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Evidence of inability of human cytomegalovirus to reactivate Kaposi's sarcoma-associated herpesvirus from latency in body cavity-based lymphocytes

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ABSTRACT

Background: Kaposi's sarcoma-associated herpesvirus (KSHV; also known as human herpesvirus 8 (HHV-8)) has been determined to be the most frequent cause of malignancies in AIDS patients. It is associated primarily with Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL), as well as with multicentric Castleman's disease (MCD).[2] The switch from the latent to the lytic stage is important in the maintenance of malignancy and viral infection. So far, the mechanism of its reactivation has not been fully understood.
Objectives: Human cytomegalovirus (HCMV) and KSHV might infect the same cells, and it was found by other groups that several viruses could reactivate KSHV from latency. We investigate whether HCMV infection could reactivate KSHV from latency in body cavity-based lymphocyte (BCBL-1) cells.
Study design and results: Laboratory strains of HCMV cannot infect B cells. In this article, we demonstrate that the UL131-repaired HCMV (vDW215-BADrUL131) derived from AD169 strain is able to infect B lymphocytes. We directly infected KSHV latent cells including BCBL-1 with vDW215-BADrUL131 to evaluate the ability of HCMV to reactivate KSHV. Inconsistent with previous reports in human fibroblast cells, our results provide direct evidence that HCMV is unable to reactivate KSHV from latency-to-lytic infection in BCBL-1 cell lines. As a control, herpes simplex virus type 1 (HSV-1) was shown to be able to reactivate KSHV.
Conclusions: Our observations, different from others, suggest that reactivation mechanisms for KSHV might vary in different cells.

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1. Background and objectives

Kaposi's sarcoma-associated herpesvirus (KSHV or human herpesvirus 8, also known as HHV-8) is the most frequent cause of malignancy in AIDS patients. This newly identified virus is associated with Kaposi's sarcoma and several B-cell malignancies such as primary effusion lymphoma as well as multicentric Castleman's disease (MCD).[2,6,7,19,25] KSHV infection in permissive cells including endothelial and fibroblast cells experiences a burst of lytic gene expression at a very early stage and subsequently causes a latent infection that can switch to a lytic infection upon reactivation.[4,13,26] Latent infection persists in a majority of cells, and only a small percentage of latent infection can be reactivated to the lytic cycle.[8] KSHV can successfully infect monocytes, endothelial/spindle cells, B cells and epithelial cells. The virus replicates primarily in KSHV-infected B cells and spindle cells.[5] Earlier studies have revealed that the KSHV latency-to-lytic switch is important in viral pathogenesis, secondary infection to maintain the amount of infected cells and tumourigenesis.[17] Information regarding reactivation of KSHV latency is mostly from *in vitro* reactivation by using chemicals or changing environment (such as hypoxia) or transfect-activating genes including RTA (replication transcriptional activator) and so on.

Herpesviruses including HCMV and KSHV are ubiquitous, and HCMV infects large populations worldwide, and exists latently in the infected host. Indeed, KSHV and HCMV are often detected simultaneously in the same patient; more importantly, both viruses can be isolated from blood cells.[3,9,12,15,16,22,29] HCMV IE1 can inhibit histone deacetylase (HDAC) activity,[21,27] which mimics the function of trichostatin A (TSA) or sodium butyrate (NaB); it is reasonable to speculate that HCMV can cause the lytic switch of KSHV infection if HCMV infected the cells that harbour the KSHV genome. Clinical data so far are still ambiguous regarding whether the mixed infection could result in KSHV reactivation. Recent studies showed

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that Tat of human immunodeficiency virus (HIV) could activate KSHV lytic infection through JAK/STAT signalling and that co-culture of HHV-6-infected T cells with KSHV latent B cells resulted in KSHV reactivation.[14,34] Vieira et al. clearly showed that an HCMV laboratory strain (AD169) of infection in established KSHV harboured a human fibroblast (HFF) cell line that can reactivate KSHV to produce viral particles,[30] and, more recently, the group mapped that UL112/113 is the viral component responsible for the reactivation of KSHV in HFF.[33] It is necessary to know whether HCMV can reactivate KSHV in BCBL-1 cells—our objective in the current studies.

2. Study design and results

HCMV laboratory strains (AD169 and Towne) lost their infectivity other than in human fibroblast cells due to mutation in the gene locus of UL131-128.[10] AD169 has one nucleotide insertion in UL131 that causes an amino acid (aa) frame shift and a functional defect of UL131. The Towne strain has an aa frame shift in UL130 and also causes defective tropism to other cells.[1,20] Clinical (wild) strains of HCMV might infect a wide range of cells. Fibroblast, endothelial, epithelial and blood cells are all susceptible to HCMV infection; it was reported that B cells isolated from 40% of the patients with active HCMV infection have viral DNA.[11] Remarkably, clinical (wild) strains of HCMV isolated from patients and then propagated in fibroblasts for just a few passages lose their ability to infect any cells other than the fibroblast cells. The fact that laboratory and clinical (wild) strains of HCMV are not equally infectious also serves to emphasise the difficulties inherent in the study of HCMV pathogenesis. The hypothesis that UL131-128 should be the determinant of cell tropism and that mutation of the gene locus is the mechanism by which HCMV loses its tropism to many cells in the laboratory was supported by recent molecular studies of HCMV tropisms.[31,32] In the studies, the investigator removed one nucleotide (nt) insertion of UL131 based on AD169. Due to the recovery of the mutation, the repaired AD169, namely vDW215-BADrUL131, was found to be able to infect not only fibroblast cells, but also endothelial and epithelial cells. Therefore, after repair of the mutations, the viral tropism can be recovered. However, it is still unknown whether the repaired HCMV could infect blood cells such as B lymphocytes.

First, we infected B lymphocytes (BJAB, B lymphocyte without KSHV latency; and BCBL-1, B lymphocyte with KSHV latency) and human fibroblast cells (Mrc-5 and HFF) with a laboratory strain of HCMV (AD169) at a multiplicity of infection (MOI) of 5 for 72 h; the whole-cell lysates were then applied to run polyacrylamide gel elctrophoresis (PAGE). Finally, we performed a Western blot to detect HCMV proteins. Compared with the infection of HCMV in human fibroblast cells (in which HCMV can express viral proteins as seen in IE1/2, MCP and pp28), no viral protein could be detected in BJAB or BCBL-1 cells (Fig. 1A). We subsequently infected BCBL-1 and BJAB cells with vDW215-BADrUL131, a repaired HCMV, at an MOI of 5 for different times. As can be seen in Fig. 1B, viral proteins from different stages can be detected. Both MCP and pp28 (UL99) are late proteins; pp28 (also called true late gene) production was proved to be DNA-replication dependent; such production suggests that DNA replication occurred as well. Therefore, the production of pp28 implies a successful infection of vDW215-BADrUL131 in B lymphocytes. Finally, we performed the plaque formation unit (PFU) assay. First, we infected BCBL-1 with HCMV AD169 or vDW215-BADrUL131 at MOI of 1 for different days as indicated in Fig. 1C. The medium and cells were collected and viral particles were released from cells by thaw and freeze for three cycles. After centrifugation, the supernatants were used to infect human fibroblast cells for counting viral plaques. The results in Fig. 1C show that vDW215-

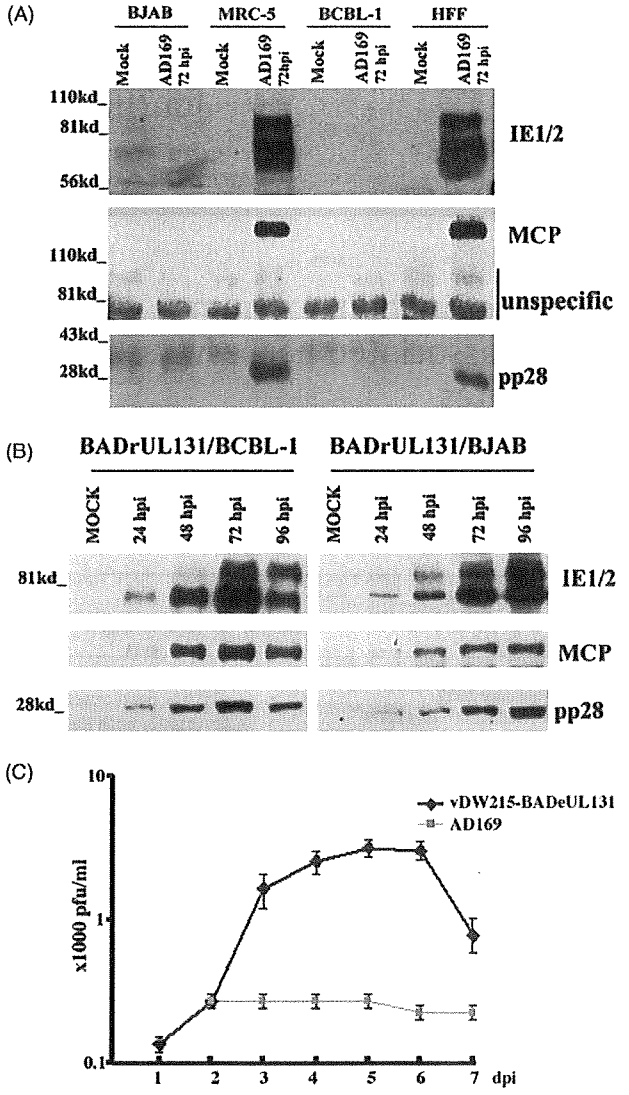

Fig. 1. Infectivity of laboratory strain and UL131-repaired HCMV in B cells. (A) After infection of laboratory strains of HMCV in cells for the time as indicated, cells were collected and lysed in Lamile buffer and applied in 7.5% PAGE; the viral proteins were detected with Western blot assay. (B) UL131-repaired HCMV, vDW215-BADrUL131 infection in B cells were detected by Western blot assay. (C) BCBL-1 cells in 12-well plates were infected with HCMV AD169 or vDW215-BADrUL131 at MOI of 1 and the medium was changed at 12 h after infection. The cells and medium was collected as the days post infection as indicated and the viral particles were released by thaw and freeze for three cycles. After centrifugation, the supernatant were applied for PFU assay. Data from triplicates were obtained and statistically analyzed; the error bars stand for the standard error from the three independent experiments.

