Induction of Epstein-Barr Virus Kinases To Sensitize Tumor Cells to Nucleoside Analogues

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The presence of Epstein-Barr virus (EBV) in the tumor cells of some EBV-associated malignancies may facilitate selective killing of these tumor cells. We show that treatment of an EBV+ Burkitt’s lymphoma cell line with 5-azacytidine led to a dose-dependent induction of EBV lytic antigen expression, including expression of the viral thymidine kinase (TK) and phosphotransferase (PT). Azacytidine treatment for 24 h modestly sensitized the cell line to all nucleosides tested. To better characterize EBV TK with regard to various nucleoside analogues, we expressed EBV TK in stable cell clones. Two EBV TK-expressing clones were moderately sensitive to high doses of acyclovir and penciclovir (PCV) (62.5 to 500 μM) and to lower doses of ganciclovir (GCV) and bromovinyldeoxyuridine (BVdU) (10 to 100 μM) compared to a control clone and were shown to phosphorylate GCV. Similar experiments in a transient overexpression system showed more killing of cells transfected with the EBV TK expression vector than of cells transfected with the control mutant vector (50 μM GCV for 4 days). A putative PT was also studied in the transient transfection system and appeared similar to the TK in phosphorylating GCV and conferring sensitivity to GCV, but not in BVdU- or PCV-mediated cell killing. Induction of EBV kinases in combination with agents such as GCV merits further evaluation as an alternative strategy to gene therapy for selective killing of EBV-infected cells.

EBV encodes a TK that shows sequence and functional homology with HSV-1 TK and encodes a 243-amino-acid N terminus whose function is unknown (22, 26). The EBV protein, like its HSV-1 homologue, but unlike the homologues in HSV-2 and varicella-zoster virus, has both TK and thymidylate kinase activity (6, 19). The substrate specificity of the EBV TK with regard to GCV has been the subject of conflicting reports, although there is general agreement that GCV inhibits EBV lytic replication (19, 24). In addition to EBV TK, EBV also encodes a second kinase. The open reading frame in BGLF4 encodes a protein that is homologous to other herpesvirus P Ts (5, 47). The EBV protein autophosphorylates and phosphorylates viral protein substrates, including the EBV early antigen EA-D and a DNA polymerase accessory factor (8).

In EBV-associated malignancies, there is little expression of lytic cycle genes, including the TK gene. Studies from several laboratories, including our own, however, have shown that CpG methylation of the episome plays an important role in the regulation of EBV gene expression. Viral genomes are methylated in a variety of EBV-associated tumors, including BL, Hodgkin’s disease, nasopharyngeal carcinoma, and a subset of posttransplant lymphomas (15, 23, 35, 43, 49). In vitro, inhibitors of DNA methyltransferase lead to lytic induction in some BL cell lines (3, 35, 39). We sought to determine whether azacytidine would activate expression of viral kinases and thus sensitize EBV+ tumor cells to killing by antiviral nucleoside analogues such as GCV.

**MATERIALS AND METHODS**

**Chemicals.** 5-Azacytidine, (E)-5-bromovinyldeoxyuridine (BVdU), and acyclovir (ACV) were purchased from Sigma (St. Louis, Mo.). 3-BVdU was a gift from Erik De Clercq (Katholieke Universiteit Leuven, Leuven, Belgium). GCV and penciclovir (PCV) were purchased from Hoffmann-La Roche Inc. (Nutley, N.J.) and SmithKline Beecham Pharmaceuticals (Philadelphia, Pa.), respectively. Azidoacytosine thymidine (AZT) was purchased from Calbiochem (La Jolla, Calif.). Hyposaxitine-aminopterin-thymidine (HAT) was purchased from Life Technologies (Gaithersburg, Md.) as a 100× lyophilized supplement and diluted in water.

