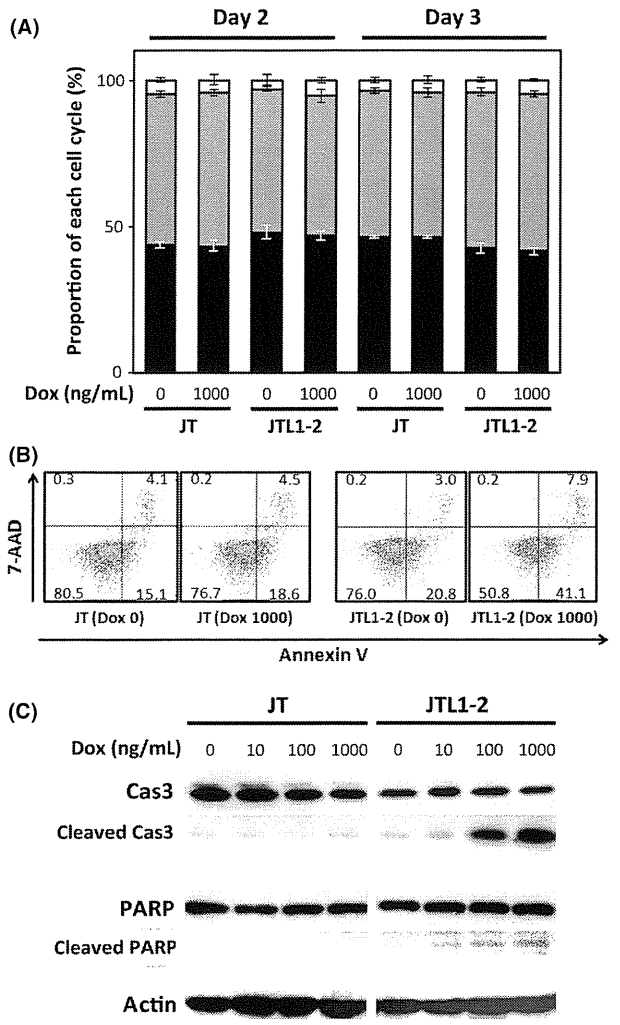

Figure 3. Cell cycle and apoptosis in JT and JTL1-2 cells. (A) Cell cycle analysis of JT and JTL1-2 cells was performed 2 and 3 days after induction with Dox (0 or 1000 ng/mL). Experiments were performed in triplicate and data are presented as means with standard errors. Black, gray, and white represent the ratio of cells in G1, S, and G2/M, respectively. (B) To assess the apoptosis, 2 days after the Dox induction (0 or 1000 ng/mL), JT and JTL1-2 cells were stained with 7-AAD and Annexin V and analyzed by FACS. The numbers in the corner of each quadrant indicate the percentage of cell events within the quadrant. Early apoptotic cells were defined as those positive for Annexin V but negative for 7-AAD. (C) Cell extracts harvested 2 days after Dox induction were analyzed by western blotting for the apoptosis markers, caspase-3 (Cas3) and poly(ADP-ribose) polymerase (PARP).

CAEBV-derived cells (relative quantity to $\beta 2$ m: SNT16, 0.14 in Fig. 1A; SNT13, 0.0090 [17]; SNT15, 0.022 [17]). However, JTL1-2 induced with 1000 ng/mL Dox expressed slightly higher levels of LMP1 compared to the other CAEBV cell lines tested (Fig. 1A). Therefore, JTL1-1 and JTL1-2 express an adequate range of LMP1 levels to evaluate its role in T cells.

Expression of LMP1 failed to increase cell proliferation and growth signal intensity

After successfully generating T cell lines that express LMP1 in response to Dox, the cell proliferation rates were analyzed in the presence of 0, 10, 100, or 1000 ng/mL Dox (Fig. 2A). The parental cell line, JT, had the fastest growth rate of the three cell lines, with the untreated controls reaching about 20×10^5 cells by day 4 (please take notice that the scale of y-axis is different in JT cells). The growth of JT cells slowed with increasing concentrations of Dox, suggesting that higher concentrations of Dox might be slightly toxic to the cells. Compared to JT and JTL1-1 cells, the growth of JTL1-2 cells was inhibited significantly after abundant LMP1 expression had been triggered by high concentrations of Dox (Fig. 2A). This suggests that LMP1, the major oncogene of EBV, may not confer a growth advantage to T cells, at least in Jurkat cells, under conditions of exogenous expression.

We then measured the activities of AKT and NFκB signaling pathways, which are activated by LMP1 in B cells. When LMP1 was expressed in a dose-dependent manner by increasing concentrations of Dox, the phosphorylation of AKT in JTL1-2 cells decreased (Fig. 2B). We also assessed the levels of the p65 component of NFκB and IκBα, the major inhibitor of NFκB. We found that the p65 levels were comparable, but that the expression of IκBα increased concurrently with LMP1 expression in JTL1-2 cells (Fig. 2B). The mRNA expression of IκBα, as assessed by microarray analysis, was also upregulated in JTL1-2 cells but not in JTL1-1 cells (data not shown).

These unexpected observations reveal that LMP1 inhibits cell growth and the activation of key signaling pathways, such as AKT and NFκB, in Jurkat cells, particularly when LMP1 is expressed abundantly. This contradicts previous studies that found that LMP1 induces cell proliferation through these pathways in B cells.

LMP1-induced apoptosis in JTL1-2 cells at high concentrations of Dox

Because of the unexpected effects of LMP1 on the growth of JTL1-2 cells, we assessed the cause of the decreased growth rate. Therefore, cell cycle and apoptosis were examined in JTL1-2 cells in the presence or absence of Dox (Fig. 3). We here did not examine cell cycle and apoptosis in JTL1-1 cells because cell growth inhibition rate of the JTL1-1 cells by Dox addition was almost comparable to the parental control cell line, JT (Fig. 2A).

Propidium iodide staining followed by FACS analysis showed that the ratio of cells in G1, S, and G2/M were comparable between JT and JTL1-2 cells, with or without Dox, after 2 or 3 days of incubation (Fig. 3A).

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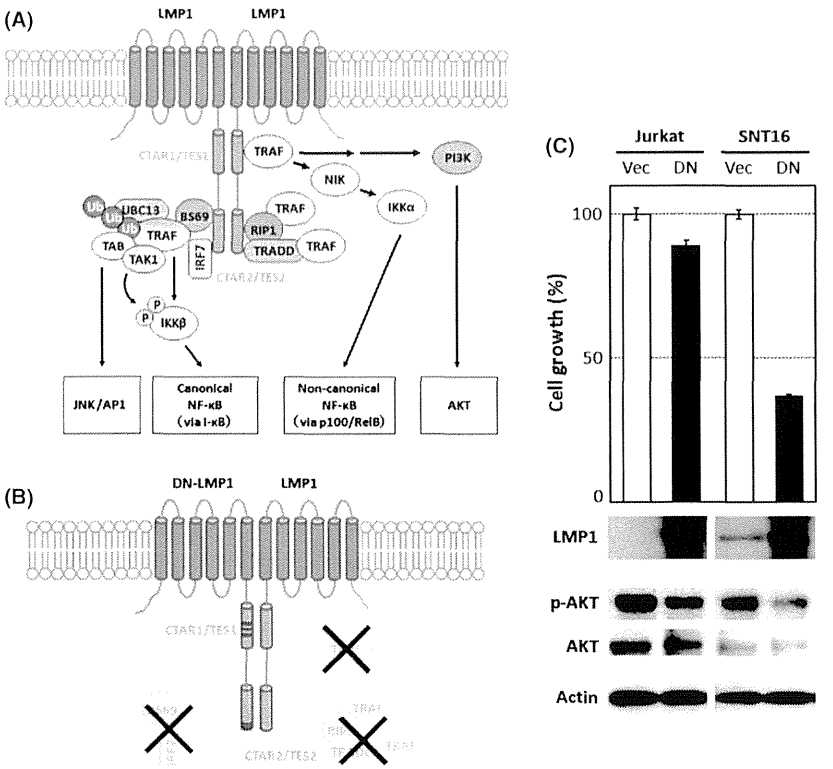

Figure 4. Dominant negative LMP1 inhibits proliferation of CAEBV T cells. (A) An illustration of the LMP1 signal pathway. LMP1 molecules form an oligomer that is required for its signaling activity (the oligomer is designated as a dimer for simplification). (B) DN-LMP1 has point mutations that modify the PXQXT motif in the TES1 domain to AXAXT and the YYD in the tail of TES2 to IID, resulting in the dysregulated signaling activity. (C) The growth rates of Jurkat and SNT16 cells were assessed after transient transfection with empty vector (Vec) or DN-LMP1 (DN). Experiments were performed in triplicate and data are presented as means with standard errors. Western blotting results for the expression of LMP1, phospho-AKT (pAKT), AKT, and actin are shown underneath the growth bars.

