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The Role of the Epstein-Barr Virus Lytic Cycle in Tumor Progression: Consequences in Diagnosis and Therapy

Emmanuel Drouet

Abstract

The Epstein-Barr virus (EBV) reactivation corresponds to the activation of EBV global replication involving not only the origin of the latent viral replication but also that of the origin of lytic replication. During this reactivation, a minority of B cells infected with EBV in its latent form enter the lytic phase. During this phase, all EBV proteins are produced, enabling the assembly of complete virions that lyse their host cells and infect neighboring cells (lytic cycle). This horizontal EBV transmission seeks to increase the pool of EBV-infected B cells. This chapter seeks to review the role of the lytic EBV proteins (particularly that of the ZEBRA protein) *in tumor development*. This protein is the main transcription factor of EBV, expressed during the activation of the lytic cycle. Recently, we demonstrated that this immediate early protein can be detected in the soluble state (s-ZEBRA) in the serum of patients with posttransplant lymphoproliferative disorder. We highlighted the role of ZEBRA in EBV pathogenesis in transplanted subjects, not only as a key protein in the activation of EBV replication but also as a protein “toxoid” released into the extracellular milieu. This release could result in increased secretion of immunomodulatory cytokines and that of angiogenesis-promoting factors conducive to tumor progression.

Keywords: tumor progression, tumorigenesis, EBV, lytic cycle, Zta/ZEBRA protein, lymphoma

1. Introduction

Epstein-Barr virus (EBV) is a member of the herpesvirus family and only infects primates, with tropism for B cells and epithelial cells, which establishes a lifelong persistent infection in over 90% of the world's population [1]. After the resolution of the primary infection episode, EBV enters a latent phase. Following primary infection, the virus establishes lifelong persistence within the host memory B-cell compartment utilizing restricted latent gene expression programs [2–4]. Like all herpesviruses, EBV can choose between two alternative lifestyles: latent or lytic replication. EBV lytic replication, which is required for horizontal spread of the virus from cell to cell, and from host to host, occurs in both epithelial cells and B cells [5–7]. During latency, EBV exists in a dormant state where only a viral gene subset is expressed, facilitating the episomal persistence of the viral genome [8]. However,

under certain physiological conditions, both viruses undergo lytic reactivation leading to expression of the full lytic gene complement, followed by the assembly and egress of infectious virions [1].

Lytic replication is mediated by the virally encoded DNA polymerase using the oriLyt replication origin and results in the release of infectious viral particles [9]. In contrast, during latent viral infection, only a viral gene subset is expressed. The genome is replicated once per cell cycle using the cellular DNA polymerase and the oriP replication origin, and the progeny virus is not released. Latent EBV infection allows the virus to persist for the life of the host and to avoid detection by the immune system [1]. In immune-competent hosts, the outgrowth of EBV-immortalized B cells is prevented through a robust expansion of EBV-specific memory T cells directed toward both lytic and latent antigens [10]. Thus, EBV-positive individuals with either congenital or acquired immune deficiencies are highly susceptible to viral reactivation and malignant transformation. Furthermore, EBV-seropositive patients treated with immunosuppressive medications following organ/stem cell transplantation are at significant risk of developing posttransplant lymphoproliferative disorder (PTLD), an often fatal B-cell lymphoproliferative disease (LPD) [11, 12].

Importantly however, EBV (as other gammaherpesviruses, e.g., KSHV) can also undergo abortive lytic reactivation, resulting in the expression of early lytic genes without subsequent virion assembly and cell lysis [13]. Although numerous efforts to understand these disorders' molecular basis have focused on viral latency, EBV lytic cycles are now widely accepted as major contributors to oncogenesis and could be important targets in the development of new therapeutic or diagnostic approaches [14, 15]. Thus, in this chapter, we will discuss how lytic replication, particularly via the immediate-early/early antigens or IEAs/EAs, augments the pathogenesis of EBV-associated malignancies, especially in the lymphoma setting or with respect to the treatments that potentially target the lytic replication cycle.

2. Arguments of the pathogenic role of lytic infection in EBV-associated malignancies

EBV establishes a lifelong infection in B lymphocytes achieved through a highly regulated viral gene expression program. Numerous studies have been focused on the contributions of EBV latent infection in the pathogenesis of EBV-induced malignancies. These studies have revealed that the membrane LMP1 protein is the major essential oncoprotein [1, 16]. In recent years, the viral lytic cycle was shown to play an essential role in carcinogenesis through several potential mechanisms. EBV lytic infection may increase the total number of latently infected cells by enhancing transmission of the virus from cell to cell and thus constitutes an essential aspect of viral pathogenesis. A small subset of lytically infected cells was detected in biopsies of EBV-associated malignancies [13, 17–22], suggesting a potential role of viral lytic infection in promoting tumor growth in vivo. Furthermore, several studies have indicated that the viral lytic cycle in a fraction of B cells promotes the transformation of B lymphocytes in vitro [23] and growth of B-cell lymphoma in vivo [24, 25] through the release of paracrine growth factors and angiogenic factors [26]. Focusing on the study of NPC, Wu et al. revealed that recurrent EBV reactivation promotes genome instability, invasiveness, and tumorigenesis of NPC cells. Another Wu et al. finding was that the contribution of the lytic cycle is more marked than that of the latent infection [27, 28]. Additionally, lytic replication enhances in NPC cells the secretion of vascular endothelial growth factor (VEGF), a factor contributing to angiogenesis and subsequent nasopharyngeal carcinoma's