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 Discussion: The presence of EBV in tumor cells of some EBV-associated malignancies may facilitate selective killing of these tumor cells. We show that treatment of an EBV+ Burkitt’s lymphoma cell line with 5-azacytidine led to a dose-dependent induction of EBV lytic antigen expression, including expression of the viral thymidine kinase (TK) and phosphotransferase (PT). Azacytidine treatment for 24 h modestly sensitized the cell line to all nucleosides tested. To better characterize EBV TK with regard to various nucleoside analogues, we expressed EBV TK in stable cell clones. Two EBV TK-expressing clones were moderately sensitive to high doses of acyclovir and penciclovir (PCV) (62.5 to 500 μM) and to lower doses of ganciclovir (GCV) and bromovinyldeoxyuridine (BVdU) (10 to 100 μM) compared to a control clone and were shown to phosphorylate GCV. Similar experiments in a transient overexpression system showed more killing of cells transfected with the EBV TK expression vector than of cells transfected with the control mutant vector (50 μM GCV for 4 days). A putative PT was also studied in the transient transfection system and appeared similar to the TK in phosphorylating GCV and conferring sensitivity to GCV, but not in BVdU- or PCV-mediated cell killing. Induction of EBV kinases in combination with agents such as GCV merits further evaluation as an alternative strategy to gene therapy for selective killing of EBV-infected cells.
to a 10-fold working concentration. HAT diluted in water was added diluted 1:10 to media.

Plasmids. The plasmid pEBVTK was generated by subcloning the BamHI fragment of pUCX (from J. R. Arrand, Christie Hospital and Holt Radium Institute, Manchester, United Kingdom) into the BamHI site of the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, Calif.). In the recombinant plasmid, the EBV TK gene is expressed from a cytomegalovirus promoter. The pcDNA3 parent was used as the vector control.

The plasmid pEBVPT was generated by PCR amplification of the EBV PT gene using Pfu polymerase (Stratagene, La Jolla, Calif.), followed by cloning into pcDNA3 at the BamHI site. The EBV TK was encoded by the open reading frame within the BGLF4 fragment of EBV. Genomic DNA isolated from EBV-positive cells was used as template in the PCR amplification. The primers used for amplification of the 1.3-kb BGLF4 insert were 5′-AGTCAAGATTCATGGA TGTGAATATGCTGCCGA-3′ and 3′-AATCAGATCTTCTCGAGCT CATCACCAGTC-3′.

The plasmid pEBVmutTK was generated by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions, with minor changes. The plasmid was amplified using 25 µg of pEBVTK as template and complementary oligonucleotides, in which the sequences, with the exception of a single point mutation, are identical to that of the wild-type EBV TK, as primers. The sequences of the primers used to generate pEBVmutTK were 5′-CGATTTGCTGTCGACCTCCGTGGTTTT TGG and 5′-GGAAACACCCGAGGTGCACAACTTAAAT-3′. Input plasmid DNA was digested with DpnI at 37°C for 1 h. The remaining plasmid DNA containing a point mutation in the EBV TK gene was then transformed into E. coli XL1-Blue competent cells (Stratagene).

The plasmid pHSVTK was generated by PCR amplification of the HSV-1 TK gene from plasmid pHSV-106 (from G. Hayward, Johns Hopkins University) containing the HSV-1 gene (6) inserted at the BamHI site of pBR322 (Life Technologies). Amplification was performed using Pfu polymerase and the following primers: 5′-TATGATCCATGTGGATTCGACT and 3′-5′-AGTCAAGATTCATGGA TGTGAATATGCTGCCGA-3′. The amplified HSV-1 TK gene was next cloned into the BamHI site of pcDNA3. Cloned sequences were confirmed by complete sequencing of both strands.

Cell culture and transfection. Rael and CA46 are EBV+ and EBV− BL cell lines, respectively (24). These were maintained in 1640 RPMI (Gibco BRL) with 10% fetal bovine serum, 100 µg of penicillin/ml, 100 µg of streptomycin/ml, and 100 µM t-glutamine. The cell line 143B is derived from a human osseosarcoma and was passed in 15 µg of bromodeoxyuridine (Sigma) per ml to maintain the cellular TK phenotype (24). 293T cells, a cellular TK− cell line, are derived from human kidney epithelial cells transformed with adenovirus E1A and EIB as well as the simian virus 40 T antigen (40). These cells were obtained from W. Burns (Medical College of Wisconsin). 143B and 293T cells were maintained in Dulbecco’s modified essential medium supplemented with 10% fetal calf serum, 100 mM nonessential amino acids, 100 U of penicillin/ml, and 250 µg of streptomycin/ml.