BADrUL131 can productively infect BCBL-1 cells while AD169 failed to produce any viral particles. Taken together, the data here provided evidence that HCMV is able to infect B lymphocyte after the tropism is recovered from AD169, and the presence of KSHV has no effect on the infection.

Although most of those individuals with latently infected KSHV never suffer from any KSHV-associated diseases, such individuals run a risk of developing KS, PEL or MCD, with the greatest risk factor for these conditions being immunodeficiency, which occurs in AIDS patients, transplant recipients and patients with viral infections. The HCMV genome encodes some products that have immunomodulatory effects that can interfere with the host's immune system. In addition, the above observation demonstrates the fact B lymphocytes can be infected by HCMV as well as by KSHV.

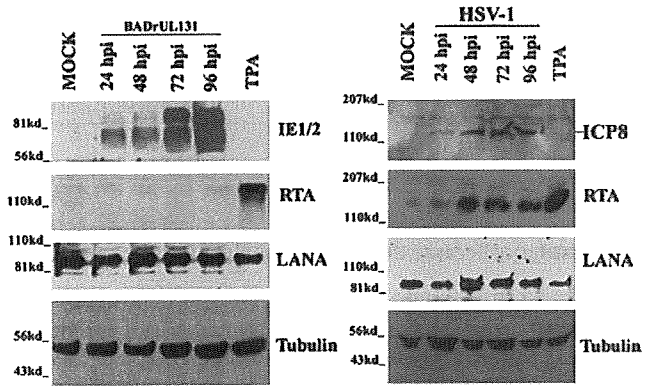

Fig. 2. KSHV reactivation in BCBL-1 cells by HCMV, HSV-1, and TPA. BCBL-1 cells were infected with vDW215-BADrUL131 (left) or HSV-1 (right) for the time indicated, and the cells were treated with TPA for 48 h and were collected and lysed in Lamile buffer; cellular proteins and viral proteins were detected with Western blot assay.

Several recent studies on the pathogenesis of KSHV have shown that a number of viruses (including HHV-6, HSV-1, HIV and HCMV) can induce KSHV lytic infection.[14,23,30,34] HCMV has been the focus of our interest not only because it can infect the same type of cells as KSHV – as has been shown by many laboratories – but also because it infects a large population (about 50–90%). An observation by Vieira et al. that HCMV can reactivate KSHV from latency might explain the virus–virus interactions.[30]

It is important to study KSHV latency disruption by HCMV in B lymphocytes because HCMV and KSHV can be detected in blood samples from the same patient, which suggests a co-infection. We infected BCBL-1 cells with vDW215-BADrUL131 HCMV at an MOI of 5 for different times as indicated in Fig. 2A. TPA-treated BCBL-1 was used as positive reactivation control. The whole-cell lysates were collected at the times indicated; Western blot was performed to demonstrate HCMV and KSHV proteins. An increase in IE1/2 production is evidence of active HCMV infection in BCBL-1 cells; faint bands above the major bands indicate small ubiquitin-like modifier (SUMO)-modified IE1/2. KSHV reactivation is indicated by the presence of RTA, a gene essential for KSVH reactivation; RTA can only be produced (and is the first gene expressed) after reactivation, as shown in TPA-treated BCBL-1 cells. The absence of RTA in HCMV-infected BCBL-1, then, suggests that HCMV failed to reactivate KSHV. Latency-associated nuclear antigen (LANA), a protein produced during KSHV latency on BCBL-1 cells and cellular protein (tubulin), was used as a sample loading control. As a control, we infected BCBL-1 cells with a wild-type strain of HSV-1, as shown in Fig. 2B, and ICP8 was used to indicate HSV-1 infection. We demonstrated that HSV-1 infection in BCBL-1 cells resulted in the

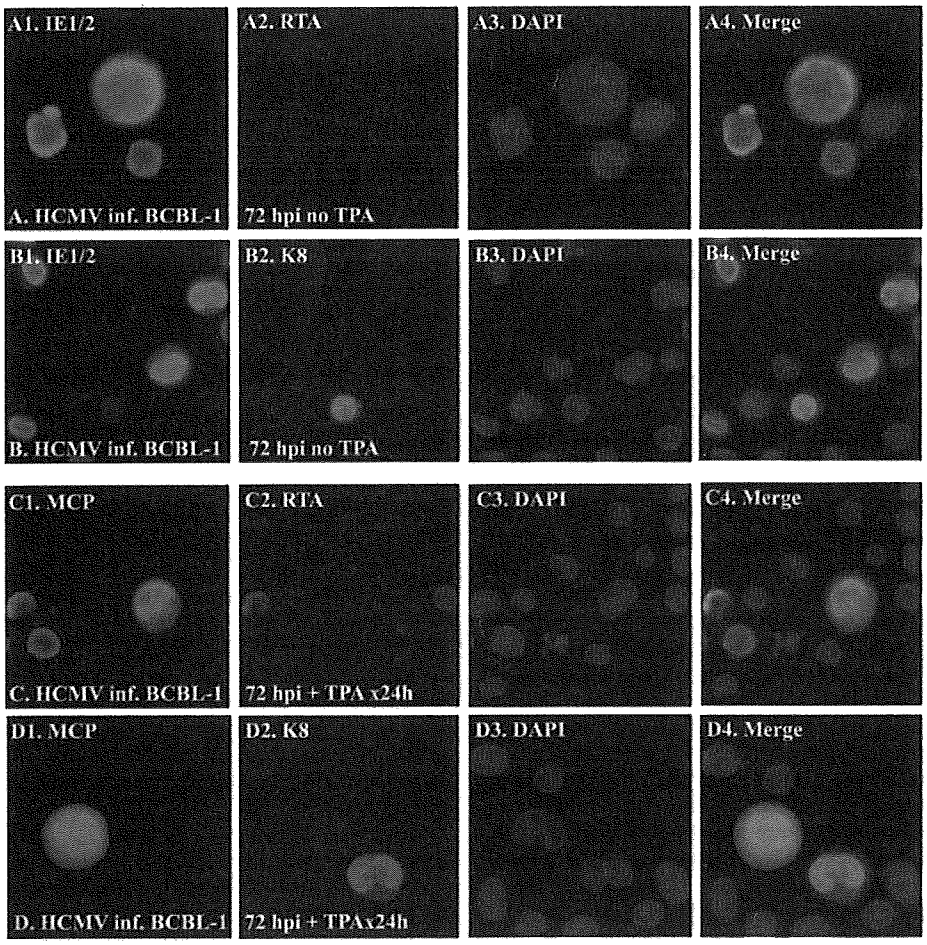

Fig. 3. Immunofluorescence to detect HCMV infection in BCBL-1 cells. BCBL-1 cells were infected with vDW215-BADrUL131, untreated (A and B) or treated (C and D) with TPA, and were washed with PBS and fixed with 1% paraformaldehyde and cytospun to slides for immunofluorescence assay using different antibioses, as indicated, to detect viral proteins: IE1/2 (A1 and B1) and MCP (C1 and D1) of HCMV in green; RTA (A2 and C2) and K8 (B2 and D2) of KSHV in red. Total cells were shown with DAPI blue (A3 to D3). Three different colors were merged and shown in A4–D4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
KSHV reactivation rate in HCMV infected cells versus non-infected cells. Relationship between HCMV infection and KSHV reactivation. Forty-eight hours after infection of BCBL-1 cells with vDW215-BADrUL131 at an MOI of 5, we added TPA in the cell culture for 24 h. The cells were cytospun to slides for detection with immunofluorescence with anti-HCMV IE1/2 and KSHV RTA antibody. Cells were chosen at random and counted.

HCMV infection state	Total number of cells counted	Number of RTA positive cells	Reactivation rate (%)
IE1/2 positive	300	35	11.7
IE1/2 negative	500	59	11.8

Student' *t*-test: $P > 0.05$.

reactivation of KSHV as indicated by the presence of RTA, which is consistent with the report from another group.[24] A question about the clinical significance of the observation of interaction of HSV-1 with KSHV still remains. An animal model might be needed to determine whether HSV-1 can reactivate KSHV.

Furthermore, we visualised the HCMV infection in BCBL-1 cells by immunofluorescence. First, when the BCBL-1 cells were infected with vDW215-BADrUL131 at an MOI of 5 for 72 h, the positive infection was shown by the production of IE1 (Fig. 3A1 and B1) and major capsid protein (MCP) in the nuclei (C1 and D1) in green. KSHV protein production was shown in red using anti-RTA polyclonal antibody (Fig. 3A2 and C2) and anti-K8 monoclonal antibody (Fig. 3B2 and D2), which signal the reactivation of KSHV because they are both lytic-stage proteins. The total cells in the microscope field were shown by 4′,6-diamidino-2-phenylindole (DAPI) staining in blue (A3–D3). The immunofluorescence studies showed that neither RTA nor K8 was detected in most of the HCMV positively infected cells. Three single colours were merged (A4–D4) to be able to visualise the relationship between HCMV infection and KSHV reactivation. It has been noticed that there is a positive cell in Fig. 3B2 (positive K8, in red) in which HCMV IE1/2 is much less than other cells in the same microscope field (Fig. 3B1). In BCBL-1 cell cultures that have not been stimulated, KSHV was reactivated (called leaking) in less than 1% of the cells; Fig. 3B1 depicts one of them. This immunofluorescence study further demonstrated that HCMV infection failed to reactivate KSHV in BCBL-1 cells.