(NPC) metastasis or relapse after remission [29]. Clinical and epidemiological studies have revealed that individuals with elevated plasma EBV DNA load and antibody titers against the lytic viral capsid antigen (VCA) and early antigen (EA) display a high risk of NPC [30]. These studies have also shown that fluctuation of EBV antibody titers occurs prior to the onset of NPC [31–34]. Attention has also focused on the diagnostic and prognostic value of IgG antibody against the IEA ZEBRA (see further) in patients with NPC [33–37] and in Hodgkin disease (HD) patients as well [38]. More recently we reported that the replicative form of EBV, as investigated using anti-ZEBRA IgG titers, correlates with poorer outcomes in women with breast cancer [39]. All in all, these studies investigating serological lytic EBV markers have emphasized the prominent role importance of the lytic infection in EBV malignancies. Recently, the identification by mass spectrometry of 44 different EBV proteins in Burkitt lymphoma cells undergoing EBV replication has increased the knowledge base on EBV lytic replication, possibly highlighting different targets for future therapeutic strategies [40].

3. The role of some lytic EBV proteins in the tumorigenesis and focusing onto the Zta/ZEBRA IE transactivator

The role of the lytic EBV proteins was highlighted in some recent reviews [13, 14]. Overall, the authors clearly demonstrated the role of these lytic EBV proteins in tumorigenesis. They also showed that EBV reactivation may aid virus transmission within the tumor microenvironment to establish latency and drive cellular proliferation. Having said that, the likely predominant role of the EBV lytic cycle is likely to provide the necessary paracrine, anti-apoptotic, and immunomodulatory signals required for tumorigenesis (**Table 1**). From this point on, we will focus on the critical role of the IE ZEBRA *trans*-activator, because this protein has a key role in the balance between the EBV latent and lytic cycles. The switch from latent to lytic infection is mediated by the IE protein ZEBRA (Orf BZLF1) and R (Orf BRLF1) [6, 41–45]. ZEBRA and R proteins are transcription factors that activate one another's promoters, as well as their own promoters [8]. In combination, ZEBRA and R induce expression of all early (E) lytic viral proteins, thereby enabling the viral replication. The ZEBRA protein belongs to the bZIP family of transcription factors and is homologous to c-jun and c-fos which bind to the consensus AP1 motif as well as to atypical AP1-like motifs known as Z-responsive elements (ZREs) [42, 44, 46]. The EBV genome is not methylated in virions. However, in cells with long-term latent infection, the majority of the EBV genome proves to be highly methylated [47–51]. DNA methylation, which plays a critical role in modulating the expression of both cellular and viral genes, induces transcriptional repression through multiple different mechanisms including prevention of transcription factor binding to DNA [48, 52–56]. Surprisingly, while DNA methylation of the EBV IE BRLF1 promoter (Rp) inhibits its activation through cellular transcription factors, it enhances ZEBRA's ability to activate the R [48]. This unusual effect of Rp methylation on ZEBRA activation is due to the enhanced ability of ZEBRA to bind to the methylated, versus unmethylated, forms of two atypical CpG-containing Rp ZRE sites and requires serine residue 186 in the basic DNA domain of ZEBRA [48, 49]. Several properties of ZEBRA are listed in **Table 2**. ZEBRA was shown to inhibit antiviral cytokine signaling [57, 58] and disrupt T-cell recognition of MHC-II molecules [59]. As ZEBRA is able to activate host cellular genes (i.e., the immunomodulatory genes IL-10 and IL-13 [60, 61]), it was also found that EBV reactivation may contribute to the growth of latently infected cells [25, 62] by promoting the release of B-cell growth factors [26].

EBV lytic gene	Cellular homologs	Lytic function	Mechanisms of oncogenesis	Oncogenic function	Clinical and diagnostic relevance (Ref)
ZEBRA (Zta, BZLF1)	c-Fos, c-Jun	IEA, transactivator in cooperation with Rta	Promotes secretion of proangiogenic factors, VEGF and cytokines (IL-6, IL-8, IL-10, IL-13), toxoid protein	Angiogenesis, B-cell growth, immunomodulation	Expression in PTLD, B-cell lymphomas, Hodgkin lymphomas, NPC, detection of s-ZEBRA in PTLD patients' serum, specific antibodies in NPC/HD/PTLD patients [84]
BHRF1 and BALF1	Bcl-2	EA, delay cell death	Inhibition of apoptosis	Cell survival	
BILF1	G protein-coupled receptor	EA immunoevasin	MHC class I downregulation		
BNRF1		EA nucleocapsid trafficking	Interacts with centromeres to cause centrosome overduplication	Genome instability	
BALF3	Terminase	EA DNA synthesis and incorporation into virions	DNA damage	Genome instability	
BGLF4		EA viral DNA replication and nuclear import	DNA damage	Genome instability	
BARF1	C-fms receptor	EA immunomodulator	Modulates expression genes involved in apoptotic signaling	Cell survival	Most abundant EBV transcripts in NPC, detection of s-BARF1 in the NPC patients' serum [110] and specific antibodies [111]
BGLF5		Host cell shutoff (DNase), TLR-9 downregulation	DNA damage	Genome instability	Expression in NPC biopsies [112]. Specific antibodies in NPC [113] patients
BcRF1	Il-10	Immunoevasin	Downregulates interferon gamma	Cell survival	Soluble vIL10 in NHL patients' serum [114]

