[CANCER RESEARCH 56, 969-972, March 1, 1996]

Advances in Brief

Switching Viral Latency to Viral Lysis: A Novel Therapeutic Approach for Epstein-Barr Virus-associated Neoplasia

Marina I. Gutiérrez, Jean-Gabriel Judde, Ian T. Magrath, and Kishor G. Bhatia[1]

Lymphoma Biology Section, Pediatric Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892-1928

Abstract

We describe an EBV-driven lytic system (LySED) that can be used to specifically target therapy to EBV-containing tumors. This system takes advantage of the transactivating properties of EBNA-1, a latency protein expressed in all EBV-containing cells, to drive the expression of *Zta*, a gene sufficient for inducing the EBV lytic cycle. Thus, EBV provides both the target and the executor for mediating tumor-specific cell death, markedly increasing the specificity of the system. Transfection of EBV-positive cell lines with the LySED construct resulted in a switch to lytic cycle and subsequent cell death, even in the presence of an inhibitor of EBV thymidine kinase (acyclovir) without an increase in virion production. In contrast, growth of EBV-negative B-cell lines was not affected.

Introduction

EBV is a herpes virus that has been associated, as a latent virus (1), with a growing number of malignant diseases including BL[2] (2), Hodgkin's disease (3), nasal lymphomas (4), nasopharyngeal carcinoma (5), peripheral T-cell lymphoma (6), and, recently, some breast carcinomas (7). Thus, regardless of its potential pathogenic role, EBV provides a practical target for molecular therapy of neoplasia.

EBNA-1 is a protein required for the replication and maintenance of the EBV genome, and is therefore consistently expressed in all EBV-containing cells (1). EBNA-1 also acts as a transcriptional activator (8). The regulatory elements that afford EBNA-1 both of these properties lie in a region of EBV known as *oriP* (9). The minimal element within *oriP* consists of a FR (10) sequences which not only confers EBNA-1-dependent expression on an adjacent promoter, but can also act as a transcriptional repressor in EBV-negative cells (11). Thus, EBNA-1-dependent *oriP* expression vectors provide a powerful tool for targeting molecular therapy to EBV-containing tumor cells.

Zta, another transactivating protein encoded in the EBV genome, is sufficient to switch the latent virus into a lytic cycle (12–14). Thus, Zta expression in an EBV-containing cell will lead to cell lysis.

We have utilized an EBV-dependent FR-based vector (11) to force the expression of Zta in EBV-containing (EBNA-1-expressing) tumor cells to induce them to undergo self-destruction. We present data to demonstrate the feasibility and specificity of this strategy.

Materials and Methods

DNA Constructs. Zta cDNA was isolated from P3HRI by reverse transcription of total RNA primed with poly(dt). Zta DNA was obtained by amplification of the cDNA using primers 5′-CTGAAGATGATGGACCCAAACTCG-3′ and 5′-TTAGTAAACGAGGCGTGAAGCAGG-3′, which yielded a product of 780-bp equivalent to the full-length Zta cDNA. The amplified Zta was cloned in the pFR-tk plasmid (11). This pFR-tk plasmid contains an SV40 promoter driving the puromycin gene for selection of transfectants. It also contains a transcription cassette made from a minimal tk promoter placed downstream of the 20-bp FR from the *OriP* region of the EBV genome. Zta was then completely sequenced to determine the integrity of the cDNA.

Transfection of Cell Lines. EBV-positive and negative BL cell lines or a lymphoblastoid cell line (Pin) were used. Twenty μg purified plasmid were transfected into 1×10^7 cells by electroporation. Twenty-four to 48 h after transfection, the cells were plated in 24-well plates at a density of $1–2 \times 10^5$ cells/ml in medium containing 2.5 μg/ml puromycin. Clonogenicity was scored 1–3 weeks after transfection.

Western Blotting for Detection of Zta and Early Viral Lytic Antigen Expression. Protein extracts from an equal number of cells harvested 24–48 h after transfection were electrophoresed in 7.5% acrylamide Tris-HCl minigels (Bio-Rad, Hercules, CA). After electrotransfer to nitrocellulose membranes, the blots were incubated with monoclonal antibodies to EA(R) and EA(D) (Novocastra Lab, Newcastle upon Tyne, United Kingdom) or Zta (DAKO, Carpinteria, CA). Horseradish peroxidase-coupled secondary antibodies were used with the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL).