We next queried whether HCMV could affect KSHV reactivation by TPA in BCBL-1 cell. After we infected BCBL-1 cells with vDW215-BADrUL131 at an MOI of 5 for 48 h, we added TPA in the cell culture for 24 h. The cells were cytospun to slides for detection with immunofluorescence with anti-HCMV MCP antibody (Fig. 3C and D) or with anti-IE1/2 (Table 1) and KSHV RTA or K8 antibody. As shown in Fig. 3C, HCMV infected cells (MCP positive: C1, left) can be still reactivated by TPA (RTA in red, C2). We also randomly counted the cells in two groups: HCMV infected (shown by IE1/2 in green) and non-infected to see whether there is any difference between the KSHV reactivation rates. As shown in Table 1, we counted 300 HCMV-infected cells in which 35 cells were reactivated (as marked with RTA staining). In 500 non-infected cells, 59 cells were reactivated. No significant difference was found between the two groups using Student's *t*-test.

3. Discussion

Human herpesviruses mostly cause latent infection in immune-competent populations and results in disease when reactivated by many different environmental conditions.[28] Several herpesviruses might infect the same person because many of them have high infection incidence. KSHV infection in the general population is not as high as other herpesviruses, but it is the first important pathogen causing cancer in AIDS patients. HCMV infects most of the population and can be occasionally reactivated.[18] Mixed infection of KSHV and HCMV intrigued us to investigate the interaction of the two viruses. The finding that HSV-1 infection can switch KSHV infection to the lytic stage is further demonstrated in this study; however, interactions between HCMV, KSHV and HIV might be of more importance because those viruses could infect the same kind of cells.

The facts that HCMV IE1 and IE2 can interact with HDAC and inhibit HDAC activities and that HDAC inhibitors can reactivate KSHV in cell culture suggest that HCMV infection might be able to reactivate KSHV infection. As a matter of fact, in the human fibroblast cell system, it has been reported recently that HCMV UL112/113 molecule can lead KSHV infection from latency to lytic stage in human fibroblast cells.[33] It is necessary to know whether HCMV could also reactivate KSHV in another cell line. We infected BCBL-1 harbouring KSHV genome with vDW215-BADrUL131 HCMV and detected the state of KSHV; it is clear that HCMV infection failed to reactivate KSHV in BCBL-1 cells. We also found that KSHV reactivation by TPA was not affected by HCMV infection. We also found that the two viruses can exist in the same cells and continue to express their own gene products. The differences in the results between our experiments and the other groups' regarding the effect of HCMV infection on KSHV reactivation imply a different mechanism of reactivation of KSHV in different types of cells.

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Transient inhibition of NF-κB by DHMEQ induces cell death of primary effusion lymphoma without HHV-8 reactivation

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Primary effusion lymphoma (PEL) is a refractory malignancy caused by human herpes virus 8 (HHV-8) in immunocompromised individuals. The tumor cells of PEL are characterized by constitutive NF-κB activation. Dehydroxymethylepoxyquinomicin (DHMEQ) is a new NF-κB inhibitor and is effective on various tumor cells with constitutively activated NF-κB. Thus, in search for a new therapeutic modality of PEL, we examined the effect of DHMEQ on PEL cells. We confirmed constitutive activation of NF-κB with subcomponents of p50 and p65 in PEL cell lines. DHMEQ quickly and transiently abrogated NF-κB activation and reduced the cell viability in dose- and time-dependent manners, inducing apoptosis through activation of both mitochondrial and membrane pathways. Array analysis revealed that DHMEQ down-regulated expression levels of NF-κB target genes, such as *interleukin-6* (IL6), *Myc*, chemokine (C-C motif) receptor 5 (*CCR5*) and NF-κB1, whereas it up-regulated expression levels of some genes involved in apoptosis, and cell cycle arrest. DHMEQ did not reactivate HHV-8 lytic genes, indicating that NF-κB inhibition by DHMEQ did not induce virus replication. DHEMQ rescued CB-17 SCID mice xenografted with PEL cells, reducing the gross appearance of effusion. Thus, DHMEQ transiently abrogated the NF-κB activation, irreversibly triggering the apoptosis cascade without HHV-8 reactivation. In addition, DHMEQ could rescue the PEL-xenograft mice. Therefore, we suggest DHMEQ as a promising candidate for molecular target therapy of the PEL. (*Cancer Sci* 2009; 100: 737–746)

Primary effusion lymphoma (PEL) is a non-Hodgkin B-cell lymphoma usually associated with immunocompromised patients such as those with aquired immune defficiency syndrome (AIDS).[1,2] Despite extensive use of highly active antiretroviral treatment (HAART) and improvement of chemotherapy management in human immunodeficiency virus (HIV)-infected patients, the prognosis of patients with HIV-related PEL remains poor. The median survival does not exceed 6 months even in the most recent series.[3] The tumor cells have an intermediate immunophenotype but B-cell genotyping shows clonal rearrangements of immunoglobulin genes.[4]

Human herpes virus 8 (HHV-8) is essential for the development of PEL that is universally associated with HHV-8. Most of the PELs are also infected with Epstein–Barr virus (EBV) and the combination of the two viruses might promote full transformation. Tumor cells in PELs are latently infected by HHV-8 and express latent proteins, while a few cells undergo lytic replication. HHV-8 encodes numerous proteins homologous to critical cell cycle regulatory and apoptosis proteins, cellular receptors and their ligands. However, because of their unique properties they can escape normal regulatory pathways and hence behave differently from their cellular counterparts. Among these genes, v-cyclin, LANA, and v-IRF1 interfere with the cell cycle deregulation, while v-FLIP prevents apoptosis.[5] PELs have constitutively active nuclear factor (NF)-κB, due to v-FLIP expression, which is essential for their survival.[6–10] In addition to v-FLIP, HHV-8 proteins K1, K15, and the viral G protein-coupled receptor (v-GPCR) induce NF-κB activity, thereby supporting the central role of NF-κB signaling in HHV-8 pathogenesis.[5]

NF-κB has been implicated in inflammation, cell proliferation, differentiation, apoptosis and cell survival. NF-κB is a ubiquitously expressed family of five proteins; p65 (RelA), p50, p52, c-Rel and RelB. Different combinations of these NF-κB subunits are considered to specify target genes under different conditions.[11,12]

Dehydroxymethylepoxyquinomicin (DHMEQ) is an NF-κB inhibitor, based on the structure of antibiotic epoxyquinomicin C.[13] DHMEQ inhibits the tumor necrosis factor (TNF)-α-induced NF-κB binding activity but not the phosphorylation or degradation of I-κB. DHMEQ inhibited the TNF-α-induced nuclear accumulation of p65. Thus, DHMEQ is a unique inhibitor of NF-κB that acts at the level of the nuclear translocation.[14] DHMEQ has been reported to be effective on various hematologic and solid malignancies with constitutively active NF-κB.[15–21]

Therefore, in search for a new modality of PEL therapy based on the idea of molecular targeting, we examined effects of DHMEQ on PEL cells *in vitro* and *in vivo*. Our data demonstrated that DHMEQ could abrogate the NF-κB activation transiently and initiate the apoptosis cascade irreversibly without activation of HHV-8 replication. In addition, DHMEQ rescued the PEL xenograft mice. Taken together, we suggest DHMEQ as a promising candidate for molecular target therapy of the PEL.

Materials and Methods

Cell culture. PEL cell lines used for experiments are BCBL1, TY1 and BC1 infected with HHV-8.[22,23] BC1 was also infected by EBV. Jurkat and K562 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). These cell lines were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Bio West, Miami, FL, USA) and antibiotics (penicillin/streptomycin, Gibco), except for BC1 and TY1 cells that were cultured with 20% FBS.

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Table 1. Primers used in the real time quantitative reverse transcription–polymerase chain reaction

Gene		Sequence of oligonucleotide
DDIT3	sense	5′-GCCAAAATCAGAGCTGGAAC-3′
	antisense	5′-TCTTGCAGGTCCTCATACCA-3′
DEDD2	sense	5′-GCAGTCAAGCAGTTCTGCAA-3′
	antisense	5′-CACAGGTCACTTTGCCTTCA-3′
p21	sense	5′-GCAGACCAGCATGACAG-3′
	antisense	5′-TAGGGCTTCCTCTTGGA-3′
IL6	sense	5′-GGTACATCCTCGACGGCATCT-3′
	antisense	5′-GTGCCTCTTTGCTGCTTTCAC-3′
BIRC3	sense	5′-CAGCCCGCTTTAAAACATTC-3′
	antisense	5′-ACCCATGGATCATCTCCAG-3′
MYC	sense	5′-GCCACGTCTCCACACATCAG-3′
	antisense	5′-TCTTGGCAGCAGGATAGTCCTT-3′

Electrophoretic mobility shift assays (EMSAs) and supershift analysis. For detecting NF-κB binding, we used an NF-κB consensus oligonucleotide (Promega Corporation, Madison, WI, USA).[24] Nuclear extracts were prepared basically as described.[23] DNA-protein complexes were analyzed as previously described.[25] The supershift analysis was performed as described previously, using antibodies against p65, p50, p52, c-Rel, and Rel-B (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), or a control mouse immunoglobulin G.[26]

Assessment of cell viability and apoptosis. Cell viability was determined by color reaction with WST-8. Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), and Annexin V reactivity was examined by Annexin V using Apopcyto Annexin V-Azami-Green Apoptosis detection kit (MBL, Nagoya, Japan) according to the manufacture's protocols. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed using DeadEnd Fluorometric TUNEL System (Promega Corporation) kit and protocols. The results for both tests were analyzed using FACSCalibur machine and CellQuest software (BD Biosciences, San Jose, CA, US). Cell cycle analysis was done as described previously,[15] at 6 h after DHMEQ treatment. The resulting DNA histograms were interpreted using the FlowJo (Tree Star Inc., Ashland, OR, US) combined with the Watson Pragmatic model.