To create a stable cell line expressing the EBV TK, 143B cells were transfected with pEBVTK. Cells were seeded at 2 × 10⁶ cells/ml into six-well plates and allowed to adhere overnight. After transfection, cells were transfected using 25 ng of pEBVTK as template and complementary oligonucleotides, 105/ml of Lipofectin reagent (Life Technologies). For stable cell line formation after incubation with GCV for 4 days, while cells were extracted for phosphorylation analysis 36 h after incubation with cold GCV and 3H[GCV].

Analysis of EBV TK and PT expression. The expression of EBV TK in TK143B cells was monitored by [3H]thymidine incorporation and by immunoblotting. For [3H]thymidine incorporation, cells were first seeded at 2 × 10⁶/well into 96-well plates, pulsed for 48 h with 1 µCi of [3H]thymidine/well, and then harvested onto glass fiber filters. EBV TK mRNA was measured by scintillation counting. For immunoblotting, proteins were separated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH). These were blocked overnight in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na2PO4, and 1.8 mM KPO4) with 5% dry milk–0.1% Tween 20 and then incubated for 1 h with a polyclonal rabbit antisera (diluted 1:1,000 in PBS with 5% dry milk–0.1% Tween 20) from a rabbit immunized with a synthetic EBV TK peptide (GRHESGLAGLYKSVNDAC). After washing, the filters were incubated with goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad, Hercules, Calif.) (diluted 1:3,000 in PBS-0.1% Tween 20). The filters were washed and the proteins were detected by ECL chemiluminescence (Amersham, Arlington Heights, Ill.).

The EBV PT was detected by Northern blot analysis. Rael and CA46 cells were seeded at 5 × 10⁴ cells/ml to adhere overnight. After transfection, treated and untreated cells were harvested by trypsinization with trizol (Life Technologies). Five micrograms of RNA was electrophoresed through a 1% agarose–2.5% formaldehyde gel. Following electrophoresis, the RNA was transferred to a Hybond-N+ nylon filter (Amersham Pharmacia Biotech Limited) in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate). A probe specific for the EBV TK was generated from the BamHI fragment of pEBVTK using α-32PdCTP and the Ready-to-Go DNA labeling kit (Pharmacia Biotech, Buckinghamshire, United Kingdom). Prehybridization and hybridization were carried out at 42°C in a mixture of 50% formamide, 1% bovine serum albumin, 5% SDS, 0.5 M sodium phosphate, and 100 µg of salmon sperm DNA/ml. After hybridization, the membrane was washed once in 3× SSPE (1× SSPE is 0.18 M NaCl, 1 mM EDTA, 10 mM NaH2PO4)–0.1% SDS at 65°C, once in 0.3× SSPE–0.1% SDS at 60°C, and once in 0.1× SSPE–0.1% SDS at 42°C. Detection of the EBV PT was performed by exposing the membrane to Kodak XAR-5 film at −70°C with screens.

In vitro viability assays. Stable cell clones were seeded into 96-well plates, cultured overnight, and incubated with the indicated nucleoside analogue for 7 days. Transiently transfected 293T cells were seeded 24 h after transfection and incubated with GCV for 4 days. Rael (EBV−) and CA46 (EBV−) cells were seeded into 96-well microtiter plates and treated with 0.5 µM 5-azacytidine for 24 h. After 24 h, the plates were centrifuged to pellet the cells, the medium was removed, and the cells were resuspended in medium containing 5% FBS. For 143B, 293T, and Rael cells incubated with the MTJ solution for 4 h at 37°C. The formazan crystals were solubilized in 0.1 N acid isopropanol. Plates were assayed at 580 nm using a microplate reader (Cambridge Technologies, Inc., Watertown, Mass.). Absorbance readings were standardized on untreated cells. Each experiment was repeated three times, with each MTT absorbance value representing replicates of six wells. Each bar in the figures represents the average of three experiments. Error bars represent the standard errors of the means.

