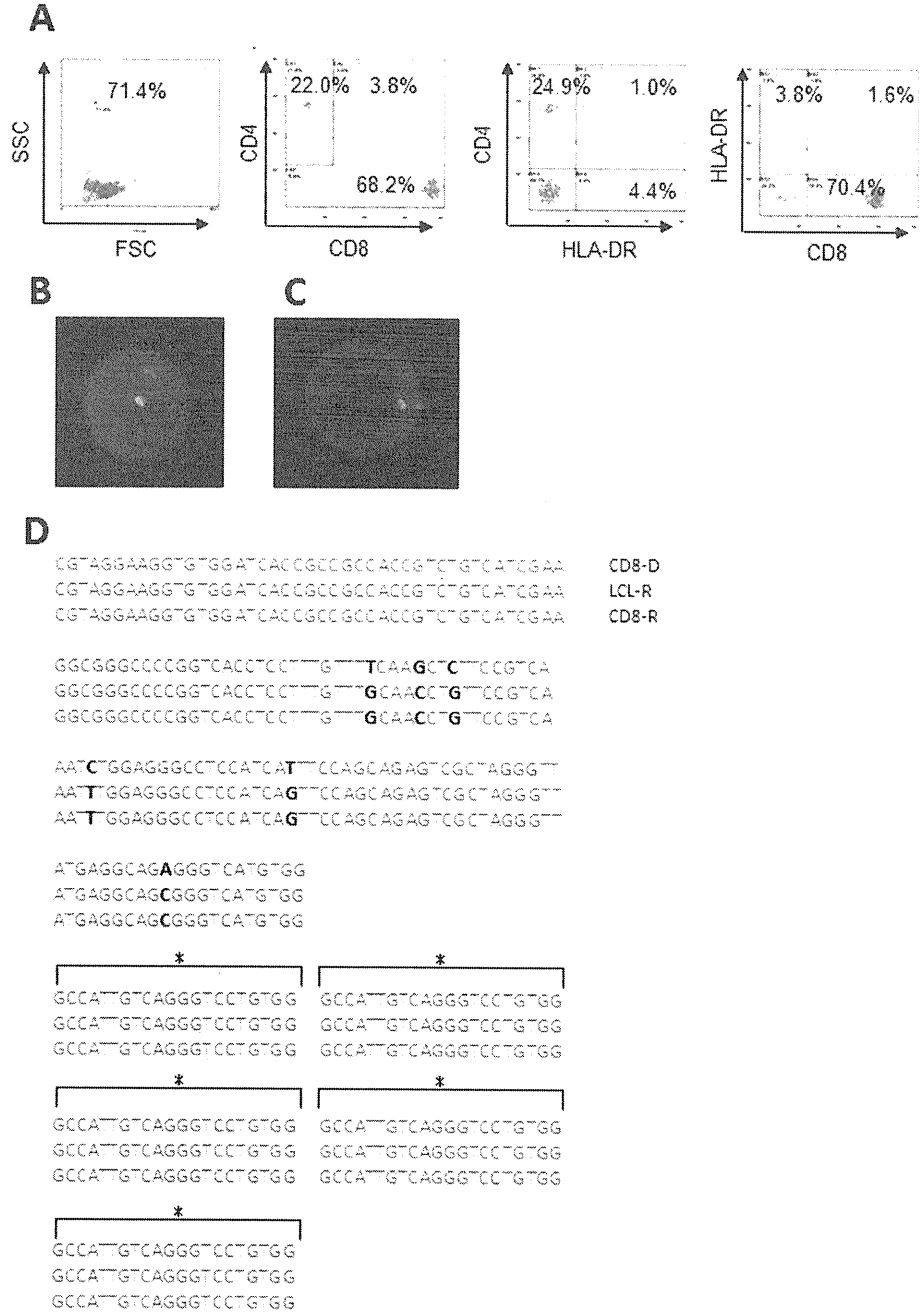

Figure 2. Analysis of Epstein-Barr virus-infected T cells 3 years after BMT at recurrence of chronic active EBV infection. A: Analysis of peripheral blood mononuclear cells by flow cytometry at the diagnosis of recurrence. B, C: Fluorescence *in situ* hybridization (FISH) analysis. Red and green signals 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.