Caspase activity detection. Activation of caspases-3, -8, and -9 were assessed by Carboxyfluorescein FLICA Apoptosis Detection Kit (Immunochemistry Technologies, Bloomington, MN, USA) according to the manufacturer's instructions. Cleavage of the caspases was confirmed by detection of cleaved products by immunoblot analysis using antibodies specific to cleaved products of caspase 3 and 9, or reactive to both cleaved and uncleaved caspase-8. The antibodies used are: cleaved caspase-3 (Asp175) antibody, caspase-8 (1C12) mouse mAb, and cleaved caspase-9 (Asp330) antibody (human-specific; all form Cell Signaling Technology, Beverly, MA, USA). Immunoblotting analysis was done basically as described previously.[18]

Microarray experiment. Total RNA was extracted from BC1 and BCBL1 cells treated with or without DHMEQ (10 μg/mL) for 6 h, using Trizol. Whole Human Genome Oligo Microarray Kit (Agilent Technologies, Santa Clara, CA, USA) was used to evaluate the gene expression in PEL cell lines according to the manufacturer's protocols. Whole data analyses were conducted using the software GeneSpring (Agilent Technologies). First, Student *t*-tests were performed in order to extract genes with expression levels significantly different between samples with and without DHMEQ treatment ($P < 0.01$). Secondly, gene ontology analyses were performed on that group of genes in BC1 in order to categorize the selected genes at the significant levels of $P < 0.01$.

Reverse transcription–polymerase chain reaction (RT-PCR) and real-time RT-PCR. Total RNA was extracted from the cells using the ISOGEN Kit (Wako Chemical Industry, Osaka, Japan). First-strand cDNAs were synthesized using 2 μg of the total RNA and 500 ng Oligo $(dT)_{12-18}$ using SuperScript™ II RT (Invitrogen Japan, Tokyo, Japan). RT-PCR was done for 25 cycles by Gene *Taq* enzyme (Wako Chemical Industries) with 10 pmoles of HHV-8 gene-specific primers. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The primers for ORFK13 (*v-FLIP*), ORF72 (*v-cyclin*), ORF73 (*LANA*), ORF50 (*Rta*), ORFK9 (*v-IRF*) and ORF74 (*v-GPCR*) are the same as those used in the previous reports.[27–29]

For the real-time RT-PCR, cDNAs were synthesized by PrimeScript RT Reagent Kit (TAKARA Bio Inc., Shiga, Japan). PCR was performed with a SYBR Premix Ex*Taq* (TAKARA Bio Inc.) and the primer sets are listed in Table 1. Reactions were performed with a Thermal Cycler Dice Real Time System (TAKARA Bio Inc.) and analyzed using the manufacturer's software. For quantification, the expression levels of six genes were normalized with that of *GAPDH* gene.

***In vivo* therapeutic effect of DHMEQ.** Twenty male CB17 SCID mice were obtained from CLEA Japan Company. Mice at 5 weeks old were injected with 4×10^6 TY1 cells intraperitoneally. In the treatment group, DHMEQ dissolved in 0.5% carboxymethyl cellulose (CMC) (Sigma, St. Louis, MO, USA) solution was administered into the intraperitoneal region at a dose of 8 mg/kg, beginning 1 day before the inoculation and 3 times a week thereafter for 1 month. In the control group, mice were treated with dimethyl sulfoxide (DMSO) dissolved in 0.5% CMC solution by the same procedure. Mice were observed for 3 months and survival curves were calculated by Kaplan and Meier's method. The statistical significance was examined by Cox-Mantel test. A *P*-value < 0.05 was considered to be statistically significant.

Results

NF-κB activation was abrogated in PEL cell lines by DHMEQ. NF-κB is constitutively activated in primary PEL cells and cell lines derived from them.[9,30] Thus, we first confirmed NF-κB activation by EMSA and analyzed its subcomponents by supershift assays, using TY1, BCBL1 and BC1 cell lines. The results confirmed constitutive activation of NF-κB in BCBL1 and BC1 cell lines as previously reported,[9,30] and NF-κB activation was first confirmed in TY1 in this experiment. Supershift analyses showed that NF-κB bands were composed of p50, p65 and RelB in all cell lines, indicating constitutive activation of both canonical and non-canonical pathways. Furthermore, it was clearly demonstrated that the major NF-κB binding signals are composed of two bands, the lower one containing p50 and upper one p65 (Fig. 1A).

We next tested the effects of DHMEQ treatment of these cells. Results of EMSA demonstrated loss of DNA binding of NF-κB after 1–3 h. However, all cell lines showed significant recovery of NF-κB activities after short periods. TY1 and BCBL1 cells lost NF-κB binding activity at 1 h; however, recovery was evident at 3 or 6 h with almost full recovery at 24 h. BC1 cells recovered almost the initial level of NF-κB activity after 24 h (Fig. 1B). These results indicated that DHMEQ could transiently inhibit NF-κB activity in PEL cell lines with partial or full recovery of the activities in 1 day. In other lymphoid cell lines, recovery of NF-κB activity after DHMEQ treatment was not observed in 1 day,[16–18] although a weak binding of NF-κB could be detected sometimes at later time points (unpublished observation). Thus, relatively quick recovery of NF-κB activity appears to be a unique feature of PEL cell lines.

DHMEQ reduced cell viability in PEL cell lines. Next we examined the cell viability after 3 days incubation with different

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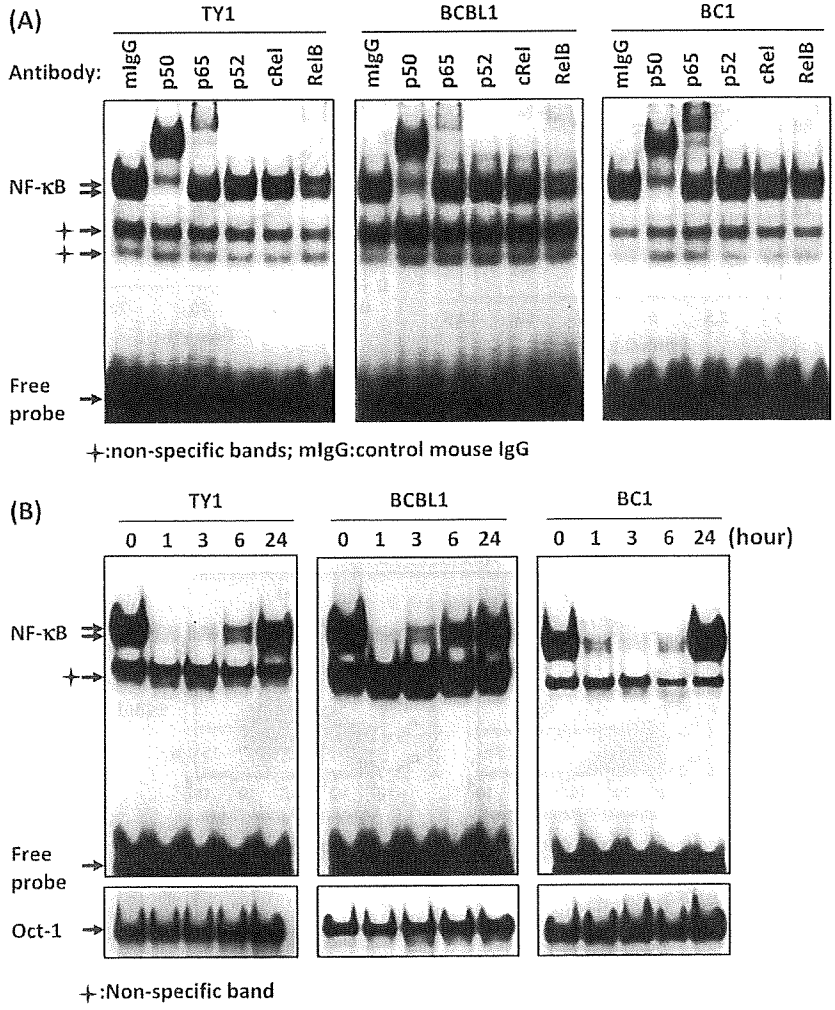

Fig. 1. Dehydroxymethylepoxyquinomicin (DHMEQ) inhibited constitutive NF-κB binding activity in primary effusion lymphoma (PEL) cell lines. (A) Sub-components of NF-κB activity in PEL cell lines. Nuclear extracts (1 μg) of cells were subjected to supershift analysis with indicated antibodies. (B) Effect of DHMEQ on NF-κB binding activity. PEL cell lines (TY1, BCBL1 and BC1) were treated with DHMEQ (10 μg/mL) for indicated hours. Nuclear extracts were examined for NF-κB binding activity by electrophoretic mobility shift analysis (EMSA) with a radio-labeled NF-κB-specific probe. Lower panels: results of Oct –1 probe as controls.

concentrations of DHMEQ (2.5, 5, 7.5 and 10 μg/mL). The results demonstrated a dose-dependent decrease in the cell viability for all PEL cell lines, but not for a control cell line, K562, without NF-κB activation. Peripheral blood mononuclear cells (PBMCs) and primary B-cells were shown to be resistant to DHMEQ treatment,[15,16,26] and most B-cell lines without HHV-8 or EBV were sensitive to DHMEQ when they have activated NF-κB.[16,17] The decrease in the cell viability was also time-dependent as we measured the cell viability at 24, 48, and 72 h after DHMEQ (10 μg/mL) treatment (Fig. 2). Thus, DHMEQ decreased the PEL cell viability in a dose- and time-dependent manner.