Southern Blot Analysis for Detection of Replicative EBV Genomes. Genomic DNA was prepared from cells harvested 3–4 days after transfection, digested with *Bam*HI, electrophoresed into 0.8% agarose gels, transferred to nylon membranes, and hybridized with a viral terminal repeat probe (5.2-kb *Bam*HI fragment; Ref. 15). Autoradiographs were obtained after 1–4 h of exposure.

Results

Zta Expression from the FR-tk Promoter-Enhancer Is Restricted to EBV-containing Cells. Fifteen EBV-positive and 4 EBV-negative cell lines were used to test the specificity of EBV-containing cells to support the induction of Zta from the pFR-tk-Zta construct. Twenty-four to 48 h after transfection with either the vector alone (pFR-tk) or with the Zta construct (pFR-tk-Zta), cells were harvested and Zta expression was determined in all 19 cell lines using Western blot analysis. Zta expression was detected in EBV-positive cell lines transfected with the pFR-tk-Zta construct. No detectable expression of Zta was observed in EBV-negative cell lines (data not shown). Similar results were also obtained when total RNA from the transfected cell lines was used for analysis of Zta expression.

Zta Expression in EBV-positive BL Cells Results in the Expression of Early Lytic Antigens and in Viral Replication. Having shown that the expression of Zta is restricted to EBV-containing cells, we next determined whether the EBNA-1-regulated expression of Zta from pFR-tk-Zta would induce the expression of the cascade of genes involved in the lytic cycle. As seen in Fig. 1 and Table 1, all of the cell lines that contained EBV, with the exception of PA682PE, demonstrated a marked induction of early lytic antigens, EA(R) and EA(D) proteins. Although expression of EA(R) and EA(D) was observed in some BL cell lines (AS283, JLP119, PA682BM, P3HRI, and Ag876

Received 11/27/95; accepted 1/17/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Lymphoma Biology Section, Pediatric Branch, National Cancer Institute, NIH, Building 10, Room 13N240, 10 Center Drive, MSC 1928, Bethesda, MD 20892-1928. Phone: (301) 496-2321; Fax: (301) 480-5648.

[2] The abbreviations used are: BL, Burkitt's lymphoma; EBNA-1, EBV nuclear antigen 1; FR, family of repeat; tk, thymidine kinase; EA (R), early antigen restricted; EA (D), EA diffuse.

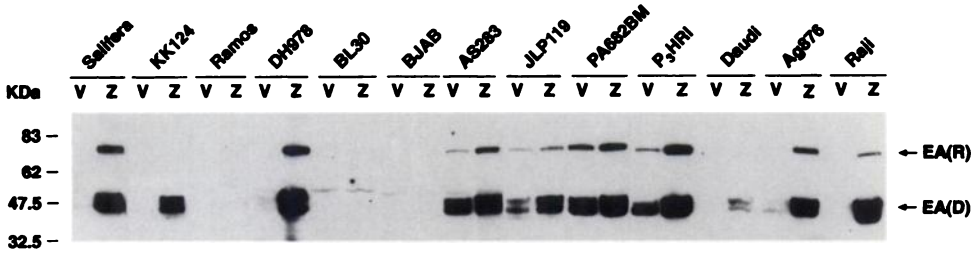

Fig. 1. Induction of EA(D) and EA(R) detected by immunoblotting. Equal number of cells transfected with the Zta construct (*Z lanes*) or the vector alone (*V lanes*) were harvested for protein assays. Early antigens are induced after pFR-tk-Zta transfection in all EBV-positive cells (Salifera, KK124, DH978, AS283, JLP119, PA682BM, P3HRI, Daudi, Ag876, and Raji). Some constitutive expression (*V lanes*) is also detected in several cell lines: AS283, JLP119, PA682BM, P3HRI, and Ag876. No induction is detected in EBV-negative cells (Ramos, BL30, and BJAB).

in Fig. 1) prior to Zta transfection, both proteins were significantly overexpressed after Zta transfection. These data indicate that EBNA-1-dependent expression of Zta is sufficient to trigger the lytic cycle in the majority of EBV-containing cell lines.