To monitor apoptotic cell death, in the Figure 3B, JT or JTL1-2 cells were stained with Annexin V, an early apoptosis marker that detects the abnormal localization of phosphatidylserine on the cell membrane, and 7-AAD, which enters cells and intercalates into nuclear DNA when the integrity of cell plasma membrane has been damaged in the later stages of apoptosis. The levels of both markers were similar in JT and JTL1-2 cells without Dox treatment (Fig. 3B). However, the proportion of Annexin V (+)/7-AAD (−) cells, indicative of early apoptosis execution program, increased to 41.1%, and the number of Annexin V (+)/7-AAD (+) cells, indicative of late apoptosis, also increased to 7.9% in JTL1-2 cells incubated with Dox (Fig. 3B).

To confirm these observations, we carried out western blotting for caspase-3 and poly (ADP-ribose) polymerase (PARP). Caspase-3 is a cysteine protease that plays a major role in apoptosis. Caspases cleave target proteins, including PARP, during the execution of apoptosis. Western blotting indicated that the increased apoptotic cell death in JTL1-2 cells was correlated with increased cleavage of caspase-3 and PARP, whereas the total levels of these proteins were unchanged (Fig. 3C). In addition, the proapoptotic gene, Jun was induced and the antiapoptotic gene, Bcl-2, were suppressed in our microarray analysis (data not shown). These results suggest that the inhibition of cell growth in JTL1-2 cells was due to the induction of apoptosis by abundant expression of LMP1.

DN-LMP1 inhibits proliferation of CAEBV-derived cell line

In the Jurkat T-cell background, the EBV major oncogene LMP1 did not enhance cell proliferation, and even more, it inhibited cell growth by inducing apoptotic cell death, particularly when high levels of LMP1 were produced. Because these data contradict published studies describing a proliferative role for LMP1, we used a more physiologically relevant cell line, SNT16, which is an EBV-positive cell line that was isolated from a CAEBV patient [14]. The EBV in SNT16 features latency type II, and so endogenous LMP1 is produced [16]. To assess whether LMP1 is

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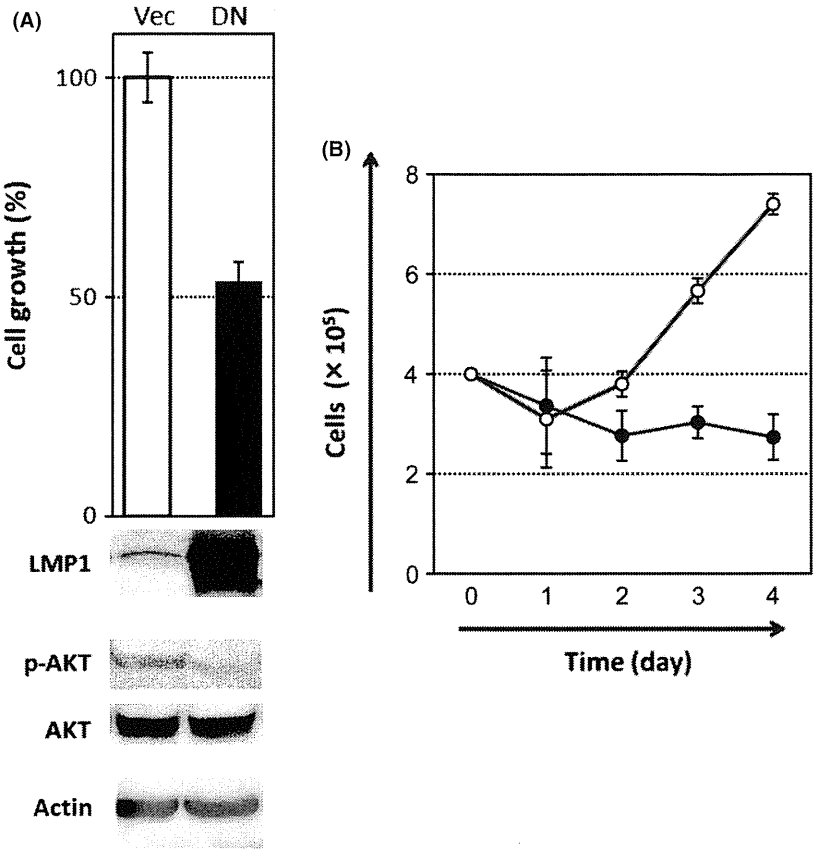

Figure 5. Dominant negative LMP1 inhibits proliferation of CAEBV NK cells. (A) As in Figure 4, the growth rates of KAI3 cells were assessed after transient transfection with empty vector (Vec) or DN-LMP1 (DN). Experiments were performed in triplicate and data are presented as means with standard errors. Western blotting results for the expression of LMP1, phospho-AKT (pAKT), AKT, and actin are shown underneath the growth bars. (B) As in (A), KAI3 cells were transfected with empty vector (white circles) or DN-LMP1 (black circles). Cell numbers were counted on indicated days after transfection.

necessary for T-cell proliferation in SNT16 cells, we attempted to knockdown LMP1 by siRNA and shRNA, but were unable to due to unknown technical difficulties. Therefore, we inhibited LMP1 activity using a DN form of LMP1. An LMP1 mutant with artificial point mutations in the CTAR1/TES1 and CTAR2/TES2 domains acts as a DN to inhibit the function of native LMP1, because these domains are the sites that dock to signaling mediators, such as TRAF proteins (Fig. 4A and B) [11, 18, 19].

To test the effects of LMP1 in CAEBV, we transfected SNT16 cells with a vector expressing DN-LMP1. It is important to note that the expression of mutant LMP1 was higher than native LMP1 (Fig. 4C). As a control, Jurkat cells were also transfected with DN-LMP1, in parallel. SNT16 cells transfected with DN-LMP1 grew significantly slower than empty vector controls, by approximately 40% (Fig. 4C). In contrast, Jurkat cells transfected with DN-LMP1 grew only slightly slower than control (Vec), suggesting that the DN-LMP1 had little effect on the proliferation of cells lacking endogenous wild-type LMP1. The phosphorylation of AKT was correlated with the growth rate of both cell lines, suggesting that the DN-LMP1 blocked the native LMP1 signaling pathway by suppressing AKT phosphorylation (Fig. 4C).

In order to extend these results, we then tested KAI3, an EBV-positive NK cell line derived from a CAEBV patient. Expression of DN-LMP1 caused significant decrease in growth of KAI3 cells, which correlated with weak phosphorylation of AKT (Fig. 5A). When cell proliferations were monitored daily, the difference became more apparent (Fig. 5B). These results suggest that LMP1 enhanced the proliferation of T/NK cells in CAEBV, similar to its effects in B cells or NPCs.

Discussion

LMP1 is an EBV-encoded oncogene that stimulates cell growth at least in B cells and NPC. Here, we demon-

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strated that LMP1 regulates cell proliferation in cell lines derived from CAEBV, whereas LMP1 gave no proliferative advantage to an EBV-negative cell line.