DHMEQ induced apoptosis in PEL cell lines. After 24 h of DHMEQ treatment (10 μg/mL), Annexin V reactivity was examined. Propidium iodide (PI) staining was included to discriminate the necrotic cells from apoptotic ones. Flow cytometry demonstrated a significant shift of the cell population toward Annexin V-positive areas in all cell lines, although some of the cells shifted toward PI-single positive areas (Fig. 3A). Summarized results are shown in Table 2, where results of three triplicate independent experiments are presented with the mean and SD. Furthermore, TUNEL assay demonstrated DNA fragmentation in all cell lines at 48 h (Fig. 3B). These results provided evidence for apoptosis induction by DHMEQ treatment in PEL cell lines.

DHMEQ activated both membrane and mitochondrial caspase pathways. Apoptosis can be induced via membrane and/or mitochondrial stimuli, each of which has their own specific pro-caspases to become active after receiving stimuli. Carboxyfluorescein FLICA Apoptosis Detection Kit revealed cleaved products of caspases-3, -8, and -9 at the same time after 6 h of DHMEQ treatment (Fig. 4A). Immunoblot analyses clearly

Table 2. Quantitative results of staining primary effusion lymphoma (PEL) cell lines with Annexin V and propidium iodide (PI)

	DHMEQ	BCBL1 (%)	TY1 (%)	BC1 (%)
UL	(–)	0.71 ± 0.14	1.96 ± 1.07	0.55 ± 0.09
	(+)	7.13 ± 3.82	6.06 ± 0.50	5.04 ± 1.60
UR	(–)	1.94 ± 0.57	2.84 ± 1.92	1.96 ± 1.02
	(+)	45.92 ± 17.04	37.89 ± 15.36	38.53 ± 20.95
LL	(–)	95.37 ± 0.29	90.91 ± 4.07	93.10 ± 2.13
	(+)	34.70 ± 11.14	33.62 ± 0.82	43.58 ± 12.14
LR	(–)	1.96 ± 0.45	4.28 ± 1.61	4.37 ± 2.20
	(+)	12.34 ± 2.97	22.41 ± 14.32	12.83 ± 9.69

PEL cells incubated with or without dehydroxymethylepoxyquinomicin (DHMEQ) (10 μg/mL) for 24 h and stained with Annexing V and PI. Then, analyzed with flow cytometry. Upper left (UL), upper right (UR), lower left (LL) and lower right (LR) quadrants are responsible for PI positive only, PI and AnnexinV positive, PI and Annexin V negative, Annexin V positive only cell populations, respectively. Results are mean value ± SD of 3 triplicate independent experiments.

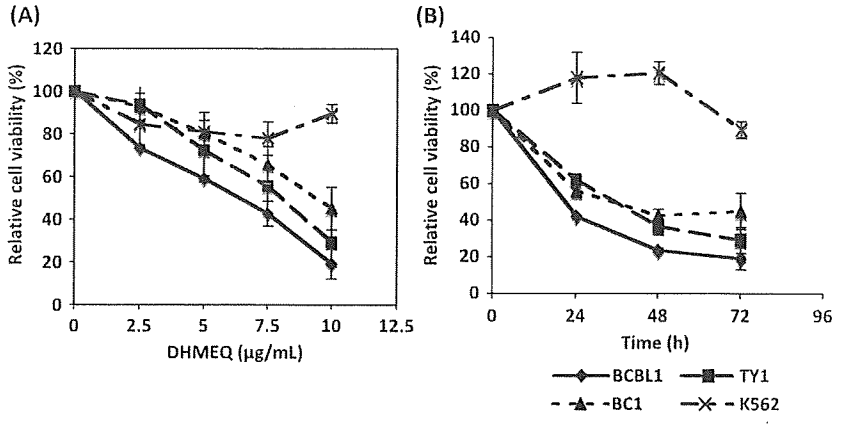

Fig. 2. Dehydroxymethylepoxyquinomicin (DHMEQ) reduced viability of primary effusion lymphoma (PEL) cells. PEL cell lines and K562 cells were treated with indicated concentrations of DHMEQ for 72 h (A) or were treated for the indicated hours with 10 μg/mL of DHMEQ (B). K562 cell line, without NF-κB activation, was used as a control. The cell viability was determined by WST-8 (Dojindo). The mean percentages of triplicate experiments compared with untreated cells are shown with standard deviation (SD).

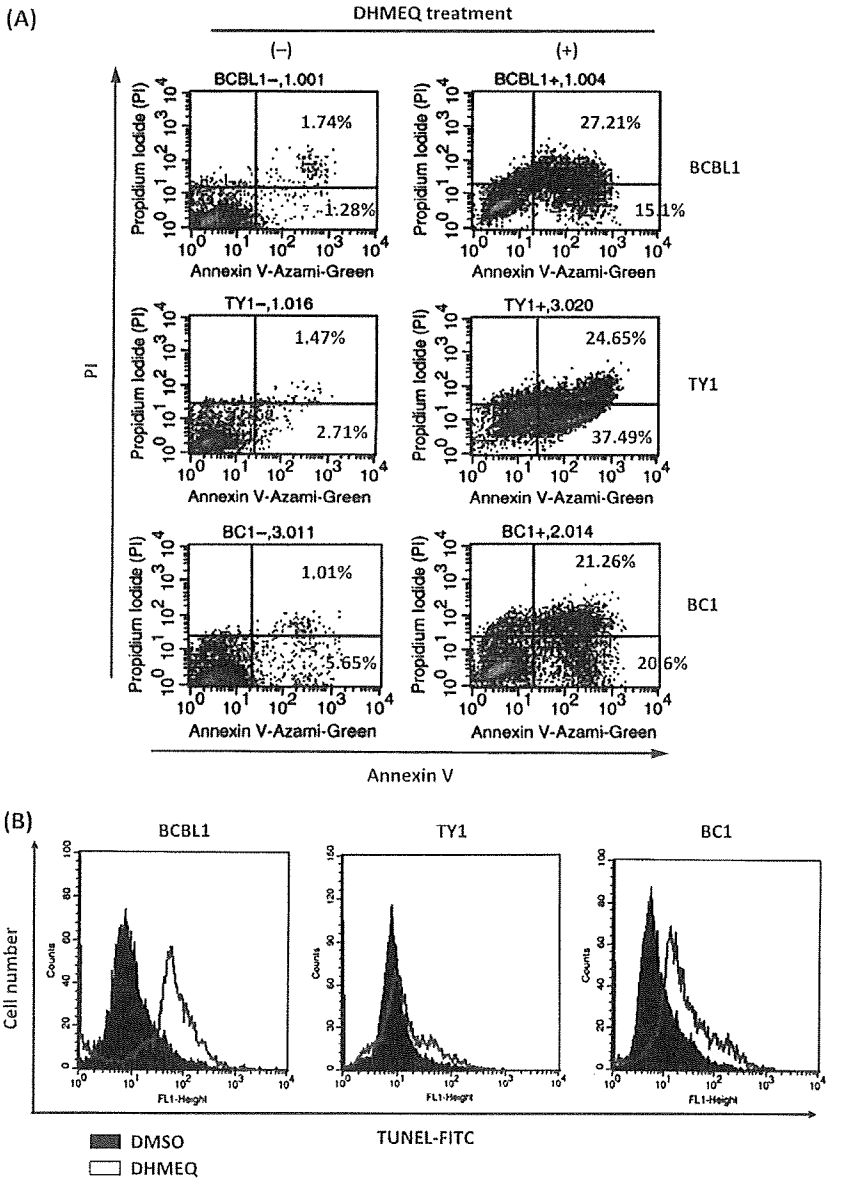

Fig. 3. Dehydroxymethylepoxyquinomicin (DHMEQ) induced apoptosis in primary effusion lymphoma (PEL) cell lines. (A) Annexin V reactivity in PEL cell lines after DHMEQ treatment. A representative result of three triplicate independent experiments is presented. PEL cell lines were treated with or without DHMEQ (10 μg/mL) for 24 h, and binding of Annexin V and intercalation of propidium iodide (PI) were analyzed by flow cytometry. (B) DNA fragmentation in the PEL cell lines after DHMEQ treatment. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was done for PEL cell lines after 48 h incubation with or without DHMEQ (10 μg/mL).