We next studied whether the lytic cycle initiated by EBNA-1-dependent induction of Zta would proceed to EBV replication and cell lysis. Southern blot analysis showed a DNA ladder of bands 500 bp apart, representing linear EBV genomes, in six of the seven cell lines tested, indicating that EBV replication had, indeed, been induced (e.g., compare *Lanes V* and *Z* in Fig. 2*B*). Although some EBV replication that was independent of Zta transfection was observed in some cell lines (AS283 and P3HRI, *Lanes V* in Fig. 2*B*), Zta overexpression induced a marked increase in the intensity of replicative EBV DNA. In PA682PE, in which Zta transfection failed to induce early antigens, EBV replication was also not induced. The possibility that EBV in this cell line is dysfunctional in replicative properties is supported by our observation that the *Bam*HI NJ het region of EBV (including the terminal repeats) is partially deleted (unpublished data).

Transfection of FR-tk-Zta Suppresses Clonogenicity of EBV-positive Cell Lines but not of EBV-negative Cells. To ensure that the lytic pathway initiated by pFR-tk-Zta transfection proceeded to the ultimate end point, *i.e.*, cell death, we examined the effect of EBNA-1-based Zta expression on the clonogenicity of EBV-positive *versus* EBV-negative cells.

As can be seen in Table 1, expression of Zta completely abolished the clonogenicity of all EBV-positive cell lines except PA682PE, the one cell line in which EAs and EBV replication were not induced.

Table 1 *Induction of early lytic antigens and cell survival of EBV-positive and EBV-negative cells transfected with either pFR-tk or pFR-tk-Zta*

Cell line (EBV status)	EA(D)/EA(R) induction	No. of clones/well	
		ZTA	Vector
P3HRI (+)[a]	+	0	>100
AS283 (+)	+	0	>100
Pin (+)	+	0	>100
Raji (+)	+	0	70
JLP119 (+)	+	0	90
KK124 (+)	+	0	>100
Ag876 (+)	+	0	>100
DH987 (+)	+	0	>100
NA (+)	+	0	>100
PA682PE (+)	–	>100	>100
PA682BM (+)	+	nd	nd
Sfa (+)	+	nd	nd
Daudi (+)	+	nd	nd
BJAB/B95.8 (+)	+	nd	nd
DW6 (+)	+	nd	nd
BJAB (–)	–	35	61
CA46 (–)	–	63	>100
Ramos (–)	–	16[c]	20[c]
BL30 (–)	–	8[c]	8[c]

[a] +, associated with EBV; –, EBV negative.
[b] nd, not done.
[c] Clones per 24-well plate.

There was, however, no difference in the number of clones obtained following transfection of either pFR-tk or pFR-tk-Zta in the EBV-negative cells. Although pFR-tk-Zta transfection repeatedly resulted in a complete abolition of clonogenicity in the EBV-positive cell lines, this appeared to be a direct result of Zta transfection; we were unable to demonstrate any significant bystander effect (data not shown).

Since Zta was expressed only in the EBV-containing cell lines, it was still possible that cell death could result from Zta-mediated toxicity and was thus independent of the requirement of the EBV genome to express the cascade of genes necessary for cell lysis. To rule out this possibility, we used a vector in which Zta was constitutively expressed from a strong EBNA-1 independent promoter, the elongation factor 1α promoter-enhancer (16). Clonogenicity was completely abolished in EBV-positive cells. In the EBV-negative cell lines, there was no difference in the number of clones obtained either with the vector control (pEF) or with the pEF-Zta, demonstrating that overexpression of Zta in the absence of functional EBV genomes is not cytotoxic.

Zta-mediated Cell Death of EBV-positive BL also Occurs in the Presence of Acyclovir. One readily apparent complication resulting from switching EBV from latency to lytic cycle in the tumor cells would be the acute viral load generated in the host. At present, it is not known whether the pathways leading to cell death and to viral replication diverge at some point following Zta-dependent activation of the lytic cycle. It seems likely, however, that cell death is not a direct consequence of viral DNA replication. If this is so, pFR-tk-Zta-mediated cell death could still occur even in the presence of inhibitors of viral DNA synthesis and hence virion production. We, therefore, tested the LySED system in the presence of acyclovir (17). Following transfection with the Zta construct, cells were selected either in the presence of puromycin alone or in the presence of puromycin and 100 μM acyclovir. EA(R) and EA(D) induction, expression of Zta, clonogenicity, and viral replication were assessed as before (Table 2).

Whereas induction of EA(R) and EA(D) was not affected by acyclovir (Fig. 2*A*), replication of EBV DNA was severely restricted (Fig. 2*B*). However, even in the absence of productive viral particles, clonogenicity of the EBV-positive cell lines was completely abolished (Fig. 2*C*).

