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Induction of Lytic Epstein-Barr Virus (EBV) Infection by Synergistic Action of Rituximab and Dexamethasone Renders EBV-Positive Lymphoma Cells More Susceptible to Ganciclovir Cytotoxicity In Vitro and In Vivo

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The purposeful induction of the lytic form of Epstein-Barr virus (EBV) infection combined with ganciclovir (GCV) treatment has been advocated as a novel strategy for EBV-positive B-cell lymphoma. We demonstrated that rituximab had a synergistic effect with dexamethasone on induction of the lytic EBV infection in CD20-positive lymphoma cells. Addition of GCV to the dexamethasone/rituximab-treated cells was more effective than dexamethasone/rituximab alone in killing EBV-positive lymphoma cells in vitro and in lymphoma-bearing nude mice but not in EBV-negative cells. These data suggest that induction of the lytic EBV infection with dexamethasone/rituximab in combination with GCV could be a potential virally targeted therapy for EBV-associated B-cell lymphoma.

Epstein-Barr virus (EBV) is an oncogenic herpesvirus associated with a number of human malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, and posttransplantation lymphoproliferative diseases (17). The presence of the EBV genome in virtually all of the malignant cells suggests that novel therapies to specifically kill EBV-infected cells could be employed to treat these malignancies. Since the majority of EBV-infected tumor cells carry the EBV genome in a latent form, antiviral therapy has not been proven useful for treatment of the diseases. One approach would be to induce EBV lytic infection in tumor cells (10), which may make the cells susceptible to antiviral drugs, such as ganciclovir (GCV) (15, 24). GCV, itself a cytotoxic prodrug, is converted into a more active cytotoxic form by the EBV lytic proteins (15, 24). The switch from latent to lytic infection is mediated by the transcriptional effects of the immediate-early protein encoded by the EBV BZLF1 gene, which is not expressed during latency (12). The immediate-early protein can induce the full component of early viral lytic genes, such as the BMRF1 gene (12). In the search for effective treatments to induce the lytic EBV infection, we found that rituximab, a chimeric anti-CD20 monoclonal antibody, has a synergistic effect with a glucocorticoid, dexamethasone, on induction of lytic EBV infection in latently EBV-infected B-lymphoma cells. Furthermore, addition of GCV to the dexamethasone/rituximab-treated cells led to enhanced cytotoxicity in EBV-positive cells but not in EBV-negative cells.

In this study, we used the CD20-positive lymphoma Akata cells. Akata cells carry the EBV genome, but only 1 to 2% of EBV-positive cells express lytic antigens (23). An EBV-negative cell clone was isolated from the parental Akata cells by the limiting-dilution method as previously reported (22). Thus, the isogenic EBV-positive and EBV-negative Akata cells were considered to be suitable for our study. Cells were incubated in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 in air and maintained in log growth phase. Cells were used for experiments only when viability exceeded 95%. We first evaluated the effects of dexamethasone on induction of the EBV lytic form. Dexamethasone was purchased from Sigma (St. Louis, MO). Cells were treated with various concentrations of dexamethasone (1 to 100 nM), and 3 days later, viral immunofluorescence was performed to quantitate the number of cells expressing a viral lytic cycle antigen, early antigen (EA). For indirect immunofluorescence, cells were washed with phosphate-buffered saline (PBS), spotted onto glass slides, and fixed in acetone. The cells were reacted with a mixture of monoclonal antibodies (MAbs), R3/C844, against the EBV EA-diffuse component (EA-D) and the EA-restricted component (EA-R) (9). After being washed in PBS, the slides were incubated with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (IgG) (Dako, Glostrup, Denmark). The slides were examined by fluorescence microscopy. At least 1,000 cells were counted for each determination. Dexamethasone-treated cells had 3 to 15% of cells expressing the lytic proteins (Fig. 1A). We then evaluated the effects of rituximab on induction of lytic EBV infection. Rituximab was provided by Zenyaku Kogyo Co. (Tokyo, Japan). Rituximab alone, up to the concentration of 100 μg/ml, did not significantly induce lytic infection. However, combination of dexamathasone with rituximab resulted in synergistic induction: immunofluorescence analysis showed that addition of rituximab (100 μg/ml) enhanced the number of cells expressing the lytic proteins