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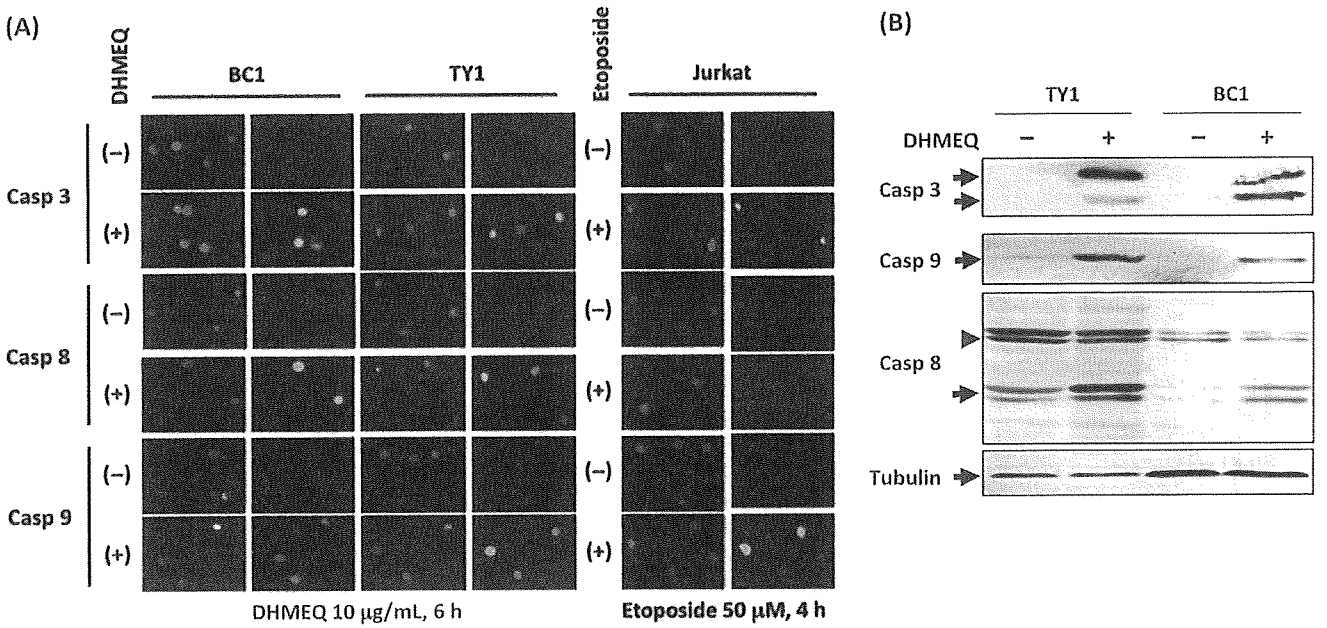

Fig. 4. Both membrane and mitochondrial caspase pathways were activated by dehydroxymethylepoxyquinomicin (DHMEQ) treatment of primary effusion lymphoma (PEL) cells. (A) Cleaved products of caspases were detected after DHMEQ treatment (10 μg/mL) for 6 h. Caspase 3, responsible for the common pathway, caspase 8, as an indicator for the membrane pathway, and caspase 9 as an indicator for the mitochondrial pathway. Jurkat cell line treated with etoposide 50 μM for 4 h served as a control. DNAs were stained by Hoechst 33258 (blue fluorescence). Green fluorescence indicated cleaved products of caspases bound to FLICA peptides. (B) Immunoblot analysis of caspase cleavage. TY1 and BC1 cells were treated with 10 μg/mL of DHMEQ for 6 h. Samples of 30 μg of whole cell lysates were examined. Positions of cleaved forms of caspase 3 and 9 are indicated on the left of the upper two panels (arrows). In the third panel, uncleaved and cleaved forms of caspase 8 are indicated on the left (arrowhead: uncleaved). An immunoblot of α-tubulin served as a control (bottom panel).

demonstrated production of cleaved products of caspase-3, -8 and -9 at the same time after 6 h of DHMEQ treatment (Fig. 4B).

Taken together, the results suggested that both caspase pathways became active in the PEL cell lines as a result of incubation with DHMEQ for 6 h.

DHMEQ modulated NF-κB target genes, apoptotic and cell cycle regulating genes. Whole human genome expression analysis was done to obtain a comprehensive view of DHMEQ effect on PEL cell lines, and to confirm the efficient targeting of the NF-κB pathway. DHMEQ down- or up-regulated expression levels of 72 and 71 genes, respectively, in BC1 and BCBL1 cell lines (Fig. 5A). Down-regulation was observed in NF-κB target genes, such as *IL6*, *Myc*, *CCR5*, *BCL-xL*, *cIAP2* and *NF-κB1*. *Bcl-xL*, *c-IAP2*, and *NF-κB1* are also anti-apoptotic genes. Other anti-apoptotic genes, such as *Birc5* and *IGF1R*, were down-regulated. However, some anti-apoptotic genes including *SSP1*, *VEGF*, *MIF* and *BAG3* were up-regulated. Some of pro-apoptotic genes such as *DEDD2*, *CDKN1A* and *APOE* were up-regulated, whereas *TNFSF10* was down-regulated. Most of the genes involved in cell cycle arrest were up-regulated, the examples of which were *CDKN1A*, *CDKN1B*, *PPP1R15* A and *DDIT3* (Supporting Information).

For validation of above results, we performed real-time RT-PCR analysis. For this purpose, we selected three up-regulated and three down-regulated genes. The results provided evidence that validate the results of expression array analysis, showing up- or down-regulation of the selected genes (Fig. 5B). Furthermore, the levels of up- or down-regulation appeared to well correlate with those obtained by the expression array analysis described above.

Since the genes involved in cell cycle arrest were induced by DHMEQ, we next examined effects of DHMEQ treatment on the cell cycle regulating using three cell lines. The results showed significant accumulation of the cells in G2/M phase at 6 h of treatment (Fig. 5C,D).

Gene ontology analysis on the genes the expression levels of which were significantly altered by the addition of DHMEQ in BC1 cells (P-value < 0.01) revealed that the majority of the genes in the following categories were down-regulated: (i) negative regulators of NF-κB import to the nucleus; (ii) IκB kinase and NF-κB cascade; (iii) negative regulators of apoptosis; (iv) DNA repair; and (v) cell cycle checkpoint. In contrast, the majority of the genes in the following categories were up-regulated: (i) positive regulators of apoptosis; (ii) cell cycle arrest; and (iii) regulators of cyclin-dependent protein kinase activity (Fig. 5E).

Taken together, we observed a trend toward induction of pro-apoptotic and cell cycle arrest genes, concomitant with suppression of NF-κB target, anti-apoptotic and DNA repair genes.

DHMEQ did not induce HHV-8 reactivation. Semi-quantitative RT-PCR was done to examine changes in the viral gene expression after the DHMEQ treatment. Some viral genes known as 'lytic genes' were selected as well as those with important viral functions. The results demonstrated low or undetectable levels of lytic gene expression without significant changes until 14 h of treatment, suggesting that NF-κB inhibition by DHMEQ treatment did not lead to viral production in these cells (Fig. 6). Expression levels of *v-FLIP*, *v-cyclin*, and *LANA* were slightly up-regulated after 3–6 h of DHMEQ treatment, returning to the basal levels after 14 h. It is worthy of note that *v-FLIP*, a potent viral activator of NF-κB pathway, showed almost constant levels of expression irrespective of DHMEQ treatment. Taken together, we could not obtain evidence for induction of virus proliferation that was previously reported as an effect of NF-κB inhibition in PEL cell lines.[31]