Discussion

In EBV-associated neoplasms (2–7), as in normal infected B-cells (1), EBV exists in a latent, *i.e.*, nonreplicating, viral form in the majority of cells. Overt EBV replication is associated with cell death and would therefore be incompatible with tumor growth. Cell death in this context occurs by mechanisms not well characterized, but is nonetheless dependent on *EBV* genes. Since virions are produced in a small fraction of the cells of EBV-associated lymphomas (3,18), it is reasonable to believe that the lytic pathway is intact in these tumor

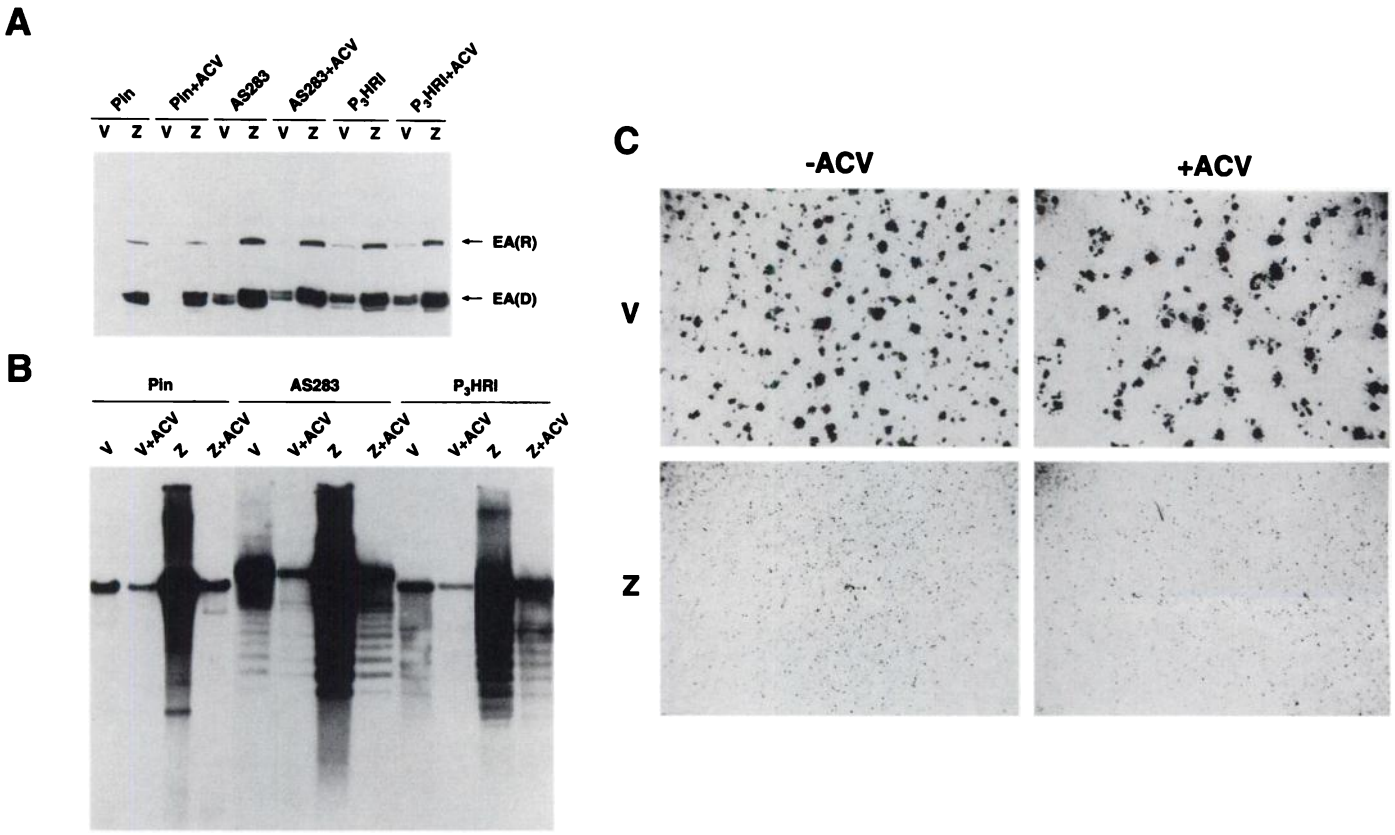

Fig. 2. *A*, Immunoblot for detection of EA(R) and EA(D) expression. SDS-PAGE-separated and electroblotted proteins were reacted with anti-early antigen monoclonal antibodies and visualized using the enhanced chemiluminescence system. Following transfection, cells were plated either in the absence of acyclovir or presence of 100 μM acyclovir (*+ACV*). *V lanes*, proteins from cells transfected with pFR-tk and *Z lanes*, proteins from cells transfected with pFR-tk-Zta. P3HRI and AS283 show EA(D) and EA(R) expression in the absence of exogenous Zta (constitutive expression). *B*, Southern blot analysis using DNA prepared from cells transfected with the Zta construct (*Z lanes*) or with the vector alone (*V lanes*) and plated in selective media containing 100 μM acyclovir (*+ACV*) or no acyclovir. All three cell lines transfected with Zta show a marked induction of EBV replicative DNA. A marked reduction of EBV replication is seen in the presence of acyclovir. P3HRI and AS283 show spontaneous EBV replication (*V lanes*), inducible by Zta (*Z lanes*). Both of these are inhibited by acyclovir. *C*, P3HRI transfected cells were plated in selective media with acyclovir (*+ACV*) or without acyclovir (*−ACV*). Cells (one well each) were photographed 1 week after transfection. Cells transfected with pFR-tk (*V*) show >100 clones, while cells carrying pFR-tk-Zta (*Z*) show no clones.

cells. EBV consequently provides a natural weapon that can be harnessed to cause specific lysis of EBV-containing tumor cells by switching latent EBV to lytic EBV. There is indeed a natural precedent for this type of tumor control mechanism. In the Lucké virus-induced adenocarcinoma of the frog, neoplasms induced in the tadpole by the Lucké virus grow during the warm summer months when the virus is not produced, but regress in winter, when low temperatures induce virus replication (19).