During the preparation of this manuscript, Ndour et al. reported that a DN-LMP1 inhibited the cell growth and tumorigenesis of a T cell line artificially transformed with EBV [20, 21]. In contrast, we here used SNT16 and KAI3, T and NK cell lines, respectively, derived from patients with CAEBV, which are more physiologically relevant models [14]. In SNT16 and KAI3 cells, EBV establishes a latent infection, expressing specific protein-coding genes including LMP1, LMP2, and EBNA1. Our results suggest that LMP1 is a necessary component of the proliferative machinery, although it is possible that LMP2 and EBNA1 also play a role. Interestingly, LMP1 and LMP2 may cooperatively promote carcinoma development in a mouse carcinogenesis model [22]. The cooperation model also explains the induction of TRAF2 by LMP2 [23]. It is possible that LMP1 and LMP2 also act synergistically during T/NK-cell proliferation because no proliferation was stimulated in Jurkat cells expressing only LMP1 (Fig. 2). Therefore, the contribution of other viral factors should be considered.

Due to unknown reasons, phosphorylated AKT in EBV-negative Jurkat cells decreased slightly by DN-LMP1 (Fig. 4C). This decrease in AKT phosphorylation by DN-LMP1 might be caused by unintended influence on the AKT signaling molecules, or simply by massive expression of the protein. Anyway, the decrease in AKT phosphorylation levels in Jurkat was not potent enough to reduce the proliferation rate.

Despite a general understanding that LMP1 is an oncogene, adverse effects of LMP1 on cultured cells have also been reported in B cells, NPC cells, and other epithelial cells [24–26]. It has been suggested that high levels of expression of LMP1 inhibited proliferation, and so the suppressed growth and apoptosis observed in JTL1-2 cells in our study might also be explained by the abundance of LMP1. Consistent with this, LMP1 could simultaneously induce and inhibit apoptosis in B cells, depending on the context [27]. In LMP1, the C-terminal domains suppress the proapoptotic effects of transmembrane domains. Therefore, it is possible that overexpressed LMP1 in JTL1-2 cells induces apoptotic cell death by causing aggregation of the protein rather than by exerting a direct proapoptotic effect.

Expression of LMP1, either low or high levels, did not promote the proliferation of Jurkat cells, suggesting that LMP1 does not enhance the growth of these cells, regardless of the expression level. There are two possible explanations for this. One is that the intrinsic growth signals in Jurkat cell are already maximal, and so LMP1 is unable to further promote cell growth. The other is that LMP1 requires an additional factor to exert these effects. For example, LMP2 or gene products induced by the transcriptional activity of EBNA1 were not expressed in our Jurkat system.

In summary, LMP1 alone was not sufficient to enhance proliferation, at least in Jurkat cells. Therefore, LMP1 may require additional factors to promote cell growth. Nevertheless, our results suggest that LMP1 plays a central role in the lymphoproliferative disorder CAEBV. Targeting LMP1 and other factors, such as LMP2A, may facilitate effective, specific drug development for the treatment of CAEBV.

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Conflict of Interest

None declared.

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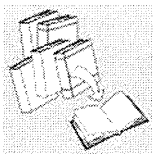
REVIEW



Modes of infection and oncogenesis by the Epstein–Barr virus

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SUMMARY

The EBV is a human γ-herpesvirus associated with various neoplasms. It is responsible for causing cancers of B, T, and NK cells as well as cells of epithelial origin. Such diversity in target cells and the complicated steps of oncogenesis are perplexing when we speculate about the mechanisms of action of EBV-positive cancers. Here, we first note three common features that contribute to the development and maintenance of EBV-positive cancers: effects of EBV oncogenes, immunosuppression and evasion/exploitation of the immune system, and genetic and epigenetic predisposition/alteration of the host genome. Then, we demonstrate the mechanisms of oncogenesis and the means by which each EBV-positive cancer develops, with particular focus on the mode of EBV infection. The EBV has two alternative life cycles: lytic and latent. The latter is categorized into four programs (latency types 0–III) in which latent viral genes are expressed differentially depending on the tissue of origin and state of cells. The production of viral latent genes tends to decrease with an increase in time, and, in an approximate manner, the expression levels of viral genes are inversely correlated with the degree of abnormalities in the host genome. Occasional execution of the viral lytic cycle also contributes to oncogenesis. Understanding this life cycle of the EBV and its relevance in oncogenesis may provide valuable clues to the development of effective therapies for the associated cancers. Copyright © 2014 John Wiley & Sons, Ltd.

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Abbreviations used

IM, infectious mononucleosis; NPC, nasopharyngeal carcinoma; LMP1, latent membrane protein 1; NF-κB, nuclear factor-kappa B; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; BCR, B-cell receptor; EBNA, EBV nuclear antigen; EBER, EBV-encoded RNA; XLP, X-linked lymphoproliferative syndrome; SAP, signaling lymphocytic activation molecule-associated protein; XIAP, X-linked inducer of apoptosis; TAP, transporter associated with antigen processing; HLA, human leukocyte antigen; CTLs, cytotoxic T lymphocytes; CAEBV, chronic active EBV; PTLD, posttransplant lymhoproliferative disorder; LCLs, lymphoblastoid cell lines; Cp, C promoter; IE, immediate-early; H3K27me3, H3 Lys27 trimethylation; PAX5, paired box 5; IL, interleukin; VEGF, vascular endothelial growth factor; TGF, transforming growth factor; STAT, signal transducers and activators of transcription; C/EBP, CCAAT enhancer-binding protein; HRS, Hodgkin and Reed–Sternberg; IκB, inhibitor of NF-κB; CNS, central nervous system; PEL, primary effusion lymphoma; AID, activation-induced cytidine deaminase; ENKTL, extranodal NK/T cell lymphoma; ANKL, aggressive NK leukemia; FOXO3, forkhead box O3; PRDM1, PR domain zinc finger protein 1; BLIMP1, B lymphocyte-induced maturation protein 1; JAK3, Janus kinase 3; ARID1A, AT-rich interactive domain-containing protein 1A; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

MECHANISMS UNDERLYING ONCOGENESIS BY THE EBV

The EBV is a human γ-herpesvirus. It has a double-stranded DNA genome measuring approximately 170 kb in length and encoding >80 genes. It is a ubiquitous virus infecting >90% of the population worldwide [1]. Transmission of the virus occurs through saliva, and once infected, it cannot be eliminated for a lifetime. The main reservoir of the EBV *in vivo* is memory B cells, but it also can infect NK, T, and epithelial cells.

Besides infectious mononucleosis (IM), which is caused by acute infection with the virus during adolescence, the EBV is linked to several human cancers such as Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma (NPC), gastric cancer, and T/NK lymphoma [2]. Although the incidence of such cancers among EBV-positive populations is not very high, these proliferative disorders are, in general, associated with a poor prognosis.

Carcinogenesis of EBV-positive cancers is a multistep process. We here suggest the three major

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causes shown in Table 1. First, the EBV encodes oncogenes such as latent membrane protein 1 (LMP1) and LMP2A. The effects of such virus-encoded oncogenes have been reviewed extensively in other studies [3,4]. For instance, LMP1 elicits growth promoting signals, such as nuclear factor-kappa B (NF-κB), phosphatidylinositol 3-kinase/AKT, and mitogen-activated protein kinases, by mimicking CD40 signaling pathway [5]. LMP2A is structurally and functionally related to B-cell receptor (BCR) and can activate phosphatidylinositol 3-kinase/AKT, NF-κB, NOTCH, and mitogen-activated protein kinases [6]. EBV nuclear antigen (EBNA) 3A/C also plays a critical role in maintenance and formation of cancer cells by silencing tumor suppressor genes [7,8]. Elsewhere, EBV-encoded noncoding RNAs (EBERs) and microRNAs (BART and BHRLF1 miRNAs) have been implicated in oncogenesis [9–13], although the targets and mechanisms of the noncoding RNAs are not perfectly understood as yet.

Second, suppression, escape, and exploitation of host immunity contribute to the emergence of EBV-positive cancers. For example, B-cell lymphomas (BCLs) associated with AIDS or transplantation are caused by systematic immunosuppression [14,15]. X-linked lymphoproliferative syndrome type 1 (XLP-1) and XLP-2 are caused by mutation of signaling lymphocytic activation molecule-associated protein and X-linked inducer of apoptosis, respectively, both of which are linked to abnormalities in the immune system [16,17]. In these cases, EBV and tumor cells make use of the weakness in antiviral/tumor immunity and can avoid elimination by the host.