Immunohistochemistry. Lytic antigen expression was assessed in Rael cells by immunohistochemical analysis of cytospin preparations from treated or untreated cells. Cells (200 µl) diluted to 3 × 10⁶ to 5 × 10⁶/ml in PBS were cytocentrifuged to positively charged microscope slides at −20°C in acetone-methanol (1:1) for 10 min, and stored dry at −20°C. Before use, slides were hydrated in distilled H2O, and endogenous peroxidase activity was blocked. Slides were then incubated with monoclonal antibodies specific for either EBV Zta (1:200; Dako, Carpinteria, Calif.) or EBV VCA (1:500; Chemicon International, Inc., Temecula, Calif.). In transient transfection analysis, EBV TK expression was detected using a polyclonal antisera from a rabbit immunized with a synthetic EBV TK peptide (described above). Detection was performed by standard indirect detection methods. The peroxidase-based DAKO Duet system (Dako) and 0.5 µg of diaminobenzidine (Sigma/ml) were used for detection of Zta and VCA. The alkaline phosphatase-based Vectastain ABC-AP kit (Vector Laboratories, Burlingame, Calif.) was used for detection of the EBV TK.
Extraction of phosphorylated GCV and HPLC analysis. TK143b.1, TK143b.2, and V143b.1 cells (2 x 10⁶ to 3 x 10⁷) were seeded into wells of six-well plates and incubated with [3H]GCV (14.6 Ci/mmol; Moravek Biochemicals) for 6 h at 37°C. Transfected 293T cells (1.5 x 10⁶ to 2 x 10⁷) were seeded into wells of six-well plates incubated with [3H]GCV (14.6 Ci/mmol; Moravek Biochemicals) and 9 µM GCV for 36 h at 37°C. Following incubation, the cells were trypsinized, washed three times in PBS, and counted using a hemacytometer. [3H]GCV was extracted by lysing the cells in 60% methanol at −80°C for 18 h. Cell lysates were centrifuged at 13,000 g for 10 min at 4°C to remove cell debris and dried in a speed vacuum. Dried extracts were stored at −80°C until analysis.

Phosphorylated forms of GCV were separated using HPLC with a strong-anion-exchange column (Whatman Partisil 10-SAX) according to a previously described procedure (14, 45), with minor modifications. Cell extracts were reconstituted in 200 µl of HPLC-grade water and centrifuged at 13,000 g for 5 min at 4°C, and the supernatant was injected. Nucleotides were eluted with a gradient of KH₂PO₄ buffer (pH 3.5) at a flow rate of 0.5 ml/min (0.02 M KH₂PO₄ [pH 3.5]) for 10 min, followed by a linear gradient to 1 M KH₂PO₄ [pH 3.5] over 45 min and a final 15 min at 1 M KH₂PO₄ [pH 3.5]). Fractions were collected every 1 min using an ISCO fraction collector (Lincoln, Nebr.). Radioactivity was counted using Hydrofluor scintillation fluid (National Diagnostics, Atlanta, Ga.) and a Beckman 5000TD scintillation counter (Fullerton, Calif.). Picomoles of phosphorylated and nonphosphorylated drug were determined by measuring the disintegrations per minute per picomole of GCV of the extract and of the fractions after separation. Relative peak retention times for GCV metabolites were as follows: GCV monophosphate (GCV-MP), 27 to 29; GCV diphosphate (GCV-DP), 41 to 45; and GCV triphosphate (GCV-TP), 61 to 65. This procedure had a detection limit of >0.02 pmol of [3H]GCV.