The lytic cycle of EBV appears to be controlled by the transactivating protein Zta, which is both necessary and sufficient to induce the cascade of proteins that eventually cause virion production and cell lysis (12–14). We have targeted the expression of a transfected *Zta* gene specifically to EBV-containing cells by making its expression dependent on the binding of the EBV latent protein EBNA-1 to FR elements present within the vector (8–11). Furthermore, in an EBV-negative background the same FR elements act to repress transcription, increasing the specificity of the system for EBV-positive cells (11). In any event, since the expression of Zta alone, at least under these *in vitro* conditions, is nontoxic, it seems likely that any leakiness associated with the system would result in minimal toxicity. The crucial executor of cell death in LySED is the EBV lytic cycle, which can only be activated in cells that contain EBV. Thus, the LySED system contains two levels at which its specificity is restricted to EBV-positive cells.

Table 2 *Expression of EBV early antigens, survival of clones, and inhibition of replicative EBV genomes in the presence of acyclovir*

Cell line. Construct	Acyclovir	EA(D)/EA(R) expression	No. of clones/Well	Linear genomes
Pin.vector	−	−	>100	−
	+	−	>100	−
Pin.Zta	−	++	0	++
	+	++	0	−
AS283.vector	−	+	>100	++
	+	+	>100	+
AS283.Zta	−	+++	0	+++
	+	+++	0	+
P3HRI.vector	−	+	>100	++
	+	+	>100	+
P3HRI.Zta	−	+++	0	+++
	+	+++	0	+

Although nonneoplastic EBV-containing cells would also be subjected to Zta-mediated destruction, this would be unlikely to cause harm to the host. The frequency of EBV-containing B cells ranges from 1 to 10/1,000,000 B cells (20). Thus, the total number of EBV-containing cells in an individual is likely to be very small, and toxicity, if any, would be minimal.

An apparent complication resulting from the efficient use of the LySED system *in vivo* would be massive virus production. This could be a concern, especially when treating immunocompromised patients. We have circumvented this problem by showing that cell death also occurs when the virus lytic cycle is activated by pFR-tk-Zta in the presence of acyclovir, an inhibitor of viral DNA replication (17). Thus, the cell death program that results from turning on the lytic

cycle can be uncoupled from the EBV DNA replication program and hence virion production.