EA, early antigen; IEA, immediate-early antigen; HD, Hodgkin disease; NHL, non-Hodgkin lymphoma; NPC, nasopharyngeal carcinoma; PTLD, posttransplant lymphoproliferative disorder.

Table 1.
EBV lytic proteins and tumorigenic functions (summarized from Ref. [14]).

Functions of ZEBRA protein	Target	Effect
ZEBRA mediates the switch from latent to lytic infection, in combination with the second transactivator Rta	Virus	ZEBRA and Rta induce expression of all early (E) lytic viral proteins, allowing the virus to replicate [45]
ZEBRA is able to bind to the methylated DNA	Virus	Activation of EBV replication [48]
ZEBRA exhibits cell-penetrating properties, like HIV-1 Tat and dengue virus NS1	Host cell	Toxoid effect [82–84]
Activation of immunomodulatory genes IL-10 and IL-13	Host cell	ZEBRA contributes to the growth of latently infected cells by promoting the release B-cell growth factors [60, 61]
Inhibition of antiviral cytokine signaling	Immune system	Immune subversion [57]
High immunogenicity: ZEBRA elicits robust B- and T-cell responses that dominate the early immune responses in patients	Immune system	EBV homeostasis and immune control of EBV replication activation [67–69] Detection of specific IgG antibodies in EBV-associated diseases [34, 38, 75, 115, 116]
ZEBRA promotes B-cell transformation and lymphomagenesis in a humanized NOD/LtSz-scid/IL2R γ null mouse model of EBV-related disease (PTLD)	Animal model	ZEBRA might be a prominent factor in the development of EBV-associated malignancy [24, 62]
ZEBRA expression (mainly through abortive cycles) was shown in EBV-associated tumors, including Hodgkin disease and PTLD	Host tissue	Pathogenesis and tumor progression in patients [13, 17, 94, 96]
ZEBRA is also released in the extracellular milieu (as HIV-1 Tat) and detectable in the serum of transplant patients, particularly those with PTLD	Host tissue	Diagnostic and prognostic value [84]

Table 2.
Characteristics of the ZEBRA transactivator.

Scid/hu mice proved to be a good model to study the pathogenesis of posttransplant B-cell lymphomas [63–65]. Several recent studies emphasized the role of EBV lytic proteins (including ZEBRA) in promoting B-cell transformation and lymphomagenesis in a humanized NOD/LtSz-scid/IL2R γ null mouse model of an EBV-related disease [24]: The authors modeled a humanized NOD/LtSz-scid/IL2R γ null mouse that was reconstituted with CD34+ human hematopoietic progenitor cells isolated from fetal liver and also simultaneously xenotransplanted with fetal liver and thymus tissues [hNSG(thy)]. The hNSG(thy) mice were infected with EBV developing CD20-positive diffuse large B-cell lymphomas (DLBCL). To understand the contribution of the lytic cycle in lymphomagenesis, the hNSG(thy) mice were infected with a BZLF1 knockout EBV. Although no significant differences were apparent in the number of infected cells and in their tissue distribution, lymphomas were found in 6/11 (54.4%) of the mice infected with the wild-type virus and in only 2/14 (14.3%) of mice infected with the mutant virus. These data along with other studies support that the lytic cycle directly helps tumorigenic mechanisms rather than the lateral spread of viruses, thereby increasing the number of latently infected cells [66]. In line with this observation, lymphomas developing in the mice infected with wild-type viruses sometimes expressed ZEBRA and BMRF1 lytic proteins but never gp350/220 structural protein. Thus, these data support the participation of the abortive lytic cycle in lymphomagenesis (see below). Hong et al. made a similar observation [62]: This research group reported that when lymphoblastoid cell lines (LCLs) derived from wild type and virus

knockout of BZLF1 and BRLF1 IE genes were transplanted into scid mice, both mutant LCLs were shown unable to induce lymphoproliferative disease, supporting a critical role of the lytic cycle in the EBV-induced lymphoproliferation [62].

It appears clearly that ZEBRA expression is critical for viral activation, persistence, and disease pathogenesis. From an immunologic standpoint, ZEBRA expression is thus likely to represent a prime target of adaptive immune responses (T-cell and humoral responses) (Table 2). ZEBRA, highly immunogenic, elicits robust T-cell responses [10, 67–72] that dominate the early immune responses in patients [70]. B-cell epitopes were additionally described; to illustrate, the DNA-binding domain of ZEBRA (basic region, including the so-called RAK epitope) is a major target antigen for IgM antibody response in EBV primary infection (45), whereas the N-terminus part (activation domain) is mainly recognized by IgG in patients with EBV reactivation [35, 73–75].