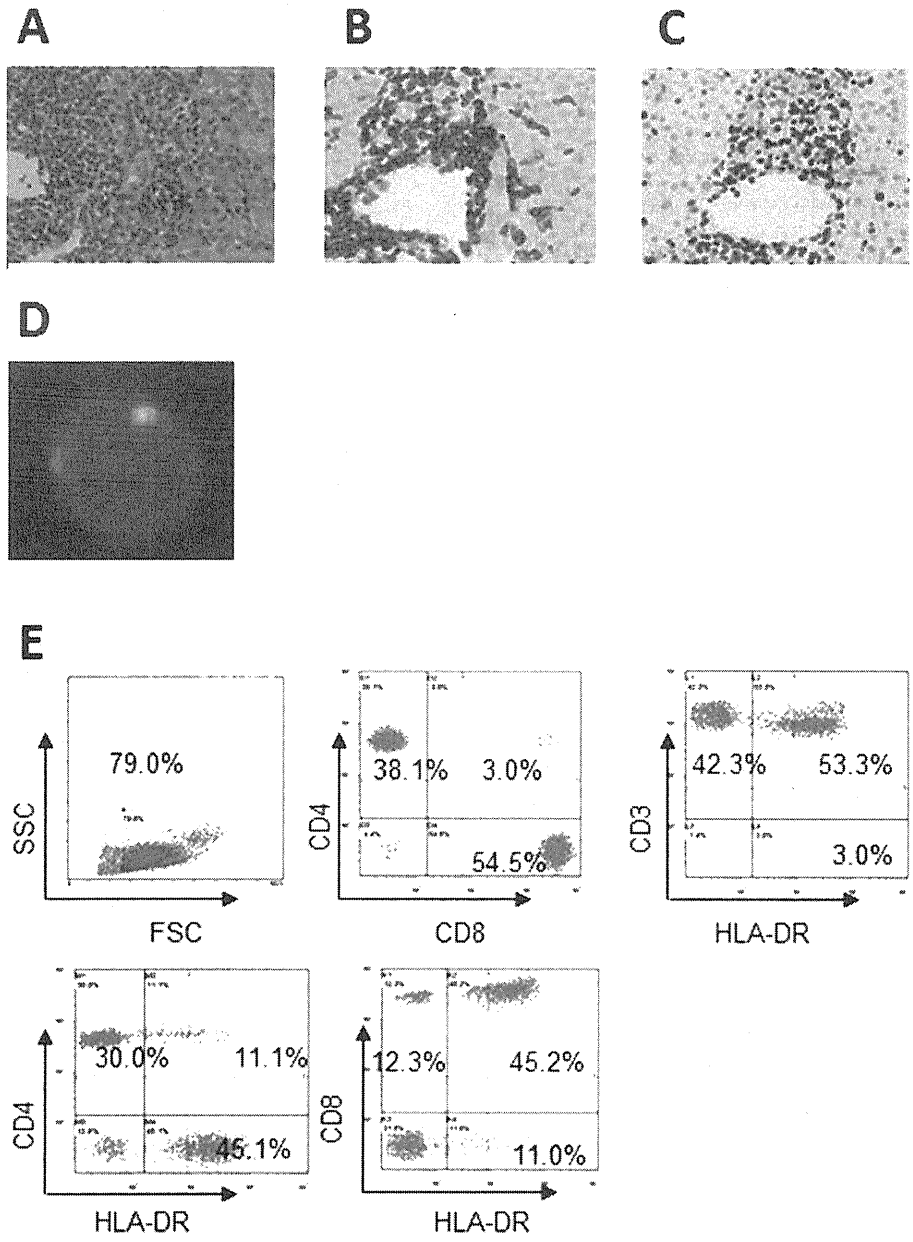

Figure 3. Analysis of Epstein-Barr virus-infected T cells at liver dysfunction development. A-C: Biopsy liver specimens at recurrence (original magnification, ×200), showing severe sinusoidal infiltration of atypical cells. A: Hematoxylin and Eosin staining. B: Stained with the anti-CD8 antibody. C: *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).

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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

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