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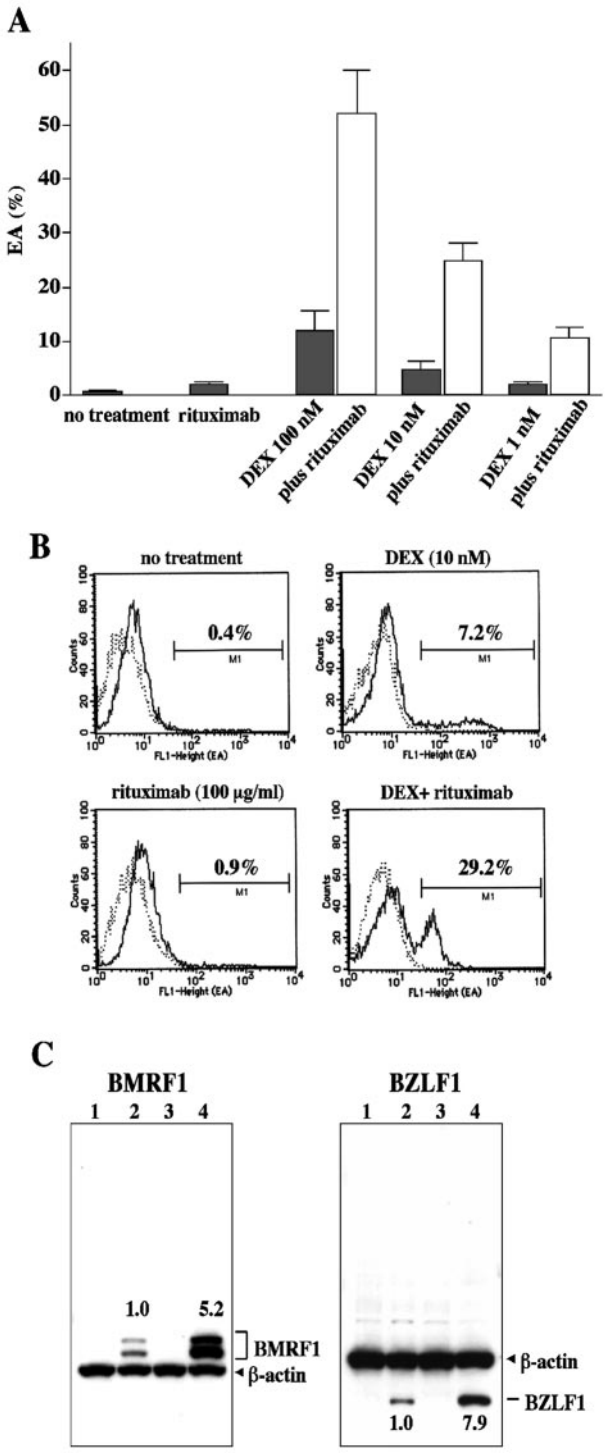

FIG. 1. Enhanced induction of lytic EBV infection by dexamethasone (DEX) and rituximab. (A) Cells were treated with no drug, rituximab (100 μg/ml), DEX at various concentrations, or DEX plus rituximab for 3 days and examined for EBV lytic EA antigens by immunofluorescence analysis. Results from four independent experiments are shown. Standard deviation bars are shown with vertical lines. (B) Induction of EA-D (BMRF1 product) was assayed by FACS analysis. The percentage of EA-positive cells is shown for each experiment. Curves defined by dotted lines represent the background fluorescence obtained on cells stained with the control antibody. (C) Immunoblot analyses to detect the BZLF1 and BMRF1 products. Lane 1, no treatment; lane 2, DEX (10 nM); lane 3, rituximab (100 μg/ml); and lane 4, DEX/rituximab. The indicated values were normalized to β-actin signal intensities and represent the ratio when signal intensity from the cells treated with DEX alone was set at 1.0.