4. ZEBRA, a deleterious protein acting as a “toxoid”

ZEBRA appears to be a multifunctional protein controlling its own expression, virus replication, cell cycle arrest, and DNA damage response in the host cells [76, 77–80]. The structure of the 245-amino acid ZEBRA protein has been resolved in its DNA-bound form [81]. Based on this finding, the protein appears divided into an N-terminal *trans*-activation region and a basic DNA-binding domain flanked by a coiled-coil dimerization region (zipper) [81]. The C-terminal domain interacts with the zipper region by forming intra- and intermolecular interactions, resulting in a hydrophobic pocket. These complex interactions are unique among the bZIP members and result in the stabilization of the ZEBRA dimer when bound to DNA [81] (Figure 1).

In 2005, we demonstrated that this multifunctional protein not only binds DNA and controls virus replication but also has the ability to penetrate lymphoid cells [82]. Moreover, we also demonstrated that ZEBRA possesses a transduction domain (C-terminus), crosses the outer membrane of live cells, and accumulates in the nucleus of lymphocytes. The protein transduction mostly occurs via direct translocation through the lipid bilayer rather than by endocytosis [83]. Soluble ZEBRA has additionally been detected in the sera of posttransplant lymphoproliferative disease patients, suggesting a possible secretion of this protein into the circulating

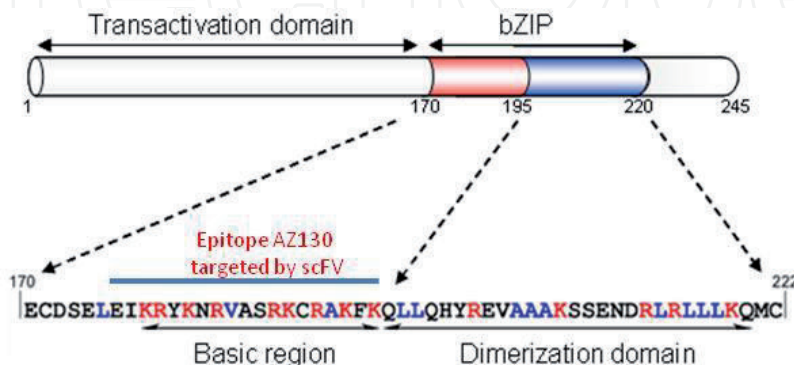


Figure 1. Schematic representation of the ZEBRA protein. The cell-penetrating peptide is located at the C-terminus (aa 178–220), enabling the cell transduction (see Ref. [83]). Two mAbs AZ125 and AZ130 specific for ZEBRA protein (recognizing the transactivation domain and the DNA-binding domain, respectively) were employed in a sandwich ELISA throughout the whole procedure for quantifying captured s-ZEBRA [84]. The DNA-binding domain of ZEBRA (basic region, including the so-called RAK epitope) is targeted by AZ130 mAb-derived scFv and could be used as possible neutralizing therapeutic drug.

blood, with further evidence toward a “toxoid” role for this lytic protein [84]. This unusual property must be compared with those of other nonstructural viral proteins like HIV-1 Tat and dengue virus (DV) NS1 [85, 86]. Therefore, we hypothesized that ZEBRA is to be involved in tumorigenesis and tumor progression, not only as an essential protein for EBV replication activation but also as a “toxin” released in the extracellular milieu. We hypothesized that early abortive replication associated with fully lytic cycles may occur within the tumor or its environment, along with eventual ZEBRA release in the bloodstream (Figure 2). In the end, this phenomenon could lead to the secretion of cytokines and angiogenesis-promoting factors, further aggravating the immunosuppressive environment (Figure 3) [26, 87].