On the other hand, EBV has intricately evolved itself to evade the host immunity. In particular, multiple genes have been reported to suppress antigen presentation. EBNA1 contains a Gly/Ala repeat sequence, through which proteasomal degradation and antigen presentation of the protein are impaired [18]. BNLF2A targets the transporter associated with antigen processing and blocks antigen presentation [19]. BGLF5 represses HLA class I synthesis, whereas BILF1 downregulates cell surface expression of the molecule [20]. Moreover, EBV can increase survival rate and promote tumor formation by playing elaborate tricks on the processes of B-cell maturation in germinal center. Such processes include selected expansion of B cells, acquisition of immune diversity (e.g. somatic hypermutation and class switching), and elimination of abnormal or self-reacting B cells. It is highly likely that at least LMP1 and LMP2A, viral functional mimic of CD40 and BCR, have tactfully evolved to modify those processes in germinal center, and thus, these EBV gene products are able to deregulate the immune system for survival [21].

In addition, the EBV exploits host immune/inflammatory systems for its survival and oncogenesis [22]. Several studies report that EBV-positive B-cell

Table 1. Oncogenesis of EBV-positive cancers

Action	Examples
Effects of oncogenes encoded by EBV	LMP1: elicits NF-κB, PI3K/AKT, MAPK, and JAK/STAT pathways, by mimicing CD40 LMP2A: elicits NF-κB and PI3K/AKT pathways, by mimicing BCR
Immunosuppression and evasion, exploitation of the host immune/inflammatory system	AIDS-associated lymphomas, PTLD, and XLP Downregulation of MHC molecule Malignant transformation alongside development in germinal center, where B cells obtain translocation or somatic mutations efficiently Growth promotion by cytokines and growth factors, induced by EBV infection
Genetic or epigenetic predisposition/alterations of the host genome	Myc translocation in Burkitt lymphoma Mutation or silencing in tumor suppressor genes, including TP53 and p16INK4A Genetic background of HLA signature

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neoplasms such as Burkitt lymphoma and Hodgkin lymphoma arise during B-cell development in the germinal center [22,23]. In addition, accumulating evidence suggests that immunological/inflammatory stimulation by the host promotes the proliferation of EBV-positive cancer [24,25]. Interestingly, EBV infection and its associated diseases are deeply linked to intense immune/inflammatory reactions. For example, severe infiltration of lymphocytes is the pathological hallmark of EBV-positive gastric cancer [26] and NPC [27]. The essential pathology of IM is hyperreaction of CTLs, which is occasionally associated with very high cytokine levels [28,29]. This is circumstantial evidence that EBV-positive cancer may exploit immunological/inflammatory reactions. Recently, Imadome *et al.* clearly demonstrated that the presence of uninfected $CD4^+$T cells from the same patient is quintessential for the successful engraftment and proliferation of EBV-positive NK, $CD8^+$T, or $\gamma\delta$T cells from chronic active EBV (CAEBV) *in vivo* [30].

Genetic or epigenetic background/alteration of the host genome is also major causalities. This category includes enhanced Ig-Myc translocation in Burkitt lymphoma [31,32] and silencing of tumor suppressor genes (e.g. $p16^{INK4A}$) by epigenetic alterations or mutations in many EBV-positive cancers [7,8,33]. It is assumed that these genetic/epigenetic alterations are caused, at least in part, by EBV, although these abnormalities may occur without the virus. In addition, several reports have demonstrated that the predisposition of individual HLA allele significantly affects the morbidity of EBV-positive proliferative disorders, particularly in NPC and Hodgkin lymphoma [34–39].

We admit that some of the three mechanisms of EBV oncogenesis overlap with others. For example, the viral oncogene LMP1 seems to act by inducing genetic/epigenetic alterations [40–42] and by modifying immune/inflammatory systems [21].

LATENT AND LYTIC MODES OF EBV

The EBV has two alternative life cycles: latent and lytic [43] (Figure 1). In the latent state, its genomic DNA exists as an episome in the nucleus, in which closed circular plasmid DNA is incorporated with histones, and produces only a limited number of viral latent genes. This silent mode of infection is advantageous for the virus to persist for long periods because only a few gene products that can be targeted by the host immune system are expressed.

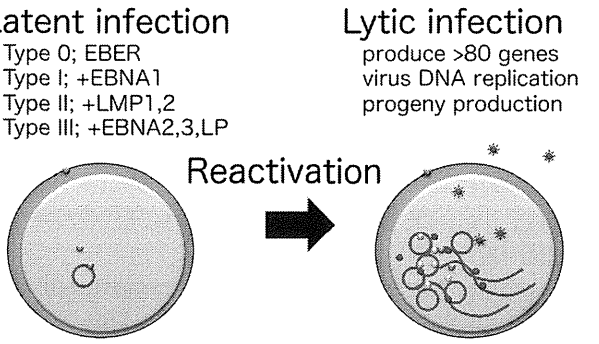

Figure 1. Life cycle of EBV. EBV infection takes two possible states: latent and lytic. During latent infection, the production of viral genes occurs from a limited number of latent genes (green). Reactivation from latency results in production of >80 genes, virus DNA replication, and the genesis of a progeny virus

The latent viral genome is replicated once at the S-phase in synchronization with the host genome and delivered to daughter cells in mitosis.

The expression pattern of viral latent genes varies with the tissue of origin, state of the cells, and immune condition [2,31,44,45] (Figure 1). Neoplasms such as Burkitt lymphoma or gastric carcinoma typically express only EBERs and EBNA1 (latency type I), whereas some types of Hodgkin lymphoma, NPC, and T/NK lymphomas produce EBERs, EBNA1, LMP1, and LMP2 genes (type II). In addition to type II genes, EBNA2, EBNA3, and EBNA-LP are also expressed in most cases of posttransplant lymhoproliferative disorder or lymphoblastoid cell lines (LCLs; type III). In type III, EBNA2 acts as a strong transcriptional coactivator for LMP1 and LMP2 promoters, as well as C promoter (Cp, promoter for EBNA2 itself). However, in latency II, other factors must take part in the activation of LMP1/LMP2 promoters because EBNA2 is absent. Memory B cells latently infected with the EBV express only EBERs *in vivo* (type 0).

In the viral lytic cycle, all the lytic genes of the EBV are expressed coordinately, including its own transcription factor or DNA polymerase catalytic subunit, resulting in amplification of its genome by >100-fold [43] (Figure 1). Execution of this dynamic cycle produces progeny virus particles, and the cells stop growing and are eventually eliminated by immunity. Although the EBV in cancer cells is mostly in the latent state, the lytic cycle of the virus also plays a key part in the development and maintenance of the cancers. Recent

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reports clearly demonstrate that lytic cycle of EBV increases B-cell transformation efficiency at cell culture levels [46,47] and development of B-cell lymphoma in a humanized mouse model [48].

PRELATENT, TRANSIENT LYTIC CYCLE IN PRIMARY INFECTION

Here, we overview the chronological timeline of EBV infection at cellular levels along with the states and changes in the expression patterns of EBV genes during oncogenesis.

For a long time, it was taken for granted that the EBV established latent infection immediately after primary infection of cells. However, in 2007, Wen *et al.* overturned this hypothesis and clearly demonstrated that BZLF1 [the immediate-early (IE) gene that induces lytic replication] is expressed when EBV-negative B cells such as Akata, Daudi, or even primary B cells are infected with the EBV [49]. This phenomenon was confirmed by the research teams of Shannon-Lowe and Tsao in the cells of epithelial origins, too [50,51]. Kalla *et al.* then demonstrated that not only the two regulator IE genes, BZLF1 and BRLF1, but also a subset of early genes such as BMRF1 are produced for ≥10 days after the infection of primary B cells by the EBV, even though lytic DNA replication and late gene expression were not detected [47] (Figure 2). Taking this phenomenon into consideration, we suggest that an abortive lytic cycle is the initial mode of EBV infection that occurs upon primary infection (Figure 3). This prelatent, transient implementation of the EBV lytic cycle, which is silenced later (Figure 2), is now accounted for by the CpG DNA methylation state of the EBV genome and the peculiar characteristics of the BZLF1 (it preferentially associates with and enhances CpG-methylated DNA motifs) [52–55]. The EBV genome in the virus particle is not associated with any form of epigenetic suppression, including CpG methylation and histone modifications. When the naked viral DNA is transported into the nucleus, IE gene expression followed by early gene expression is induced; however, the progeny virus is not generated. At this stage, BZLF1 cannot exercise its entire transcriptional ability because the viral genome is devoid of CpG methylation [56]. This short, abortive, lytic cycle of primary EBV infection provides a proliferative advantage, at least to resting naïve and memory B cells [47].