RESULTS

Induction of EBV lytic antigen expression and activity of the viral TK. Induction of lytic viral antigen expression was evaluated after exposure of the EBV⁺ BL cell line Rael to various concentrations of 5-azacytidine for 6 h. Expression of EBV Zta, VCA, and TK was dose dependent. Treatment with 15 µM 5-azacytidine induced expression of the immediate early antigen Zta in 80% of the cells and expression of the late lytic antigen VCA in 20% of the cells at 48 h (Fig. 1A). Immunoreactivity with antiserum to an EBV TK peptide was detected in induced cells as a 70-kDa protein (Fig. 1B), consistent with previous reports of EBV TK detection by immunoblotting (19, 22, 24). The antiserum worked well for the immunoblot format but not for immunohistochemical assays, and therefore it was not possible to directly assess the percentage of cells expressing TK in induction experiments.

To determine whether the expression of EBV TK might sensitize cells to nucleoside analogues, we transfected the TK⁺ osteosarcoma cell line with an EBV TK expression vector and a control vector as described in Materials and Methods. An immunoblot showed reactivity in the TK-transfected clones (TK143b.1 and TK143b.2) but not in the control clone (V143b.1) (Fig. 2A). EBV TK is functional in these cell clones, as evidenced by the ability to grow in HAT and by [3H]thymidine incorporation (Fig. 2B). We measured the sensitivity of EBV TK-expressing cells and control cells to several nucleoside analogues. TK143b clone cells were moderately more sensitive to high doses of ACV and PCV (62.5 to 500 µM) and to lower doses of GCV and BVdU (10 to 100 µM) than was the control (V143b.1 [TK⁻] clone) (Fig. 3). Some sensitivity to AZT was found, but there was no dose-response relationship. This result is consistent with a previous report that EBV TK sensitizes NIH 3T3 cells to BVdU, but poorly sensitizes them to ACV and GCV (28).

We carried out further experiments to exclude the possibility that our results were an artifact of clonal selection. The sensitivity to GCV was also assessed by the transient transfection of cellular TK⁺ 293T cells with pEBVTK. Historically, high transfection efficiencies can be achieved using this system, and no selection is employed. The sensitivity of cellular TK⁺ 293T cells transfected with pEBVTK, but not empty control vector DNA, to GCV (Fig. 4) supports the hypothesis that the difference in sensitivity to nucleoside analogues between EBV TK-expressing cells and control cells is due specifically to the presence of an overexpressed protein. Previous investigators have reported various conflicting results in this regard (6, 19, 24, 28, 53). We sought to determine whether the sensitivity of EBV TK-expressing cells to GCV reflected the specific enzymatic activity of EBV TK or reflected a nonspecific cellular toxicity, possibly associated with foreign protein overexpression, or cellular activation of GCV. To this end, we introduced...
EBVTK was associated with negligible levels (data not shown). Thus, the results of these experiments parallel the killing experiments.

A second EBV GCV kinase. Herpesviruses such as cytomegalovirus, while lacking a TK, are nonetheless able to phosphorylate GCV (21, 48). The cytomegalovirus GCV kinase (UL97) has been identified as a PT with homologues throughout the herpesvirus family (1, 47). Recently we have shown that human herpesvirus 8 open reading frames with homology to herpesvirus TKs and PTs both phosphorylate GCV (5). In order to determine whether the EBV PT homologue, BGLF4, also sensitizes cells to GCV, we transiently transfected 293T cells with an EBV PT expression vector as described in Materials and Methods. EBV PT RNA can be induced by 5-azacytidine in Rael cells (Fig. 6A). Though expression from pEBVPTK was not assessed, as shown in Fig. 6 transient transfection of the plasmid pEBVPT into 293T cells was associated with phosphorylation of GCV and sensitization to GCV-mediated cell killing (25 μM GCV for 4 days, compared with an empty plasmid control). A comparison with UL97 was not performed. In contrast to pEBVTK, the putative EBV PT did not sensitize cells to BVdU or, interestingly, to PCV (data not shown).