approximately four to five times in comparison with dexamethasone (10 nM) treatment alone (Fig. 1A). For fluorescence-activated cell sorting (FACS) analysis, cells were fixed in 4% paraformaldehyde, washed in staining buffer (PBS with 1% bovine serum albumin and 0.03% saponin), and incubated with the mouse MAb R3 (Chemicon, Temecula, CA), which recognizes polypeptides of EA-D (BMRF1 product) (16). Isotype-matched control antibody was mouse IgG_1 (Dako). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was used as a secondary antibody. Cells were analyzed by Becton Dickinson FACScan with CELLQUEST analysis software (San Jose, CA). FACS analysis also demonstrated that simultaneous treatment with rituximab and dexamethasone led to enhanced induction of EA-D (BMRF1 product), approximately four times compared with dexamethasone treatment alone (Fig. 1B). These results were confirmed by immunoblot analysis (Fig. 1C). Immunoblot analysis was performed as previously described (1), and reactive proteins were detected with the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). The AZ-69 MAb (Argene, Varilhes, France) reacts with a polypeptide of ZEBRA (BZLF1 product) (13). The MAb to β-actin (AC-74; Sigma), as an internal control antibody, was used in parallel to confirm that equal amounts of protein were loaded in lanes of the gels. Quantitation of signal intensities of the immunoblots was performed by densitometric scanning using Light-Capture AE-6962 (Atto, Tokyo, Japan). Values were normalized to β-actin signal intensities of the same lanes. Addition of rituximab to the dexamethasone-treated cells resulted in enhanced induction of EA-D (BMRF1 product) and ZEBRA (BZLF1 product), approximately five and eight times, respectively.

We next examined whether GCV is effective in killing the EBV-positive and EBV-negative cells treated with dexamethasone and rituximab. GCV was supplied from Tanabe Pharmaceutical Co. (Osaka, Japan). We isolated EBV-negative cells from the parental Akata cells. The absence of the EBV genome in the isolated clone was confirmed by PCR (Fig. 2A). FACS analysis showed that the CD20 antigen was uniformly expressed in the EBV-positive and EBV-negative cells (Fig. 2B). These cells at an initial concentration of 10^5 cells/ml were incubated with no drug or dexamethasone (1 nM or 10 nM)/rituximab (100 μg/ml) in the presence or absence of GCV (20 μM). The number of viable cells remaining after the 5-day incubation was determined by trypan blue exclusion. GCV alone at a concentration of 20 μM did not influence cell growth. Addition of GCV led to enhanced cell killing in EBV-positive cells treated with dexamethasone/rituximab (Fig. 2C): the number of cells surviving the combination treatment with GCV and dexamethasone/rituximab was reduced significantly in the EBV-positive cells but not in the EBV-negative cells. In these experiments, expression of the lytic EBV protein (BMRF1 product) in the EBV-positive cells treated with dexamethasone/rituximab was confirmed by immunoblot analysis (Fig. 2D). It may be worth noting that treatment of the cells