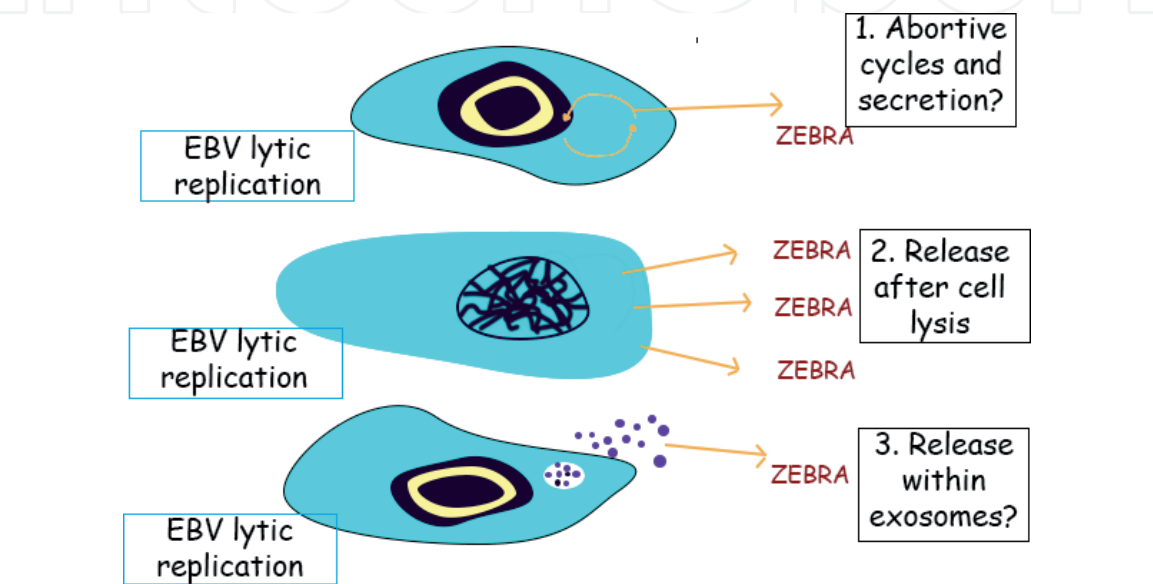


Figure 2.
Schematic representation of different possibilities of ZEBRA's release from the EBV-infected cells.

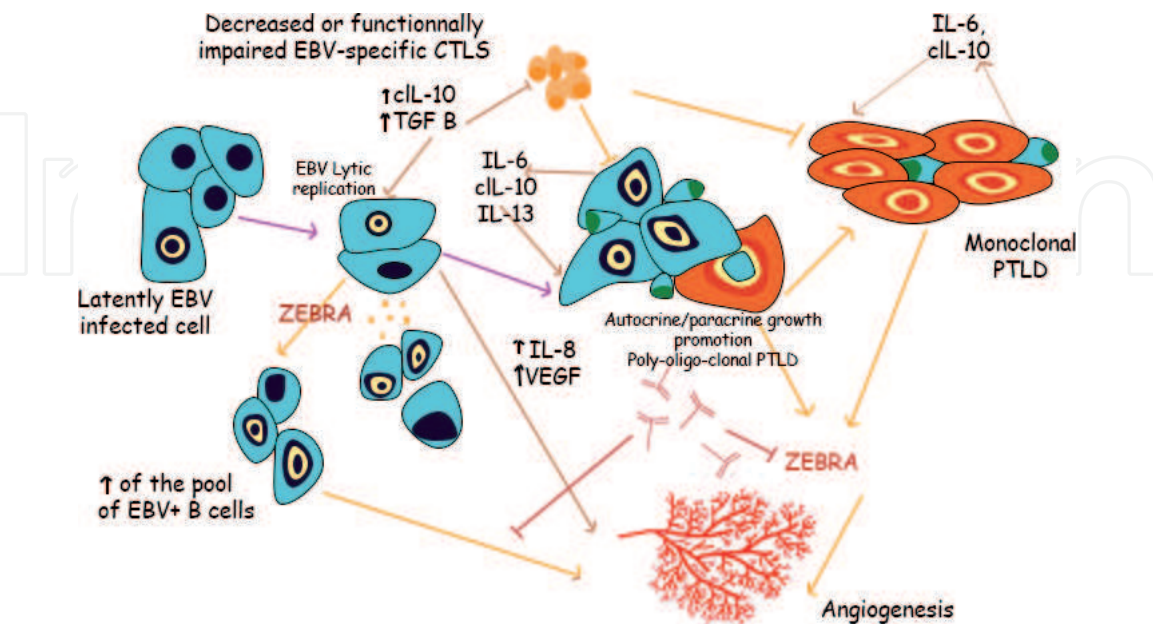


Figure 3.
Multiple effects of the ZEBRA protein on tumorigenesis and tumor progression. At any level, ZEBRA is likely to be expressed and released in the circulation as s-ZEBRA. The expression of ZEBRA and EBV replication activation may be modulated by T-cell and B-cell responses in the context of lytic/abortive cycles. There is possibility to neutralize s-ZEBRA by IgG antibodies, and ZEBRA protein could be a novel EBV-specific antigen suitable for immunotherapeutic approach.

5. Evidence of abortive lytic cycle and their role in tumorigenesis

In the absence of other lytic genes, particularly those encoding late structural proteins, without the formation of infective viral particles, BZLF1 expression is termed the “abortive lytic cycle” (**Figure 4**) [13]. The existence of abortive cycles was demonstrated in EBV-associated malignancies through the detection of either the ZEBRA protein (via monoclonal antibodies) or mRNA: Hodgkin disease [17], Non-Hodgkin lymphoma (NHL) [18, 88], NPC [20], or Burkitt lymphoma [21]. Decades ago, we revealed the early stages of EBV replication in lymphomas in scid/hu mice, assessed by the expression of ZEBRA expression, whereas the VCA expression late replicate protein proved to be weak [89]. In a recent review, the authors discussed evidence supporting an abortive lytic cycle with several lytic genes expressed, such as immunomodulatory (BCRF1, BARF1, BNLF2A, BGLF5, and BILF1) and anti-apoptotic (BHRF1 and BALF1) proteins. In their paper, the authors also discussed how the EBV immunomodulatory mechanisms result in paracrine signals that feed tumor cells. The existence of such abortive lytic cycles would reconcile the requirement of lytic expression in view of viral tumorigenesis without relying on a complete cycle that would induce cell lysis, thus releasing the newly formed infective viral particles [13].