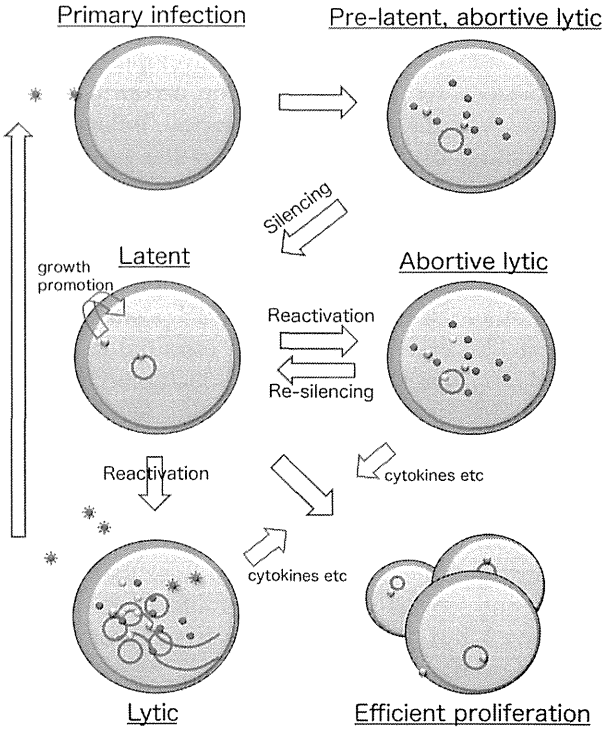

Figure 2. Progress of a complicated life cycle of the EBV that leads to the efficient proliferation of cells. Upon primary infection of EBV, the cells undergo prelatent, short lytic cycles in which only immediate-early and early genes (blue and red circles) are expressed without viral lytic DNA replication. The transient lytic state is silenced later, and only a limited number of latent genes (shown in green) is expressed. In latency, only one copy of the EBV genome (red hoop) was drawn for simplicity, but in general, one latent cell contains approximately 5–100 copies of the viral genome. A part of latent cells transits into the abortive lytic state, in which viral lytic replication may or may not be induced and then "resilenced" to the latent state again. Others may undergo the complete lytic cycle, including late gene (yellow) expression and viral genome replication and production of the progeny virus. For cancerous growth, cells may exploit cytokines released from lytic or abortive lytic cells, although latent factors such as LMP1 can also promote cell proliferation by enhancing cell signaling, modulating immune system, and inducing genomic instability

LATENCY AND THE LYTIC CYCLE CONTRIBUTE TO ONCOGENESIS

Complete silencing of this prelatent active state requires weeks. The major epigenetic players in suppression include CpG DNA methylation, histone H3 Lys27 trimethylation (H3K27me3), H3K9me2/3, and H4K20me3 [45,56–61]. This silencing of exogenous DNA is considered to be a type of innate immunity of the host because neutralizing of nucleic acids of extrinsic origin means silencing of pathogens. If we take a closer look at this stage, while the expression of lytic genes is

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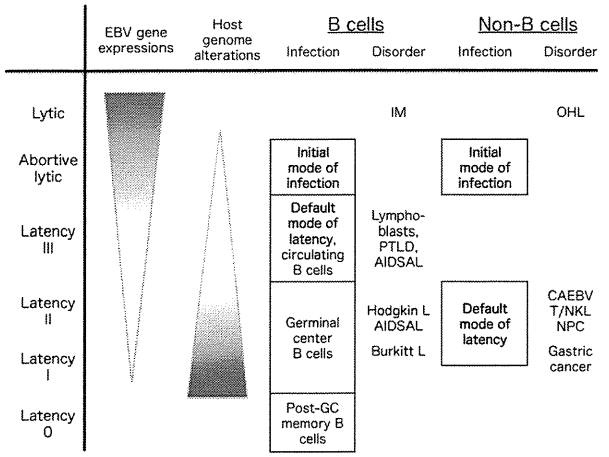

Figure 3. Mode of infection and oncogenesis of the EBV. The abortive lytic state is the initial mode of infection in B or non-B cells because of the prelatent, transient lytic infection upon primary infection. Latency III and II are the default modes of latency in B and non-B cells, respectively (could be type I for some gastric cancer cases, though). Although EBV gene expressions are silenced, slowly but steadily, the host genome accumulates genetic or epigenetic alterations for malignancies. IM, infectious mononucleosis; PTLD, posttransplant lymhoproliferative disorder; AIDSAL, AIDS-associated lymphomas; OHL, oral hairy leukoplakia; CAEBV, chronic active EBV infection; NPC, nasopharyngeal carcinoma

being eliminated, a limited number of latent genes are kept expressed (Figure 2) depending on the cell types. EBV infection of B cells *in vitro* (naïve, germinal center, or memory B cells) results in latency type III by default [62], whereas a type II pattern manifests in non-B cells such as T, NK, or epithelial cells (Figure 3) [50,63,64], although there seem to be exceptions, especially in the case of gastric carcinoma [65]. In fact, type III latency is reserved only for B cells because activation of Wp/Cp (the promoters for EBNAs, including EBNA2) seems to require transcription factors such as paired box 5, which are predominantly present only in B cells [60,66,67]. In addition, it seems likely that lymphocyte-specific factors such as PU.1 or its related factors are required for EBNA2-dependent LMP1 expression [68]. Latent infection by the EBV unambiguously contributes to EBV oncogenesis in B or non-B cells (Figure 2). For instance, a latent gene, LMP1, activates growth/survival signaling pathways [5], induces pro-inflammatory cytokines such as IL-6 and IL-8 [69,70], plays a part in immune evasion [21], and increases genomic instability [42,71].

Difference in default latency patterns between B cells and non-B cells may contribute to oncogenesis differently. EBV can efficiently transform even resting (G0) B cells in a short period, although it could increase cell growth of already immortalized cells of epithelial origin [51]. This may be accounted for by the presence of extra oncogene such as EBNA3, expressed in B cells.

The lytic cycle of the virus also makes a significant contribution to the development and maintenance of associated cancers (Figure 2) [46–48]. First, the lytic cycle of the EBV in a fraction of cells enhances expression of viral/cellular cytokines and growth factors, such as viral IL-10, IL-10, IL-8, vascular endothelial growth factor, and transforming growth factor-β, which increases proliferation of the surrounding cells latently infected with EBV [72]. Moreover, we propose the "hit and hide" hypothesis, in which the abortive lytic state of the virus enhances instability in the host genome and is subsequently "resilenced" to latency (Figure 2) [72]. Among the lytic genes, at least BZLF1 [73] (the lytic switch), BGLF4 (the only protein kinase of EBV [74]), and BGLF5 (the alkaline nuclease [75]) are reported to affect the stability of the host genome. The "hit" of the host genome, implemented during the transient lytic cycle, may increase the proliferation of the resilenced or "hidden" latent cells. Interestingly, recent paper indicated, by using humanized mouse model, that lymphoma could be developed by abortive but persistent lytic infection of EBV [76]. So, lytic cycle of the virus may not even need to be completely silenced for cancerous growth of infected cells, if lytic replication levels are low.