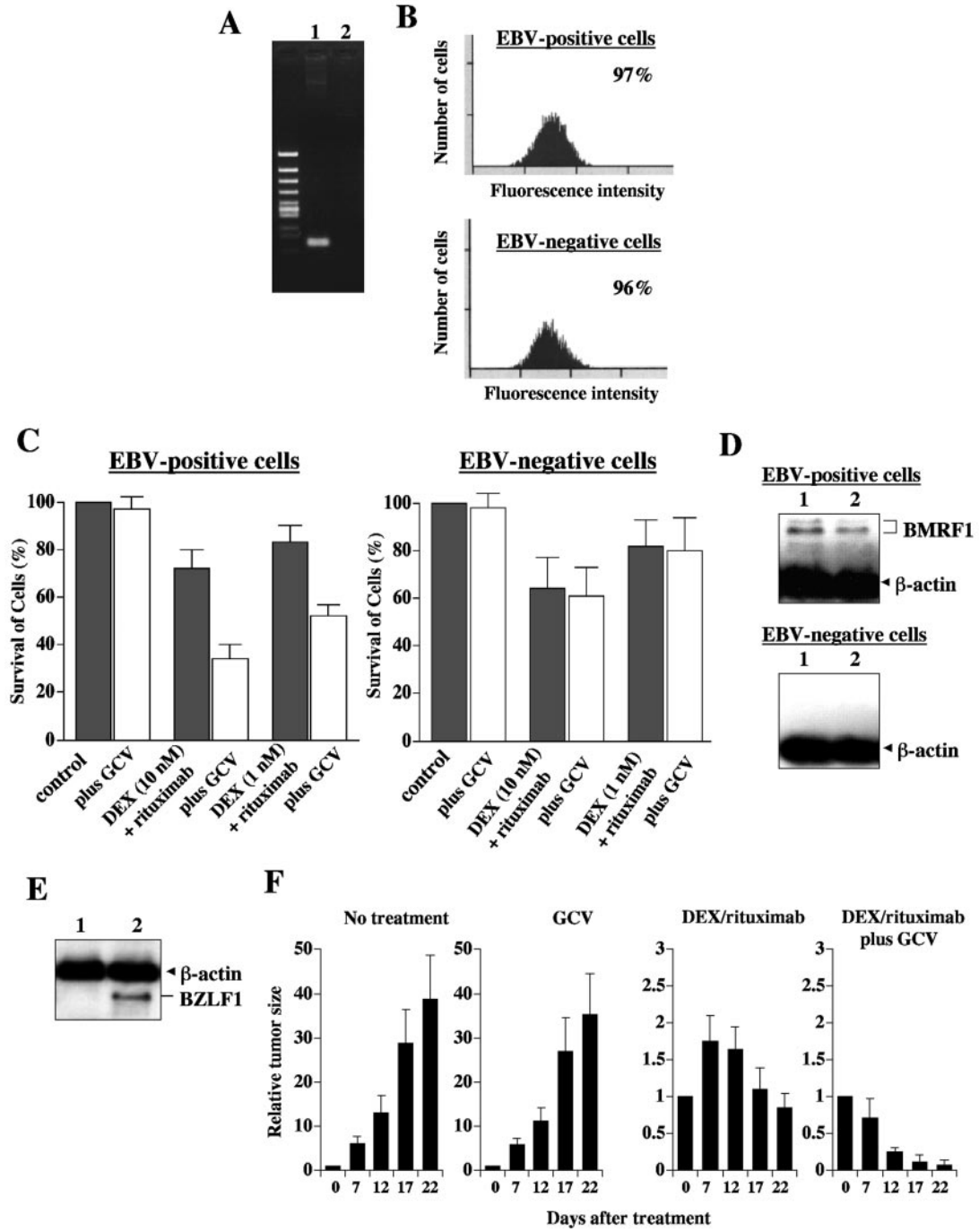

FIG. 2. Dexamethasone (DEX) and rituximab confer GCV susceptibility to EBV-positive cells in vitro and in vivo. (A) The absence of the EBV genome in the isolated clone (lane 2) from parental EBV-positive Akata cells (lane 1) was confirmed by PCR. The primers were derived from the sequences corresponding to the EBV BamHI-W region, generating a 129-bp fragment. ϕX174/HincII-cut DNA size markers are also shown. (B) FACS analysis showed that both EBV-positive and EBV-negative cells uniformly expressed the CD20 antigen. The percentages of CD20-positive cells are shown. (C) EBV-positive versus EBV-negative cells were treated with no drug or DEX (1 nM or 10 nM) plus rituximab (100 μg/ml) in the presence or absence of GCV (20 μM) for 5 days. The percentage of viable cells in comparison with untreated control cells (set at 100% viability) is shown. Error bars indicate standard deviations from three independent experiments. (D) Immunoblot analysis confirmed the expression of the lytic EBV protein (BMRF1 product) in EBV-positive Akata cells treated with DEX (1 nM) plus rituximab (100 μg/ml) in the absence (lane 1) or presence (lane 2) of GCV (20 μM) for 5 days. (E) DEX and rituximab induced lytic EBV infection in tumor cells of nude mice. EBV-positive Akata cells were transplanted into nude mice, and when tumors were formed, the mice were treated with no drug or DEX/rituximab. Three days later, the tumor extracts were examined for induction of the lytic EBV protein (BZLF1 product) by immunoblot analysis. Lane 1, no treatment; lane 2, DEX/rituximab. (F) GCV enhanced the cytotoxic effects of DEX/rituximab in nude mice. Nude mice with EBV-positive tumors (total of 10 tumors in each group from five independent experiments) were given no drug, GCV only, DEX/rituximab, or DEX/rituximab plus GCV. The relative tumor volume (set at 1.0 on day 0) in each treatment group at various time points after initiation of treatment (day 7, 12, 17, or 22) is shown. Error bars indicate standard deviations.