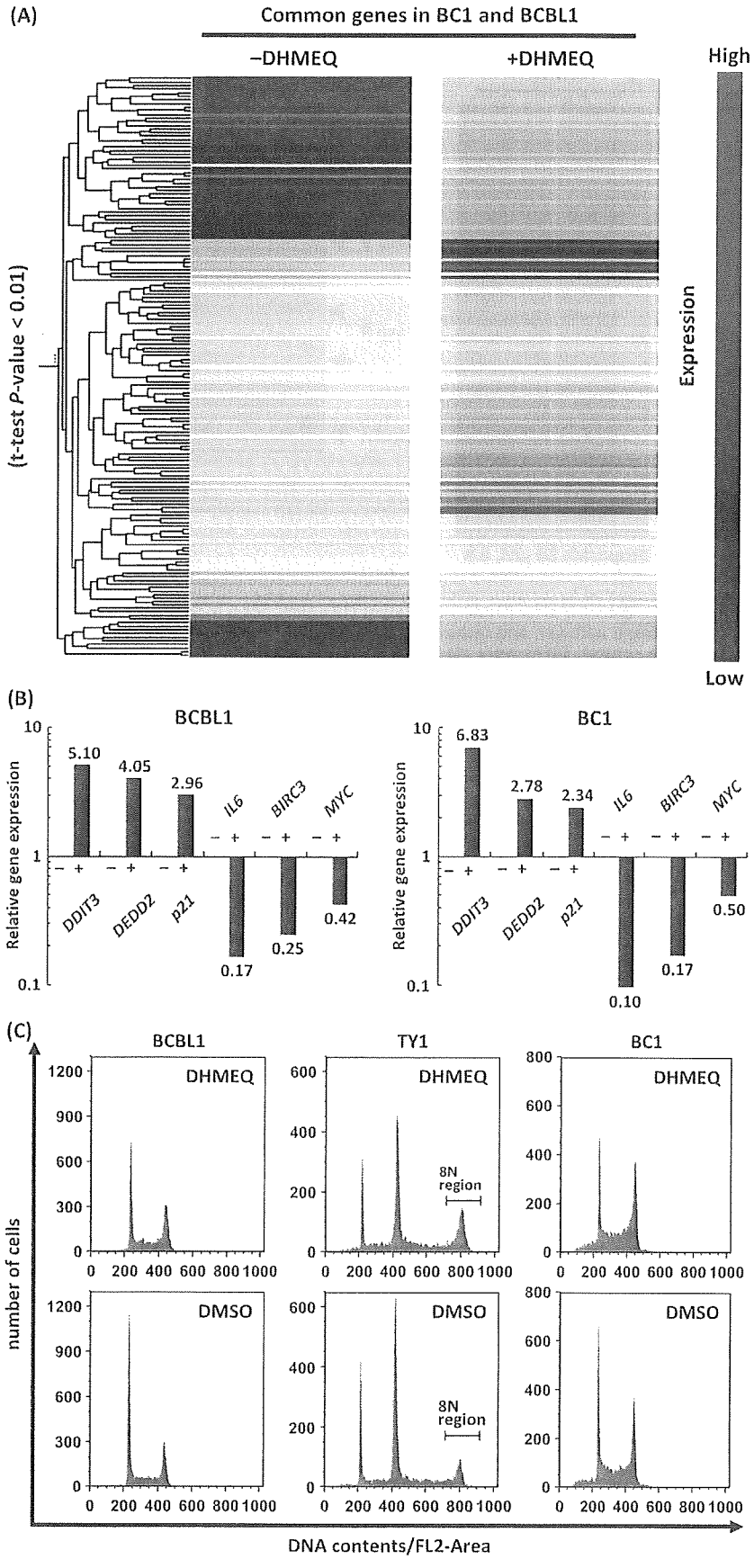

Fig. 5. Dehydroxymethylepoxyquinomicin (DHMEQ) modulated genes responsible for proliferation, cell cycle, pro- and anti-apoptosis. Whole Human Genome Oligo Microarray Kit (Agilent Technologies) was used to evaluate the gene expression in BC1 and BCBL1 with or without DHMEQ (10 μg/mL), 6 h. (A) The result of cluster analysis showed significant changes in expression levels between ±DHMEQ in both cell lines (*t*-test, *P*-value < 0.01). (B) Results of real-time reverse-transcription–polymerase chain reaction for validation of the array data. Up- or down-regulation of selected genes was confirmed in both cell lines tested. (C) Effects on cell cycle regulation. Representative results of flow cytometry after 6 h of DHMEQ are presented. TY1 cells appear to be a mixture of 2 N and 4 N populations. (D) Percentages of cells in G2/M phase. In TY1 cells, the percentage of the 8 N region was considered to represent a significant part of the cells at G2/M, and was used for comparison. Differences were statistically significant (*1, $P < 0.05$, *2, $P < 0.05$, *3, $P < 0.001$) (E) Gene ontology analysis on BC1 genes of which expression levels were significantly changed by addition of DHMEQ (*t*-test, *P*-value < 0.01). The graph shows numbers of the genes in following selected categories: a, negative regulators of NF-κB import into nucleus; b, IκB kinase and NF-κB cascade; c, positive regulators of apoptosis; d, negative regulators of apoptosis; e, DNA repair; f, cell cycle arrest: g, cell cycle checkpoint; h, regulators of cyclin dependent protein kinase activity. Positive and negative areas represent the number of up-regulated genes, and those of down-regulated genes, respectively.

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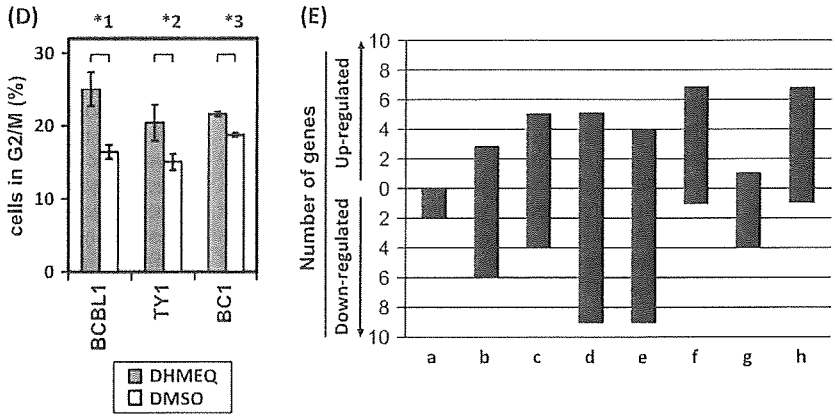

Fig. 5. Continued

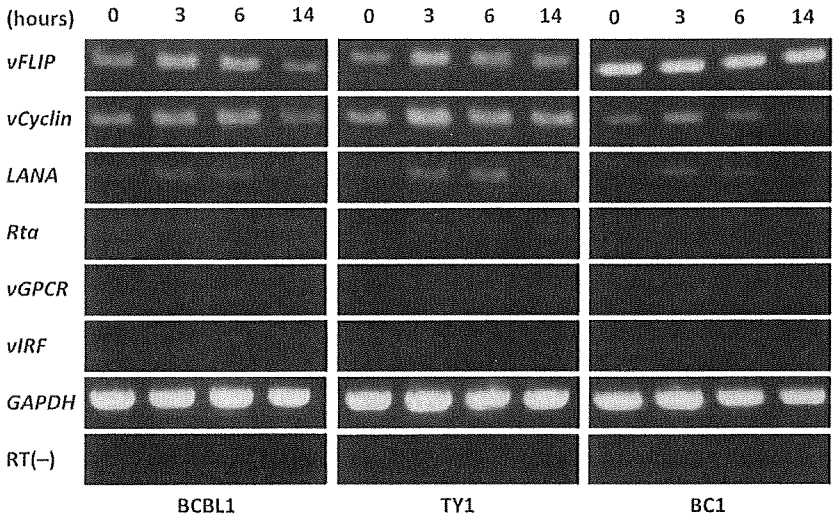

Fig. 6. Viral gene expression after dehydroxymethylepoxyquinomicin (DHMEQ) treatment showed no transition from latent to lytic phase. Viral gene expression after DHMEQ treatment (10 μg/mL) at different time points was examined by semiquantitative reverse-transcription–polymerase chain reaction. Two micrograms of total RNA were used to prepare cDNA using oligo(dT)$_{12-18}$ primer. *GAPDH* and RT(–) served as controls.

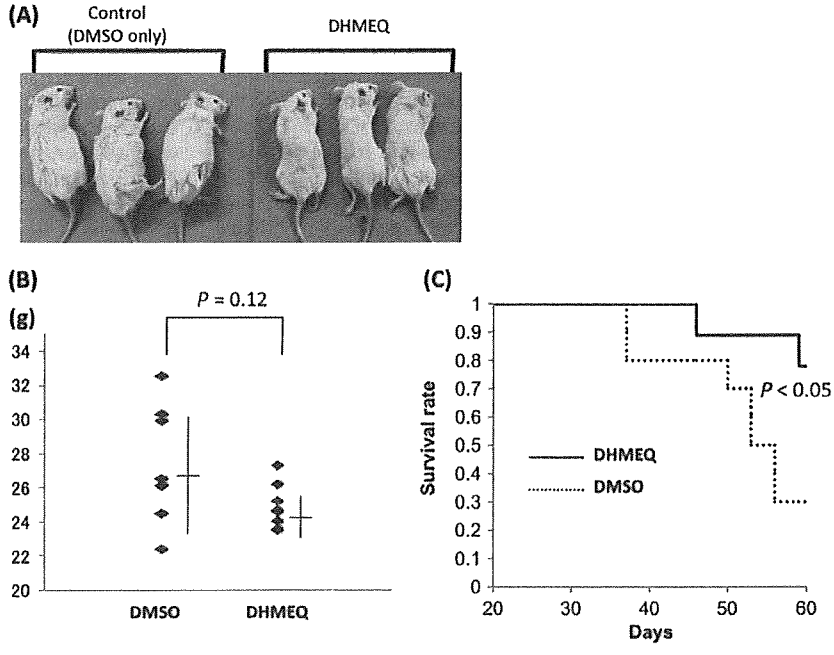

Fig. 7. Dehydroxymethylepoxyquinomicin (DHMEQ) rescued primary effusion lymphoma (PEL) xenografted mice. Mice at 5 weeks old were injected with 4×10^6 TY 1 cells intraperitoneally. DHMEQ (8 mg/kg) in 0.5% CMC was administered three times a week intraperitoneally for 1 month. Dimethyl sulfoxide in 0.5% CMC was administered to the control group. (A) Gross appearances of the mice. (B) A graph showing weights of mice with mean values. (C) Survival curves of mice with or without DHMEQ treatment.

DHMEQ showed a potent inhibitory effect on the growth of PEL cells in SCID mice. We next examined whether DHMEQ treatment could be effective against xenografted tumors in a SCID mice model. TY1 cells were injected intraperitoneally into the SCID mice and DHMEQ or vehicle alone was administered according to the protocol described in the Materials and Methods section. The gross appearance of the mice with or without DHMEQ treatment was significantly different, showing abdominal distention in vehicle-treated mice, whereas DHMEQ-treated mice were apparently normal in body shape. The body weights of the DHMEQ-treated mice were much less than those of vehicle-treated mice after 1 month, although the difference was not statistically significant (Fig. 7A,B). DHMEQ treatment rescued 8/10 xenografted mice at the point of 3 months, whereas only 3/10 survived in the vehicle-treated control group. The results are similar to those of our previous experiments in animal models of adult T-cell leukemia (ATL).[32,33] Statistical analysis showed a significant increase in the survival rate in the mice treated with DHMEQ compared with the control (Cox-Mantel test; $P < 0.05$, Fig. 7C). These results demonstrated that DHMEQ was effective against PEL cells *in vivo*, which provided supportive evidence for possible clinical application.

Discussion

The present work demonstrated that DHMEQ transiently abrogated the NF-κB activation in PEL cell lines with significant recovery of NF-κB activity after 24 h. Apoptotic cell death was observed in a few days without reactivation of HHV-8. Furthermore, DHMEQ rescued PEL-xenografted SCID mice preventing formation of tumors and effusions. Thus, we suggest DHMEQ as a promising candidate for molecular targeted therapy of the PEL.

NF-κB activity recovered significantly after 24 h of DHMEQ-treated PEL cell lines, which was not observed in other lymphoid cell lines we have so far tested,[16–18] although low levels of NF-κB recovery were observed after 24 h or later (unpublished observation). Thus, the rapid and significant recovery of NF-κB activity can be considered as a characteristic of PEL cell lines. Transient NF-κB inhibition can be an advantage for DHMEQ compared with other NF-κB inhibitors, since persistent inhibition of the NF-κB pathway may lead to adverse effects on innate and acquired immune systems where NF-κB plays a pivotal role. The mechanism for recovery of the NF-κB activity remains to be studied. It may be due to degradation of DHEMQ within the cells and/or persistence of the NF-κB activating stimuli that can overcome the effects of DHMEQ. No information is available at present as to the half-life of DHMEQ in the cell. On the other hand, persistence of high levels of NF-κB activating signals may be explained by v-FLIP, a well-known viral stimulator of the NF-κB pathway,[7,34,35] that was expressed at stable levels irrespective of DHMEQ treatment (Fig. 6).