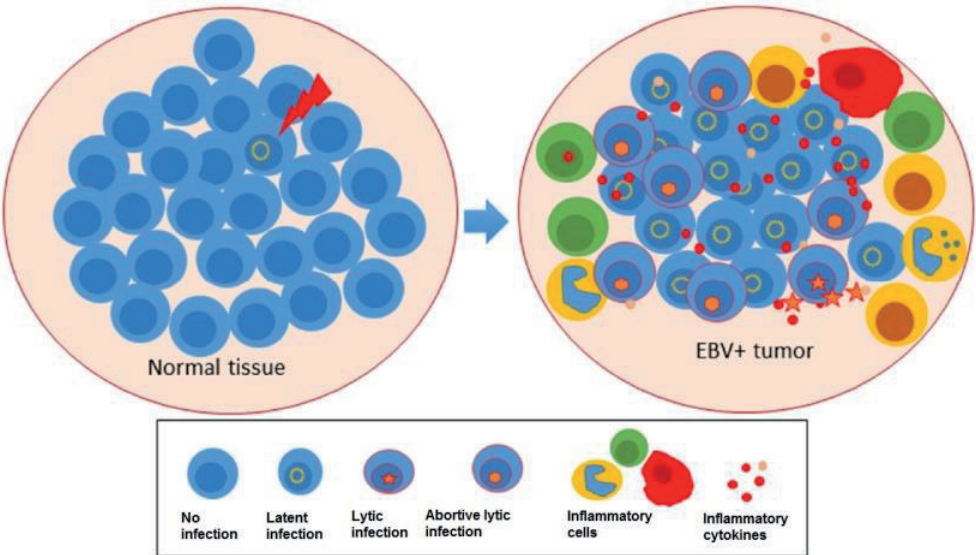


Figure 4. The existence of abortive lytic cycles (presence of ZEBRA protein, with absence of other lytic genes, particularly those encoding late structural proteins) would reconcile the requirement of lytic expression in view of viral tumorigenesis without relying on a complete lytic (from Ref. [13] with permission).

6. Consequences in terms of diagnosis and therapy

The observation of ZEBRA transduction properties and abortive cycles has prompted us to imagine the existence of a circulating ZEBRA form, especially in the serum of patients, particularly those with lymphoid tumors. Many articles have previously mentioned the existence of the lytic cycle, especially in transplant patients with PTLT. Studies conducted on EBV lytic proteins, especially the IE proteins like ZEBRA, in patients with PTLT or HIV-associated NHL are still scarce, mostly relating to the role of EBV proteins and gene products in neoplastic tissues [18, 40, 90–95]. It must be pointed out that several authors exploring BZLF1 transcripts in the peripheral blood lymphocytes (PBL) of PTLT patients demonstrated that both a high EBV genome number and strong BZLF1 mRNA expression are sensitive

markers of EBV-related PTLD [96]. In a previous study, we demonstrated ZEBRA expression in the whole peripheral blood mononuclear cells (PBMCs) from a patient exhibiting a LPD using flow cytometry. In these patients who underwent non-myeloablative allogeneic stem cell transplantation, the ZEBRA antigen was found in mostly 5% of PBMCs [97]. Moreover infected cells were detected in the peripheral blood at higher levels (e.g., 1–10 lytic-infected cells per 10^4 B lymphocytes [1, 98] versus 1 and 50 per 10^6 B cells in persistently infected healthy individuals) [99].

Recently we succeeded in detecting soluble ZEBRA (s-ZEBRA) protein in serum from transplant patients (measured by an antibody-based ELISA). The s-ZEBRA (>100 ng/mL) was predictive in 80% of PTLD patients within 10 weeks, prior to PTLD diagnosis ($p < 0.0001$) [84]. We applied this technique in both solid organ transplant patients and in hematopoietic stem cell (HSC) patients. During the HSCT patient follow-up, the availability of iterative serum samples enabled us to investigate the kinetics of s-ZEBRA appearance in comparison to that of EBV DNA qPCR and anti-ZEBRA IgG antibodies. As for patient follow-up, it was interesting to notice that the circulating ZEBRA protein could be detected during periods in which the viral DNA was not detectable by qPCR. This could be explained by certain inconsistencies observed between the qPCR and s-ZEBRA detection results (**Figure 5**). This discrepancy may be accounted for by the precocity of the ZEBRA signal measured over the course of EBV infection in this patient population. This precocity of s-ZEBRA detection (with respect to the qPCR) was independent of the PCR format, since we observed the same phenomenon in PTLD patients who were investigated by measuring the EBV load (expressed in copies/150,000 cells). In two PTLD patients, s-ZEBRA was detected at 2 and 6 weeks, respectively, prior to the PTLD episode and before the increase in qPCR signals [100]. It is interesting to note that the s-ZEBRA potentially correlated the symptomatology, as only one patient (#P3) (**Figure 5**) exhibited very high levels (3690 ng/mL) compared to the two others (#P4 and #P10) without any PTLD (162 and 300 ng/mL, respectively) (**Table 3**).