B-CELL LYMPHOMATOGENESIS AND THE EBV

Latency III is a necessary and fundamental latent mode in EBV infection of B cells (Figure 3) because this type of latency induces resting B cells (memory and naïve B cells) to proliferating lymphoblasts and expands virus-containing B cells to increase survival [22]. Here, we would like to describe that type III latency is the "default" mode of latency caused by primary infection of the EBV in B cells (Figure 3) because of several reasons. First, we assume that the infection of circulating B cells is the initial configuration for the EBV. Second, the infection of naïve, germinal center, or memory B cells by the virus under more simple conditions (*in vitro*) results in latency type III [62]. Third, some Burkitt lymphoma cells, generally associated with latency I EBV, occasionally switch to latency III (but not latency II) if cultured without immunological pressure [77].

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The EBV-positive lymphoblasts derived from naïve B cells are led to a germinal center where B cells develop into memory (or plasma) cells [1]. In the germinal center, the EBV in general exhibits latency type II (or possibly type I) [1,22] (Figure 3) because of the loss of Wp/Cp activity. Because the specific transcription factors of Cp/Wp, such as paired box 5, are still abundantly present in germinal center B cells [78], the mechanism of silencing of the promoter is elusive. EBNA2 may not be compatible with entry into the germinal center and may therefore require to be silenced [1,22,62]. In type II latency, LMP1 and LMP2 are expressed even without EBNA2, probably through the effect of signal transducers and activators of transcription (STAT), NF-κB, and CCAAT enhancer-binding protein [64,79–82].

The EBV in circulating memory B cells (which are the main reservoir of the virus) manifests latency 0 after exiting the germinal center (Figure 3). Memory B cells, the reservoir cells of the virus, will not be eliminated without cell division for years or even decades; therefore, once the virus can establish successful latent infection in memory B, it does not require viral products to support its survival.

Lines of evidence also indicate that primary infection of memory B cells by the EBV plays an important role in at least IM pathogenesis [2]. After onset of the disease, viral genes are downregulated, and the virus in the memory B cells then establishes persistent infection, featuring latency 0, presumably without going through the germinal center.

Endemic Burkitt lymphoma [1,2,22,31] in central Africa is associated with the EBV of type I latency (Figure 3). The presence of Myc translocation [83] and somatic hypermutation in the variable region of Ig [84,85] indicates that the lymphomas originate from centroblasts of germinal center B cells. Despite the fact that almost 100% of endemic lymphoma specimens are EBV positive, dependence on the virus for cell proliferation is not very high because only EBNA1 and EBERs are expressed in the lymphomas, and EBV-negative cell clones can be isolated occasionally. It is likely that other latent proteins such as LMP1 and LMP2A play important roles in the process of oncogenesis, even though these proteins are not expressed anymore in already developed Burkitt lymphomas. Immunosuppression may also be a major contributor to Burkitt lymphoma because Ig-Myc translocation is frequently found in AIDS-associated, EBV-positive BCLs (approximately 35%) [14,86] and XLP [87]. Besides Myc translocation, genetic alterations have also been reported in TP53 [88] and RB2 [89] genes.

A hallmark of Hodgkin lymphoma [1,2,23] is Hodgkin and Reed–Sternberg (HRS) cells, even though the cells represent ≤1% of tumor cells. Instead, EBV-negative, nonmalignant, normal lymphocytes (including T or B cells) occupy the tissue, indicative of an inflammatory reaction. Isolation of EBV-positive HRS cell line is technically very difficult without support by the nonmalignant surrounding lymphocytes. HRS cells have a trace of somatic hypermutations and crippling mutations, nonsense or deleterious mutations that appoint elimination by apoptosis in the germinal center, indicating that the cells also originate from the germinal center [90]. To avoid apoptosis, signaling pathways from the BCR and $CD4^+$T cells are required, and, interestingly, LMP2A and LMP1 can complement the signaling, respectively, at least partly [23,91]. Therefore, type II latency (Figure 3), in which LMP1 and LMP2 are expressed in addition to EBNA1 and EBERs, in Hodgkin lymphoma is justifiable. Despite the definite dependence on LMP1 and LMP2A, EBV association with Hodgkin lymphoma specimen is not as high as that with Burkitt lymphoma [1,2,23]. Comparative examination demonstrated that NF-κB activity in EBV-negative HRS cells was as high as that in EBV-positive HRS cells [23]. Therefore, constitutive activation of the signaling, by the EBV or any other reason, is assumed to be a requisite for Hodgkin lymphoma. Associated mutations in the inhibitor of NF-κB [92–94], CD95/FAS [95], and A20 [96] and REL amplifications [97] have been reported for Hodgkin lymphoma, all of which affect NF-κB activation. Immune system also plays an important part in Hodgkin lymphoma because particular HLA alleles are highly associated with the incidence [36–38], and approximately 5% lymphomas in patients with AIDS fall under this criterion, with almost 100% cases being EBV positive [14].

Posttransplant BCL [98] and AIDS-associated BCL [14,86] are primarily caused by severe immunosuppression. The cellular origin of most of these lymphomas is the germinal center B cells, but postgerminal center memory B cells also account, at least in part, for the disease. For example, two EBV-associated malignancies, primary CNS lymphoma and primary effusion lymphoma, likely reflect postgerminal center B cells [2,15]. Posttransplant/

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AIDS-associated BCLs are usually derived from a germinal center, probably because germinal center B cells can efficiently acquire somatic mutations there because of an abundance of activation-induced cytidine deaminase [98]. Dependence of the lymphomas on the EBV is high both *in vivo* and in cell cultures, typically featured with type III latency (Figure 3), making the most of the absence of immunosurveillance.

T/NK CELL LYMPHOMATOGENESIS AND THE EBV

Besides B cells, EBV can infect and promote proliferation of at least T and NK cells. These EBV-associated T/NK proliferative disorders [99,100] are more frequently found in East Asia, including Japan. EBV is present in almost 100% of established cell cultures, as well as in specimens of those T/NK proliferative disorders. The CAEBV [101] is a lymphoproliferative disorder with a poor prognosis that follows IM. In general, IM patients recover within weeks; however, in some rare cases, the symptoms of IM continue for more than months. Such cases of CAEBV are frequently characterized by prolonged high anti-EBV (EA-D and VCA) immunoglobulin titers, no obvious immunosuppression, and EBV positivity in NK or T cells [30]. CAEBV was named because the levels of immunoglobulins against the lytic gene products of the EBV, EA-D, or VCA are high in most cases. Although a fraction of the infected T/NK cells are possibly in a lytic state, a dominant portion of lymphocytes proliferating in the disorder shows type II latency (Figure 3), in which LMP1 and LMP2 are expressed in an EBNA2-independent manner [102,103]. It has also been reported that CTL activity against certain EBV gene products is weak [104,105], suggesting that a partially weakened host immune system may be essential.

The lymphoproliferative disorder CAEBV is thought to develop into malignant T/NK cell lymphoma, at least in some cases [106]. Two categories, extranodal NK/T cell lymphoma and aggressive NK leukemia, have been strongly associated with EBV infection [107]. EBV gene expression in these cells is type II (Figure 3), but in some cases of T/NK infection, LMP1 and LMP2 were not detectable, representing latency type I [108,109]. In fact, even in type II cases, analyses at the single-cell level showed that the expression of LMP1 and LMP2 is heterogeneous and that a significant portion of cells does not express these genes [100,109]. The contribution of these gene products to the proliferation of T/NK lymphomas remains controversial. Although dominant negative LMP1 repressed proliferation of T lymphocytes infected with the EBV ([110], our unpublished data), expression levels of LMP1 do not necessarily correlate with proliferation [111]. We assume that primary EBV infection results in latency II in T or NK cells by default, whereas LMP1 and LMP2 levels gradually decline while the infected cells accumulate mutations advantageous for their division. Therefore, we believe viral oncogenes such as LMP1 are needed for the pathogenesis of T/NK lymphomas, at least at the beginning of the oncogenic procedure. However, such a necessity may decrease after the cells acquire significant levels of epigenetic or genetic alterations. Candidates of such genomic lesions include TP53, K-RAS, β-catenin [112], forkhead box O3 (FOXO3), and PR domain zinc finger protein 1/B lymphocyte-induced maturation protein 1 [113]. Although exome sequencing has recently identified mutations in Janus kinase 3 [114], this could not be achieved in Japan [115].