Another benefit of DHMEQ may reside in targeting the translocation of p65 into nuclei. Generally, target specificity of kinase inhibitors is not strict. For example, Bay11-7083 has non-specific activities on other kinases along with tyrosine phosphorylation of a protein of unknown origin.[36] Furthermore, Bay11-7085 does not inhibit translocation of p65 into nuclei, and shows non-specific inhibition of binding of other transcription factors to its binding sequences.[37] On the other hand, DHMEQ treatment results in disappearance of p65 and p50 in the nuclei of cell lines of Hodgkin-Reed-Sternberg (H-RS) cells and ATL, not affecting binding of other transcription factors such as AP-1 and Oct1 in EMSA.[15,16] Taken together, the specific activity of DHMEQ appears to be an advantage over other NF-κB inhibitors with respect to avoiding adverse effects.

The apoptosis cascade was triggered with transient abrogation of NF-κB by DHMEQ treatment. Furthermore, subsequent recovery of NF-κB activity could not rescue the DHMEQ-treated PEL cells, suggesting that DHMEQ triggered irreversible activation of the apoptosis cascade. These observations are in line with those discussed in the context of 'oncogene addiction' and 'oncogenic shock'.[38–40] Sharma *et al.* proposed a model referred to as 'oncogenic shock' to account for the observed apoptotic outcome resulting from the acute inactivation of oncoproteins in addicted cancer cells. According to this model, proapoptotic as well as prosurvival signals are both outputs emanating from the same addicting oncoprotein, and the differential decay rates associated with these two broad classes of signals following oncoprotein inactivation, leads to a signal imbalance that contributes to cell death.[39,40] The results of our gene ontology analyses demonstrated that more anti-apoptotic genes are down-regulated at 6 h compared with pro-apoptotic genes (Fig. 5B), which suggest a condition after acute inactivation of the oncoprotein, where rapid attenuation of oncoprotein-generated prosurvival signals is associated with lingering pro-apoptotic signals.

The pathway analysis of apoptosis revealed activation of both membrane and mitochondrial pathways, which is evidenced by detection of cleaved products of caspases-8 and -9 at the same time. Previously we reported the same results using human T-cell leukemia virus type I-transformed cells and cell lines of H-RS cells and multiple myeloma.[15,16,18] Since links between the receptor and the mitochondrial pathways exist at different levels, upon death receptor triggering, activation of caspase-8 may result in cleavage of Bid, a Bcl-2 family protein with a BH3 domain only, which in turn translocates to mitochondria to release cytochrome c thereby initiating a mitochondrial amplification loop.[41,42] In addition, cleavage of caspase-6 downstream of mitochondria may feed back to the receptor pathway by cleaving caspase-8.[43] Thus, the data can be interpreted in either context, leaving the exact mechanism of DHMEQ-induced apoptosis remaining to be studied.

DHMEQ did not induce transition from the latent to lytic phase of HHV-8 (Fig. 6). There are controversies as to whether inhibition of NF-κB leads to replication of HHV-8,[31] or not.[10] Another investigator suggested that NF-κB is necessary for HHV-8 replication.[44] However, we did not find any significant differences in the expression of known lytic genes after NF-κB inhibition (Fig. 6). Thus, our results did not support the idea that DHMEQ administration may increase the risk for viral replication. Furthermore, the latent viral genes such as *LANA*, *v-FLIP*, or *v-cyclin* did not show significant changes in the levels of gene expression. RNA interference results showed that v-FLIP is essential for the survival of PEL cells.[8] Our data indicated that NF-κB activation, not v-FLIP itself, was essential for survival of PEL cells, since apoptosis was induced in the presence of stable levels of v-FLIP expression.

Inhibition of NF-κB causes profound effects on cellular gene expression by direct and indirect mechanisms. The results of expression array analysis confirmed down-regulation of many NF-κB target genes, which was mostly in accordance with previously reported results of NF-κB inhibition by Bay11-7082 in EBV-infected lymphoblastoid cell lines.[45] Although the suppressions of *IL6* and *cIAP2* were observed previously by using Bay11-7082 on a PEL cell line,[10] we could not find significant suppression in *c-FLIP*, *cIAP1*, *TRAF2* or *IκBα* as they reported. Instead, we found *Bcl-xL*, *Myc*, *CCR5*, *NF-κB1* and *FAS* among the down-regulated genes. These differences could be due to different agents used, with different specificity of activities. Suppression of other anti-apoptotic or cell cycle progression genes could be mediated by the functions of other genes, which were modulated by NF-κB inhibition. The result of gene ontology biological process analysis of the genes with significant changes in expression levels were reasonable because it showed a trend toward induction of pro-apoptotic and cell cycle

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arrest genes, concomitant with suppression of IκB kinases, anti-apoptotic and DNA repair genes. The results appear to represent the expression profile corresponding to 'oncogenic shock'.[39,40]

In vivo experiments showed that PEL xenografted mice without treatment tended to be heavier, both in appearance and weight. Although we could not find statistical significances between the two groups, the mean value of the body weight was higher in the control group, which was explained by the fact that untreated mice developed tumors and effusion in body cavities. Besides, the treated group apparently did not have either effusions or tumors. The survival rate was significantly better in the treated group, indicating that DHMEQ could rescue the PEL-xenografted mice.

In summary, the present work demonstrated that DHMEQ could abrogate NF-κB activation transiently and initiated the apoptosis cascade irreversibly without activation of HHV-8 replication. In addition, DHMEQ rescued the xenografted mice. Therefore, our data provided proof of the concept that DHMEQ can be a promising candidate for molecular target therapy of the PEL.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

List of up-regulated genes after DHMEQ (10 μg/mL for 6 h) treatment in BC1 and BCBL1 calculated by GeneSpring software (*T*-test, *P*-value < 0.01).

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Long-Term Administration of Valacyclovir Reduces the Number of Epstein-Barr Virus (EBV)-Infected B Cells but Not the Number of EBV DNA Copies per B Cell in Healthy Volunteers▽†

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Epstein-Barr virus (EBV) establishes a latent infection in B cells in the blood, and the latent EBV load in healthy individuals is generally stable over time, maintaining a "set point." It is unknown if the EBV load changes after long-term antiviral therapy in healthy individuals. We treated volunteers with either valacyclovir (valaciclovir) or no antiviral therapy for 1 year and measured the amount of EBV DNA in B cells every 3 months with a novel, highly sensitive assay. The number of EBV-infected B cells decreased in subjects receiving valacyclovir (half-life of 11 months; $P = 0.02$) but not in controls (half-life of 31 years; $P = 0.86$). The difference in the slopes of the lines for the number of EBV-infected B cells over time for the valacyclovir group versus the control group approached significance ($P = 0.054$). In contrast, the number of EBV DNA copies per B cell remained unchanged in both groups ($P = 0.62$ and $P = 0.92$ for the control and valacyclovir groups, respectively). Valacyclovir reduces the frequency of EBV-infected B cells when administered over a long period and, in theory, might allow eradication of EBV from the body if reinfection does not occur.

Primary infection with Epstein-Barr virus (EBV) is frequently asymptomatic in infants and children, but infection of adolescents and young adults can result in infectious mononucleosis. EBV is associated with several malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and lymphoproliferative disease, in immunocompromised and immunocompetent persons (6, 20).

In healthy EBV-seropositive persons, about 1 to 10 in 10^5 peripheral B cells are infected with EBV (14). The virus establishes latency in memory B cells. The level of the latent EBV load in healthy individuals remains stable over time, maintaining a "set point" for each individual (19). It is uncertain how this "set point" is maintained, but the latent EBV load is thought to reflect a balance between removal of EBV-infected cells due to the half-life of memory B cells and reinfection of new memory B cells during virus reactivation. The EBV genome replicates when B cells latently infected with EBV divide using the host DNA polymerase, which is not sensitive to the action of acyclovir. However, when the virus reactivates in latently infected B cells, EBV replicates using the viral DNA polymerase, which is inhibited by the phosphorylated form of acyclovir. Therefore, blocking production of new virus with acyclovir should decrease the latent EBV load at a rate equivalent to the half-life of memory B cells. Patients with zoster who were treated with oral acyclovir for 28 days showed no reduction in the EBV load in the blood, despite complete inhibition of EBV shedding in the saliva (23). These results suggested that antiviral therapy for longer than 28 days is necessary to detect a reduction in the EBV load in the blood.

In this study, we administered either valacyclovir (valaciclovir) (which is absorbed more effectively than acyclovir and metabolized to acyclovir) or no antiviral to healthy volunteers for 12 months and measured the level of EBV DNA in the blood every 3 months.

MATERIALS AND METHODS

Subjects. Patients at the National Institutes of Health Clinical Center and at the University of Texas Center for Clinical Studies with a history of recurrent genital herpes with three to nine recurrences a year and either a positive culture for herpes simplex virus (HSV) from the genital area or a positive serology for HSV type 2 (HSV-2) received valacyclovir at 500 mg per day for 1 year. Patients with genital herpes were required to be off HSV-suppressive therapy for 3 months before entering the study. The control group did not have symptomatic genital herpes and received no antiviral therapy. Subjects were excluded if they were receiving immunosuppressive therapy, had malignancy, or were infected with human immunodeficiency virus. Informed consent was obtained from all subjects, and the study was approved by the institutional review boards of the National Institute of Allergy and Infectious Diseases and the University of Texas Center for Clinical Studies. Blood was obtained from the valacyclovir-treated group and the control group at day 0 (before initiating valacyclovir) and at 3, 6,

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