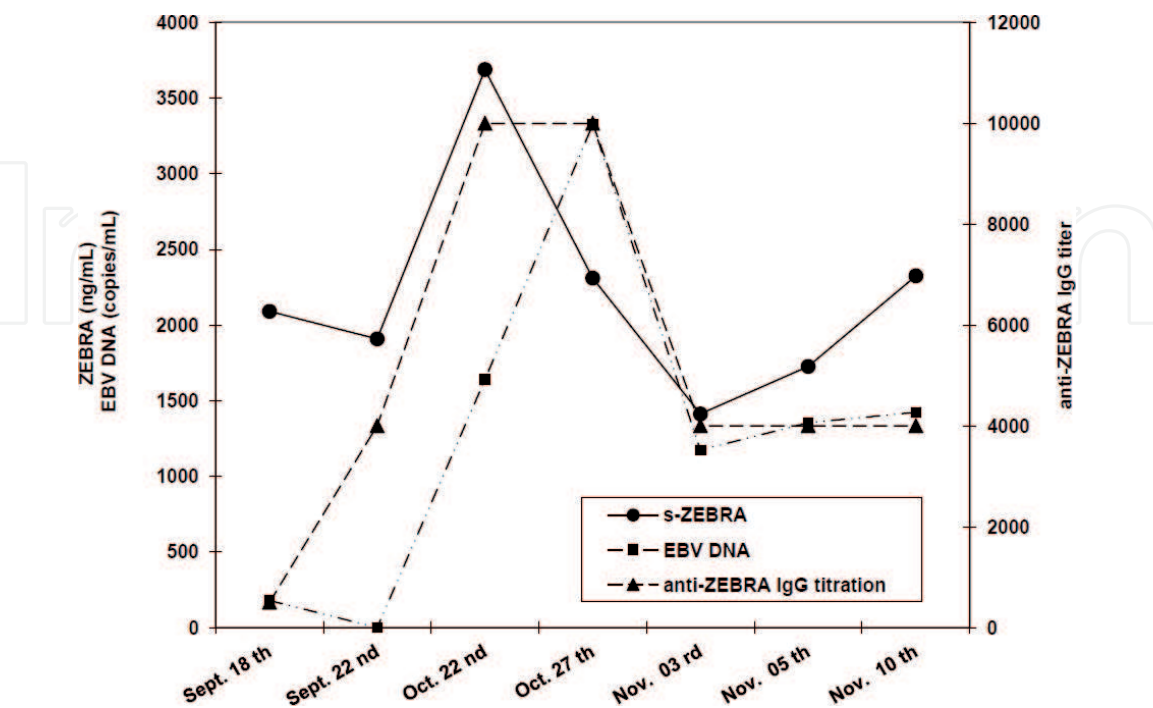


Figure 5. Follow-up of the HSCT patients #P3 (see **Table 3**): Levels of s-ZEBRA (ng/mL), titers of anti-ZEBRA IgG, and EBV DNA load (copies/mL) are noted, respectively. This patient experienced a lymphoproliferative episode (Oct. 27) and then was treated by anti-CD20 therapy, with decrease of the markers explored. The re-increase in s-ZEBRA during the course of the GvHD is worth noticing, until the patient finally died (Nov. 10).

Patients	Nr of serum samples	Means (m)			PTLD
		s-ZEBRA (OD ₄₅₀ value)	anti-ZEBRA (IgG titer)	EBV DNA load (copies/mL)	
Immunocompetent Seronegative	30	0.067	NT	0	No
Immunocompetent Seropositive	25	0.092	NT	NT	No
Infectious mononucleosis	50	0.238	24,000	NT	No
Hematopoietic stem cell transplant patients					
P1	3	0.461	2833	93	No
P2	3	0.321	3333	28	
P3 (deceased)	7	3.26	5214	1300	Yes
P4	3	0.92	2000	947	No
P5	4	0.274	2300	136	No
P6	3	0.255	200	2417	No
P7	2	0.24	2250	42	No
P8	2	0.201	4000	117	No
P9	2	0.248	4000	NT	No
P10	1	1.236	20,000	0	No
P11	1	0.323	20,000	0	No
	31	m = 0.727	m = 6012	m = 507.9	

Only one patient (#P3) experienced a PTLN episode. For patients #P3, #P4, and #P10, the OD₄₅₀ values correspond to 3690, 162, and 300 ng/mL of circulating s-ZEBRA, respectively. NT, not tested. EBV DNA load in serum was measured by qPCR (see Ref. [84]).

Table 3.
Summary of the s-ZEBRA ELISA, anti-ZEBRA IgG titration, and EBV PCR assay results in the control populations (immunocompetent EBV-seronegative/EBV-seropositive subjects, patients with primary infection) and in 11 hematopoietic stem cell transplant patients.