with dexamethasone/rituximab itself showed relative cytotoxicity (Fig. 2C). The finding is consistent with a previous report in which Rose et al. (18) demonstrated synergistic cytotoxicity against CD20-positive B-lymphoma cells in vitro by dexamethasone and rituximab.

We examined whether dexamethasone/rituximab treatment can induce lytic EBV infection in vivo. Female BALB/c nude mice, 4 to 5 weeks of age, were inoculated with 5×10^7 cells in the flanks via subcutaneous injection. Treatment was started 10 to 20 days after transplantation, when tumors reached the size of 150 to 200 mm^3. Mice were treated with one of the following: no treatment, dexamethasone (5 mg/kg of body weight on day 0, intraperitoneally)/rituximab (50 mg/kg administered on day 0, intraperitoneally), combination of rituximab/dexamethasone and GCV (100 mg/kg for 5 days, intraperitoneally), or GCV only. The tumors were measured regularly, and the tumor volume was calculated as $1/2 \times a \times b^2$, where a represents the longest axis of tumors in millimeters and b represents the axis perpendicular to a in millimeters. The tumor-bearing nude mice were treated with no drug or dexamethasone/rituximab. Three days later, mice were euthanized, and tumor extracts were analyzed by immunoblot assay (Fig. 2D). Dexamethasone/rituximab led to induction of the lytic EBV protein (BZLF1 product) in the nude mouse tumors. We next tested whether GCV can enhance the cytotoxic effect of dexamethasone/rituximab in the tumor-bearing nude mice (Fig. 2E). Treatment with GCV alone did not influence tumor growth. Treatment with dexamethasone/rituximab suppressed tumor growth, but the tumors in mice treated with dexamethasone/rituximab plus GCV were significantly smaller than those in mice treated with dexamethasone/rituximab alone. Some tumors were not totally obliterated by the dexamethasone/rituximab-plus-GCV treatments. It may be explained that the tumors contained the cells in which the lytic form of EBV infection had not been fully induced by dexamethasone/rituximab and that therefore the cells could not be susceptible to GCV cytotoxicity.

One approach to virally targeted therapies for EBV-associated lymphoproliferative diseases would be to induce lytic EBV infection intentionally, thereby converting certain antiviral drugs, such as GCV, into cytotoxic forms that kill the host tumor cells without completion of the viral replicative cycle and release of infectious EBV particles (5, 10, 25). Although several chemicals, such as phorbol esters, calcium ionophores, and sodium butyrate, induce lytic EBV infection (12), whether these chemicals can be safely used in patients remains unknown. The initial results of a phase I/II trial, in which patients were given the combination of arginine butyrate and GCV, showed that complete clinical responses were achieved in 5 of 10 patients with EBV-associated lymphoproliferative diseases previously resistant to conventional chemotherapy (4, 14), suggesting that such virally targeted strategies may be promising. We looked for reagents that can induce the lytic EBV infection and yet can be safely used in patients. Dexamethasone was previously shown to activate the lytic EBV genes (19). Here we demonstrated that the addition of rituximab to dexamethasone enhanced the efficacy of dexamethasone. Several studies have shown that rituximab can initiate signal transduction events that activate protein tyrosine kinases and increase intracellular Ca^{2+} (8, 20, 21). Since these signal pathways are necessary for the efficient induction of the viral lytic form following ligation of B-cell receptors (2, 3, 11), activation of such protein kinases as well as Ca^{2+} elevation initiated by rituximab are likely to account for enhancement of the effect of dexamethasone on inducing the lytic EBV infection.