EPITHELIAL CANCERS AND THE EBV

Nasopharyngeal carcinoma [116] is an epithelial cancer that is most frequently found in the southern part of China. Almost 100% of NPC specimens are associated with the EBV, and dependence of the carcinoma on the EBV has been demonstrated [51], although EBV genome is lost very frequently when cultured because of unknown reasons. Type II latency accounts for a significant part of the cancer, whereas LMP1 and LMP2 could not be detected in other cases [117] (Figure 3). The contribution of LMP1 to NPC development has been well studied [118]. Heterogeneity in the expression of the two latent membrane proteins indicates that these oncogenic proteins may not be required for cancerous proliferation of NPC, at least in some cases in which the cells have already obtained significant levels of mutations, similar to T/NK lymphomas. With regard to NPC, the possible candidates of such mutations have been studied extensively: loss or mutations in Ras association domain-containing protein 1A, cyclin-dependent kinase inhibitor 2A/p16^{INK4A}, and TP53 [119,120] and overexpression of BCL-2 [121] and cyclin D1 [122]. In addition to the EBV, other etiological factors have also been implicated in NPC, including consumption of salted fish and tobacco, which

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can serve as mutagens [123]. Immune system must influence NPC, too, because there is an extensive literature indicating an important role for HLA genes in the etiology of NPC [34,35,124].

The EBV can also be detected in approximately 10% (or less) of gastric cancers [125,126]. Because gastric carcinoma cell lines naturally infected with EBV are widely present, the virus presumably plays a crucial role in the cell proliferations, even at the cell culture level. EBV infection of epithelium cell cultures, including AGS, frequently leads to type II latency upon primary infection by default [50,64], although the virus can also adapt type I latency in cell cultures of gastric carcinomas [65,127]. Infiltration of lymphocytes and macrophages in both NPC and gastric cancer is very remarkable and almost inevitable, and thus, it seems like essential for the pathogenesis. Beyond mutations in other genes such as TP53, exome sequencing has identified frequent mutations or deficiencies in the AT-rich interactive domain-containing protein 1A gene, which encodes a member of the SWI–SNF chromatin remodeling family, in EBV-positive gastric cancer [128]. In addition, the research team of Fukayama has focused on epigenetic silencing of tumor suppressor genes, including $p16^{INK4A}$, $p14^{ARF}$, p73, E-cadherin, and phosphatase and tensin homolog deleted on chromosome 10, in EBV-positive gastric carcinomas, where they identified LMP2A as a contributor to epigenetic silencing [129–131].

CONCLUDING REMARKS

Analysis of human cancer specimens is important for understanding the nature of cancers. However, learning only from already developed cancers may not clearly demonstrate the mechanisms underlying oncogenesis. We reviewed the histories of the developmental procedures of EBV-positive cancers, focusing particularly on the mode of infection. Detailed clarification of the life cycle of the EBV in association with oncogenesis may provide valuable information for the establishment of novel antiviral or anticancer therapies.

CONFLICT OF INTEREST

The authors have no competing interest

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Survival rates and prognostic factors of Epstein–Barr virus-associated hydroa vacciniforme and hypersensitivity to mosquito bites

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Summary

Background Epstein-Barr virus (EBV)-associated T/natural-killer lymphoproliferative disorders form a group of diseases that includes classical and systemic hydroa vacciniforme (HV) and hypersensitivity to mosquito bites (HMB). Patients with systemic HV (sHV) and HMB often have a poor prognosis, although little is known about the prognostic factors.

Objectives To elucidate the prognostic factors of HV and HMB.

Methods We studied clinicopathological manifestations, routine laboratory findings, anti-EBV titres, EBV DNA load and EBV-encoded gene expression, including expression of BZLF1, in 50 patients with classical HV (cHV), sHV, HMB only and HMB with HV (HMB + HV), and further analysed 30 patients who were available for follow-up.

Results The median age of disease onset was 5 years (range 1–74). A follow-up study indicated that fatal outcomes were observed in three of eight patients with sHV, two of six patients with HMB only, and two of five patients with HMB + HV. The main causes of death were complications from haematopoietic stem-cell transplantation and multiorgan failure. There were no fatalities among the 11 patients with cHV. Univariate analysis revealed two poor prognostic indicators: (i) onset age > 9 years and (ii) the expression of an EBV-encoded immediate–early gene transcript, BZLF1 mRNA, in the skin lesions ($P < 0{\cdot}001$ and $P = 0{\cdot}003$, respectively).

Conclusions No prognostic correlation was observed in EBV-infected lymphocyte subsets, anti-EBV antibody titres or EBV DNA load. Late onset and EBV reactivation are both related to more severe phenotypes of the disease, and thus may predict a poor prognosis.

What's already known about this topic?

- Epstein–Barr virus-associated T/natural-killer lymphoproliferative disorders form a group of diseases that includes classical and systemic hydroa vacciniforme (HV), and hypersensitivity to mosquito bites (HMB).
- Patients with systemic HV and HMB usually present with fever, liver damage and haematological abnormalities, and often have a fatal outcome.

What does this study add?

- Our patients with classical HV showed a favourable prognosis, while approximately one-third of the patients with systemic HV or HMB died during the 10-year follow-up.
- Late onset (over 9 years of age) and an EBV reactivation signal (BZLF1 mRNA expression) were both related to more severe phenotypes of the disease, and a poor prognosis.

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Epstein-Barr virus (EBV)-associated T/natural-killer (NK) lymphoproliferative disorders (LPDs) form a group of diseases including hydroa vacciniforme (HV) and hypersensitivity to mosquito bites (HMB). EBV-associated HV lesions contain a number of EBV-encoded small nuclear RNA (EBER)$^+$ T cells, together with larger numbers of EBER$^-$ cytotoxic T lymphocytes (CTLs). Meanwhile, NK cells are absent or occur at background levels in such lesions.[1,2] Although there are no systemic symptoms or abnormalities in the routine laboratory tests of patients with classical HV (cHV), EBV DNA load and EBV$^+$ γδT cells are increased in the peripheral blood mononuclear cells (PBMCs).[3] In contrast, patients with HV-like ulcerative cutaneous eruptions often present with systemic symptoms such as fever, hepatic damage and lymphadenopathy (systemic HV, sHV), and show dense inflammatory-cell infiltrates that reach the subcutaneous tissue. As reported previously, patients with cHV may progress to sHV in the clinical course.[2]

HMB is an EBV-associated T/NK LPD characterized by intense local skin reactions and systemic symptoms, including high fever, lymphadenopathy, hepatosplenomegaly and haemophagocytic syndrome.[4] These clinical symptoms can be induced by mosquito bites, other insect bites or vaccination. Patients with HMB usually have EBV$^+$ NK-cell lymphocytosis,[3,5] and HV-like eruptions may occur over the course of the disease. We previously examined differences in cellular events between HMB and HV-like eruptions.[3] Our results indicated that many CD56$^+$ NK cells and T cells are present in the subcutaneous infiltrates in HMB, but no CD56$^+$ NK cells occur in HV dermal infiltrates.

Unlike cHV, both sHV and HMB have been reported in Asian and Latin American countries.[6–8] However, the nomenclature of EBV-associated T/NK LPDs has been controversial. Patients with sHV in the present study may be synonymous with HV-like lymphoma in the World Health Organization classification,[9] and may overlap with chronic active EBV infection (CAEBV) and EBV-associated haemophagocytic lymphohistiocytosis (HLH).[10] Because of the diagnostic value of HV-like cutaneous signs, we have used the terms HV and HMB in our classification, excluding the diagnoses of CAEBV and HLH.

Although no prognostic markers have been elucidated, previous reports of CAEBV indicate that patients with the EBV$^+$ T-cell-predominant type have a poorer prognosis than those with the EBV$^+$ NK-cell-predominant type, and that late onset may be a risk factor.[10] In the present study, we attempted to clarify cellular and molecular markers related to the prognosis of cutaneous EBV-associated T/NK LPDs in a series of patients with HV and HMB, and to verify the validity of diagnostic criteria to distinguish benign from malignant types for the purposes of prognosis.