Therefore, s-ZEBRA detection could be a potential diagnostic marker for EBV follow-up in immunocompromised patients. Given this particular setting, our findings suggest that s-ZEBRA testing could help identify patients likely to develop severe outcomes during the critical posttransplant period. Based on our findings, we assume that the circulating ZEBRA form to be a useful target for the rapid and early diagnosis of PTLN, meaning that ZEBRA antigen-capture ELISA is likely to be a good confirmatory test for assessing EBV load in this patient population. Future evaluations of the prognostic value of ZEBRA detection should focus on the sampling time and clinical phase of lymphoproliferative disease. To improve the sensitivity and specificity of PTLN diagnosis [101–103], we hypothesize that combining approaches to detect both the circulating antigen and EBV load would be likely to increase the sensitivity and reliability of tests designed to identify such malignant EBV-related diseases.

These data focused on the relevance of the lytic cycle have already attracted the attention of the EBV community due to the potential usefulness of targeting certain lytic proteins (**Figure 6**). Investigations using both in vitro and in vivo systems revealed that FDA-approved leflunomide, a teriflunomide metabolite that targets EBV replication, inhibited the earliest step of lytic EBV reactivation (BZLF1 and BMRF1 expression) and thus prevented the development of EBV-induced lymphomas in both a humanized mouse model and a xenograft model [104].

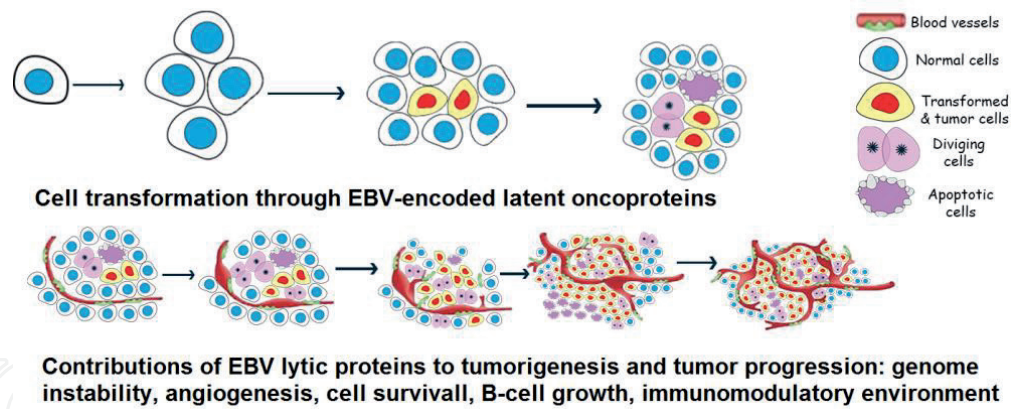


Figure 6.

Contributions of latent and lytic EBV in tumorigenesis and tumor progression. Consequences in clinical settings: In PTLN patients, EBV DNA quantification (qPCR) in blood samples corresponds to the burden of memory B cells (not proliferating lymphoblasts) infected by EBV. Thus, the increased EBV load in PBMCs, currently measured in the routine patients' follow-up, is accounted for by an increase in the number circulating EBV-positive cells (memory B cells), similar to latently infected resting B cells. Conversely, the measurement of circulating s-ZEBRA could be a novel biomarker for PTLN prognosis. The potential usefulness of targeting certain lytic proteins is also critical for managing several EBV malignancies in the future.

More recently duvelisib (a molecule inhibiting the PI3K/akt signaling pathway, thereby inhibiting BCR signaling) was shown to reduce the expression of EBV lytic genes like BZLF1 and gp350/220, in EBV-positive cell lines and cell growth, suggesting that this molecule was able to suppress the lytic EBV cycle induced by BCR signaling [105]. The histone acetylase and DNA methyl transferase inhibitors are possible avenues to suppress the ZEBRA expression and entire lytic cascade [106]. Immunotherapeutic approaches such as vaccination against IE proteins or IE-specific therapeutic monoclonal antibodies (mAbs) look likewise promising. A recent study demonstrated that vaccination of hu-PBL-SCID mice against the ZEBRA protein could enhance specific cellular immunity and significantly delay the development of lethal EBV-LPD [107]. Efforts are additionally being made to improve the quality of CD4+ T-cell line infusions responding to EBV lytic antigens [108]. Recently authors demonstrated the role of BARF1 as a novel EBV-specific antigen suitable for immunotherapeutic approach. These authors provided evidence that mAbs anti-BARF1 are likely to be a potent tool for managing several EBV malignancies [109].

In conclusion, the relevance of the lytic cycle and, particularly, the role of ZEBRA in lymphomagenesis is a new paradigm pertaining to the prevention and treatment strategies for EBV-associated cancers. Therefore, it now appears relevant to investigate the lytic EBV infection in immunocompromised patients, such as organ transplant recipients, who are highly prone to developing EBV-associated malignancies. With respect to circulating s-ZEBRA, we have made the following assumptions: (i) it may be a marker of over-immunosuppression by triggering the expression of immunomodulating cytokines; (ii) it may thus consequently play a specific role in the oncogenic process, even tumor progression. More efforts should be invested to examine the potential of drugs that target EBV lytic proteins, especially the IE proteins, such as ZEBRA.

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