In the present study we demonstrated that combination treatment with dexamethasone/rituximab plus GCV was more effective than dexamethasone/rituximab alone in killing B-lymphoma Akata cells with type 1 EBV latency in vitro and in nude mice. Importantly, such a GCV cytotoxic effect was EBV dependent. These data suggest that GCV in combination with dexamethasone/rituximab could be a virally targeted therapy for EBV-associated B-cell lymphoma. Since both rituximab and glucocorticoids have been included in treatment regimens for patients with B-cell lymphoma, our results may have clinical relevance. Recently, Feng et al. (6) reported that doxorubicin can induce lytic EBV infection in EBV-transformed B cells. Ghetie et al. (7) demonstrated that rituximab can potentiate the cytotoxic effects of doxorubicin in B-lymphoma cells. Given these observations, the strategy with rituximab plus dexamethasone/doxorubicin in combination with GCV could be a stronger virally targeted therapy for EBV-associated B-cell lymphoma. Clinical studies, therefore, are required to determine whether such strategies can be effective in patients. Our interest now points toward detailed analysis concerning whether EBV-positive lymphoma cells containing other types of EBV latency, such as type 3, also show a favorable response to combination therapy with ganciclovir and dexamethasone/rituximab.

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REFERENCES

1. **Daibata, M., E. M. Enzinger, J. E. Monroe, R. E. Kilkuskie, A. K. Field, and C. Mulder.** 1996. Antisense oligodeoxynucleotides against the BZLF1 transcript inhibit induction of productive Epstein-Barr virus replication. Antiviral Res. **29:**243–260.
2. **Daibata, M., R. E. Humphreys, K. Takada, and T. Sairenji.** 1990. Activation of latent Epstein-Barr virus via anti-IgG-triggered, second messenger pathways in the Burkitt's lymphoma cell line Akata. J. Immunol. **144:**4788–4793.
3. **Faggioni, A., C. Zompetta, S. Grimaldi, G. Barile, L. Frati, and J. Lazdins.** 1986. Calcium modulation activates Epstein-Barr virus genome in latently infected cells. Science **232:**1554–1556.
4. **Faller, D. V., S. J. Mentzer, and S. P. Perrine.** 2001. Induction of the Epstein-Barr virus *thymidine kinase* gene with concomitant nucleoside antivirals as a therapeutic strategy for Epstein-Barr virus-associated malignancies. Curr. Opin. Oncol. **13:**360–367.
5. **Feng, W., B. Israel, N. Raab-Traub, P. Busson, and S. C. Kenney.** 2002. Chemotherapy induces lytic EBV replication and confers ganciclovor susceptibility to EBV-positive epithelial cell tumors. Cancer Res. **62:**1920–1926.
6. **Feng, W., G. Hong, H. J. Delecluse, and S. C. Kenney.** 2004. Lytic induction therapy for Epstein-Barr virus-positive B-cell lymphomas. J. Virol. **78:**1893–1902.
7. **Ghetie, M. A., H. Bright, and E. S. Vitetta.** 2001. Homodimers but not monomers of Rituxan (chimeric anti-CD20) induce apoptosis in human B-lymphoma cells and synergize with a chemotherapeutic agent and an immunotoxin. Blood **97:**1392–1398.
8. **Hofmeister, J. K., D. Cooney, and K. M. Coggeshall.** 2000. Clustered CD20 induced apoptosis: Src-family kinase, the proximal regulator of tyrosine phosphorylation, calcium influx, and caspase 3-dependent apoptosis. Blood Cells Mol. Dis. **26:**133–143.
9. **Imai, S., M. Sugiura, O. Oikawa, S. Koizumi, M. Hirao, H. Kimura, H. Hayashibara, N. Terai, H. Tsutsumi, T. Oda, S. Chiba, and T. Osato.** 1996. Epstein-Barr virus (EBV)-carrying and -expressing T-cell lines established from severe chronic active EBV infection. Blood **87:**1446–1457.
10. **Israel, B. F., and S. C. Kenney.** 2003. Virally targeted therapies for EBV-associated malignancies. Oncogene **22:**5122–5130.
11. **Iwakiri, D., and K. Takada.** 2004. Phosphatidylinositol 3-kinase is a deter-