Patients and methods

Patients

Fifty patients were categorized into four groups: cHV (23 cases), sHV (12 cases), HMB only (nine cases) and HMB + HV (six cases), according to the criteria in Table 1. Briefly, patients with cHV presented with lesions defined as vesiculopapules on sun-exposed areas without any systemic symptoms or abnormalities in routine laboratory test results. Patients with sHV presented with HV-like eruptions associated with systemic symptoms such as fever and lymphadenopathy and/or abnormalities in routine blood examinations at diagnosis. In our series, one of 12 patients with sHV initially had skin symptoms without systemic symptoms, although the patient had an increased percentage of NK cells (> 30% of lymphocytes) in the blood test. HMB is defined as an intense skin response to mosquito bites, insect bites or vaccination associated with systemic symptoms and/or abnormalities in routine blood tests.

Skin biopsy materials, crusts and blood samples were obtained for diagnosis, and used for *in situ* hybridization with EBER and quantitative reverse-transcriptase polymerase reaction (RT-PCR) to detect EBV infection in skin lesions.

This study was approved by the ethical committee (the institutional review board of Okayama University Hospital, no. 419, 2011) in accordance with the 1975 Declaration of Helsinki.

Assay for Epstein–Barr virus DNA load in peripheral blood mononuclear cells

DNA was extracted from 1 × 10^6 PBMCs using a QIAamp™ Blood Kit (Qiagen, Venlo, the Netherlands), and the PCR amplification was performed using QuantiTect™ Probe PCR (Qiagen) with Roche Light cycler (Roche, Pleasanton, CA, U.S.A.). The PCR primers for this assay were selected in the BamHI M region (BMRF1). The upstream and downstream primer sequences were 5 -GTGCCAATCTTGAGGTTTTAC-3 and 5 -CACCCGGGGACTTTTATC-3 , respectively. The fluorogenic probes used were probe A, 5 -GACCTGCCGTTGGATCTTA GTG-3 , and probe B, 5 -TATTTTATTTAACCACGCCTCCGA AGA-3 . Amplification was carried out at 95 °C for 15 min, followed by 50 cycles of 95 °C for 15 s, 56 °C for 20 s and 72 °C for 15 s. The semiquantitative amounts of EBV DNA copies in patients' samples were determined from the standard curve obtained by PCR amplification of serial 10-fold dilutions of the template plasmid DNA solution.

Primer sets for reverse-transcriptase polymerase chain reaction

RNA was extracted from the samples with TRIzol™ reagent (Gibco-BRL, Gaithersburg, MD, U.S.A.), and the cDNA was amplified by PCR using EBER1-specific and BamHI A rightward transcripts (BARTs)-specific primers, as described previously.[11,12] The integrity of the RNA was checked by the parallel amplification of beta-2-microglobulin (β2-MG). To detect EBV reactivation, BZLF1 was amplified by RT-PCR, using BZLF1-specific outer primers: sense, 5 -CATGTTTCAACCGC TCCGACTGG-3 , and antisense, 5 -GCGCAGCCTGTCATTTTCA GATG-3 . Amplification consisted of 40 cycles of 94 °C for

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Table 1 Criteria for classical and systemic hydroa vacciniforme (HV) and hypersensitivity to mosquito bites (HMB)

	Classical HV	Systemic HV	HMB
Cutaneous lesion	Vesiculopapular lesion	Ulceronecrotic lesion	Swollen erythema or skin ulcer following mosquito bites, insect bites or vaccination
EBER-positive cells	+	+	+
Systemic symptoms and/or abnormality in routine laboratory findings[a]	–	+	+

EBER, Epstein–Barr virus-encoded small nuclear RNA. [a]Systemic symptoms include high-grade fever, lymphadenopathy and hepatosplenomegaly. Laboratory abnormalities include hepatic damage, haematological findings suggestive of haemophagocytic syndrome and natural-killer-cell lymphocytosis (> 30% of cells).

45 s, 64 °C for 30 s and 72 °C for 1 min. BZLF1-specific inner primers were sense, 5 -TCCCAGTCTCCGACATAACCCA-3 , and antisense, 5 -AGCAGCGACCTCACGGTAGT-3 ; amplification involved 28 cycles of 94 °C for 45 s, 58 °C for 30 s and 72 °C for 1 min. Amplification gave 167 b.p. for EBER1 cDNA, 142 b.p. for BARTs cDNA, 295 b.p. for β2-MG cDNA and 332 b.p. for BZLF1 cDNA (639 b.p. for BZLF1 DNA).

Labelling for Epstein–Barr virus-encoded small nuclear RNA *in situ* hybridization

Lymphoid cells containing EBER1 were detected by in situ hybridization on paraffin-embedded sections, as described previously.[13] The density of EBER-positive cells was classified into four subgroups according to the percentage of positive cells: 1 + (1–5% positivity in the infiltrate); 2 + (5–25%); 3 + (25–50%) and 4 + (≥ 50%).

Immunophenotyping of infiltrating cells

Deparaffinized biopsy specimens were incubated with monoclonal antibodies to CD3ε, CD4, CD8, CD20, CD30 (Dako, Glostrup, Denmark) and CD56 (Novocastra Laboratories Ltd., Newcastle upon Tyne, U.K.), as described previously.[14]

Flow cytometric analysis for lymphocyte subsets

Blood samples from the patients were reacted with fluorescence-conjugated antibodies to CD3, CD56, T-cell receptor (TCR)αβ and TCRγδ (Beckman Coulter, Brea, CA, U.S.A.), and analysed using a FACSCalibur flow cytometer and CELLQUEST software, version 5.2.1 (Becton Dickinson Co., Franklin Lakes, NJ, U.S.A.).

Statistical analysis

Analyses were performed using SPSS for Windows version 20.0 (IBM, Armonk, NY, U.S.A.). For univariate analyses, a one-sided Fisher's exact test was used to compare the categorical variable. To compare the quantitative variable, the Mann–Whitney U-test was used. For survival analysis, the Kaplan–Meier method and log-rank test were used. In all analyses, $P < 0{\cdot}05$ was considered significant.

Results

Clinical observations of the patients

Fifty patients (26 male and 24 female) were enrolled in the current study. They were classified into four groups: cHV, sHV, HMB only or HMB + HV, according to our tentative diagnostic criteria as described elsewhere (Table 1).[3] The age of onset ranged from 1 to 74 years (median 5 years). The median onset ages and the sexes of the four groups were as follows: cHV, 5 years (13 male, 10 female); sHV, 8 years (five male, seven female); HMB only, 8 years (six male, three female) and HMB + HV, 3·5 years (two male, four female). Of 23 patients with cHV, 21 (91%) had cutaneous lesions that presented within their first decade. The cutaneous signs of cHV occurred at younger ages than those of sHV and HMB + HV ($P = 0{\cdot}022$ and $P = 0{\cdot}026$, respectively).

Mucocutaneous symptoms such as conjunctivitis and oral aphthous stomatitis/gingivitis were observed in six (26%) of 23 patients with cHV and five (42%) of 12 patients with sHV, but were not observed in any of the nine patients with HMB or the six with HMB + HV. Of the 27 patients with sHV, HMB only or HMB + HV, 22 presented with systemic symptoms, including fever (22, 81%), diarrhoea (two, 7%), intestinal perforation (one, 4%), hepatosplenomegaly (11, 41%), myocarditis (two, 7%) and haemophagocytic syndrome (two, 7%) (Table 2).

Follow-up study

Of the 50 patients enrolled in the present study, 30 were included in a follow-up study; the time to follow-up ranged from 1 to 26 years (median 6·5 years). The median follow-up times for the four groups were 8 years for cHV, 7 years for sHV, 3 years for HMB only and 12 years for HMB + HV. All 11 patients with cHV were alive at follow-up, with or without disease, and nine of the 11 patients had been treated only with sunscreen. One of the two remaining patients, a

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