References

1. Rogers, R., Strominger, P. J., and Speck, S. Epstein-Barr virus in B lymphocytes: viral gene expression and function in latency. Adv. Cancer Res., *58:* 1–26, 1992.
2. Shiramizu, B., Barriga, F., Neequaye, J., Jafri, A., Dalla-Favera, R., Neri, A., Gutierrez, M., Levine, P., and Magrath, I. Patterns of chromosomal breakpoint locations in Burkitt's lymphoma: relevance to geography and Epstein-Barr virus association. Blood, *77:* 1516–1526, 1991.
3. Brousset, P., Knecht, H., Rubin, B., Drouet, E., Chittal, S., Meggetto, F., Al Saati, T., Bachmann, E., Denoyel, G., Sergeant, A., and Delsol, G. Demonstration of Epstein-Barr virus replication in Reed-Stenberg cells of Hodgkin's disease. Blood, *82:* 872–876, 1993.
4. Guerrero, I., Franklin, J., Naresh, K. N., Caceres, E., Bhatia, K., O'Conor, G., and Magrath, I. p53 expression, bcl-2 expression and EBV association in Peruvian nasal lymphomas. Blood, *84* (Suppl.)*:* 522a, 1994.
5. Chang, Y. S., Tyan, Y. S., Liu, S. T., Tsai, M. S., and Pao, C. C. Detection of Epstein-Barr virus DNA sequences in nasopharyngeal carcinoma cells by enzymatic DNA amplication. J. Clin. Microbiol., *28:* 2398–2402, 1990.
6. Suzushima, H., Asou, N., Fujimoto, T., Nishimura, S., Okubo, T., Yamasaki, H., Osato, M., Matsuoka, M., Tsukamoto, A., Takai, K., *et al.* Lack of the expression of EBNA-2 and LMP-1 in T-cell neoplasms possessing Epstein-Barr virus. Blood, *85:* 480–486, 1995.
7. Labrecque, L. G., Barnes, D. M., Fentiman, I. S., and Griffin, B. E. Epstein-Barr virus in epithelial cell tumors: a breast cancer study. Cancer Res., *55:* 49–45, 1995.
8. Reisman, D., and Sugden, B. Trans-activation of an Epstein-Barr transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Mol. Cell. Biol., *6:* 3828–3846, 1986.
9. Reisman, D., Yates, J., and Sugden, B. A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two *cis*-acting components. Mol. Cell. Biol., *5:* 1822–1832, 1985.
10. Wysokenski, D. A., and Yates, J. L. Multiple EBNA 1-binding sites are required to form an EBNA 1-dependent enhancer and to activate a minimal replicative origin with oriP of Epstein-Barr virus. J. Virol., *63:* 2657–2666, 1989.
11. Judde, J. G., Spangler, G., Magrath, I., and Bhatia, K. Use of Epstein-Barr virus nuclear antigen-1 in targeted therapy of EBV associated neoplasia. Hum. Gene Ther., in press, 1996.
12. Kieff, E. and Liebowitz, D. Epstein-Barr virus and its replication. *In:* B. N. Fields, D. M. Knipe, *et al.* Fundamental Virology, 2nd ed. New York: Raven Press, Ltd., 1991.
13. Holley-Guthrie, E. A., Quinlivan, E. B., Mar, E. C., and Kenney, S. The Epstein-Barr virus (EBV) BMRF1 promoter for early antigen (EA-D) is regulated by the EBV transactivators, BRLF1 and BZLF1, in a cell-specific manner. J. Virol., *64:* 3753–3759, 1990.
14. Rooney, C. M., Rowe, D. T., Ragot, T., and Farrell, P. J. The spliced *BZLF1* gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. J. Virol., *53:* 3109–3116, 1989.
15. Raab-Traub, N., and Flynn, K. The structure of the termini of Epstein-Barr virus as a marker of clonal cellular proliferation. Cell, *47:* 883–889, 1986.
16. Mizushima, S., and Nagata, S. pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res., *18:* 5322, 1990.
17. Wagstaff, A. J., Faulds, D., and Goa, K. L. Acyclovir: a reappraisal of its antiviral activity, pharmacokinetic properties and therapeutic efficacy. Drugs, *47:* 153–205, 1994.
18. Gutierrez, M. I., Bhatia, K., and Magrath, I. Replicative viral DNA in Epstein-Barr virus associated Burkitt's lymphoma biopsies. Leuk. Res., *17:* 285–289, 1993.
19. McKinnell, R. G., Ellis, V. L., Dapkus, D. C., and Steven, L. M., Jr. Early replication of herpesviruses in naturally occurring frog tumors. Cancer Res., *32:* 1729–1733, 1972.
20. Wagner, H. J., Bein, G., Bitsch, A., and Kirchner, H. Detection and quantification of latently infected B lymphocytes in Epstein-Barr virus-seropositive, healthy individuals by polymerase chain reaction. J. Clin. Microbiol., *30:* 2826–2829, 1992.