minant of responsiveness to B cell antigen receptor-mediated Epstein-Barr virus activation. J. Immunol. **172:**1561–1566.
12. **Kieff, E., and A. B. Rickinson.** 2002. Epstein-Barr virus and its replication, p. 2511–2573. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology, 4th ed. Lippincott-Raven, Philadelphia, Pa.
13. **Le Roux, F., A. Sergeant, and L. Corbo.** 1996. Epstein-Barr virus (EBV) EB1/Zta protein provided in *trans* and competent for the activation of productive cycle genes does not activate the BZLF1 gene in the EBV genome. J. Gen. Virol. **77:**501–509.
14. **Mentzer, S. J., S. P. Perrine, and D. V. Faller.** 2001. Epstein-Barr virus post-transplant lymphoproliferative disease and virus-specific therapy: pharmacological re-activation of viral target genes with arginine butyrate. Transplant. Infect. Dis. **3:**177–185.
15. **Moore, S. M., J. S. Cannon, Y. C. Tanhehco, F. M. Hamzeh, and R. F. Ambinder.** 2001. Induction of Epstein-Barr virus kinases to sensitize tumor cells to nucleoside analogues. Antimicrob. Agents Chemother. **45:**2082–2091.
16. **Pearson, G. R., B. Vroman, B. Chase, T. Sculley, M. Hummel, and E. Kieff.** 1983. Identification of polypeptide components of the Epstein-Barr virus early antigen complex with monoclonal antibodies. J. Virol. **47:**193–201.
17. **Rickinson, A. B., and E. Kieff.** 2002. Epstein-Barr virus, p 2575–2627. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Virology, 4th ed. Lippincott-Raven, Philadelphia, Pa.
18. **Rose, A. L., B. E. Smith, and D. G. Maloney.** 2002. Glucocorticoids and rituximab in vitro: synergistic direct antiproliferative and apoptotic effects. Blood **100:**1765–1773.
19. **Schuster, C., S. Chasserot-Gola, and G. Beck.** 1991. Activation of Epstein-Barr virus promoters by a growth-factor and a glucocorticoid. FEBS Lett. **284:**82–86.
20. **Shan, D., J. A. Ledbetter, and O. W. Press.** 1998. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. Blood **91:** 1644–1652.
21. **Shan, D., J. A. Ledbetter, and O. W. Press.** 2000. Signaling events involved in anti-CD20-induced apoptosis of malignant human B cells. Cancer Immunol. Immunother. **48:**673–683.
22. **Shimizu, N., A. Tanabe-Tochikura, Y. Kuroiwa, and K. Takada.** 1994. Isolation of Epstein-Barr virus (EBV)-negative cell clones from the EBV-positive Burkitt's lymphoma (BL) line Akata: malignant phenotypes of BL cells are dependent on EBV. J. Virol. **68:**6069–6073.
23. **Takada, K., K. Horinouchi, Y. Ono, T. Aya, T. Osato, M. Takahashi, and S. Hayasaka.** 1991. An Epstein-Barr virus-producer line Akata: establishment of the cell line and analysis of viral DNA. Virus Genes **5:**147–156.
24. **Westphal, E. M., A. Mauser, J. Swenson, M. G. Davis, C. L. Talarico, and S. C. Kenney.** 1999. Induction of lytic Epstein-Barr virus (EBV) infection in EBV-associated malignancies using adenovirus vectors *in vitro* and *in vivo*. Cancer Res. **59:**1485–1491.
25. **Westphal, E. M., W. Blackstock, W. Feng, B. Israel, and S. C. Kenney.** 2000. Activation of lytic Epstein-Barr virus (EBV) infection by radiation and sodium butyrate *in vitro* and *in vivo*: a potential method for treating EBV-positive malignancies. Cancer Res. **60:**5781–5788.