To determine whether induction of the kinases led to increased sensitivity to antiviral nucleoside analogues in EBV+ BL cell lines, we compared the effects of 5-azacytidine on an EBV+ and an EBV− Burkitt’s cell line in the presence and absence of various antiviral nucleoside analogues. EBV+ Rael cells treated with 0.5 μM 5-azacytidine are induced to express EBV TK, as demonstrated by an immunoblot (Fig. 7A). EBV− Rael cells were more sensitive than EBV− CA46 cells to the cytotoxic effects of 5-azacytidine, perhaps as a result of the induction of lytic viral antigen expression, reducing cell numbers by half in the absence of nucleosides and hampering evaluation of nucleoside effects. The data suggested that 5-azacytidine sensitized an EBV+ to BVdU and S-BvdU, and AZT (Fig. 7B). The effects were not as pronounced as in the transfected cells (Fig. 4), and with the exception of AZT, high concentrations of drug were necessary to see an effect of 50% or greater. No nucleoside that did not sensitized cells was identified.

**DISCUSSION**

The investigations presented here suggest that pharmacologic activation of viral kinase gene expression may render cells carrying EBV genomes selectively sensitive to high doses of several nucleoside antiviral agents. EBV TK appeared to sensitize cells to killing by GCV, PCV, BVdU, and AZT. A putative PT sensitized cells to GCV but not to BVdU or PCV.

The ability of EBV TK to sensitize cells to killing by GCV has been the subject of recent conflicting reports (6, 19, 24, 28, 53). Four previous studies examined the activity (phosphorylation) and substrate specificity (competition) of purified or partially purified EBV TK expressed as fusion proteins in bacteria, and all documented TK function. Three studies further examined GCV; two of the studies showed no significant thymidine competition by nor phosphorylation of GCV, whereas one study showed GCV to compete 10-fold more efficiently for EBV TK than for HSV-1 TK, a result that has not been generally confirmed. Because it was possible that purified EBV
FIG. 3. Treatment of EBV TK-expressing cells and control cells with nucleoside analogues in vitro. Cells were seeded into 96-well microtiter plates at 10^3 cells/well. After allowing cells to adhere overnight, cells were incubated with GCV (A), PCV (B), ACV (C), BVdU (D), S-BVdU (E), or AZT (F) for 7 days. A colorimetric assay measuring the conversion of MTT to formazan was used to determine the fraction of cells surviving relative to untreated controls (100% viable). Each experiment was repeated three times, with each MTT absorbance value representing replicates of six wells. Each bar represents the average of three experiments, with error bars representing the standard errors of the means.
TK lost its ability to selectively phosphorylate GCV, though not thymidine or thymidine analogues, when compared with EBV TK protein expressed in cells, we examined GCV phosphorylation as well as sensitization to various nucleoside analogues. Our results parallel those of Loubiere et al., who calculated a selectivity index as the ratio of the drug concentrations required to reduce thymidine incorporation by 50% in untransfected parental and EBV TK-transfected NIH 3T3 cells (28). The selectivity index was 4 for ACV, 12 for GCV, and 1,375 for BVdU. Although the thymidine analogue was 1,000 times more selective, sensitization to purine analogues was suggested. The goal of this study was to compare two efficient TKs for gene therapy, with EBV TK serving as a negative control. Since comparisons were made to a parental line that was neither transfected nor selected with toxic drug and in the absence of an analysis of protein expression or GCV phosphorylation, these results are less conclusive. Gustafson et al. showed that lysates from an EBV TK^+ clone of 143b TK^2 cells phosphorylated GCV twice as well as a TK^+ control clone but ~1,000 times less well than an HSV-1 TK-expressing clone analyzed under the same conditions. However, they did not see sensitization to GCV or ACV, although they used a system closely parallel to that used in our studies (19, 20). Some of the discrepancies might be explained by a loss of viral TK expression, with partial reversion of cells to a TK^- phenotype, if periodic assessment were not performed. Such reversion has sometimes been noted in HAT-selected lines (2, 17, 32). To help prevent this problem, our EBV TK-expressing cell clones were selected in HAT for only 1 week prior to experimentation. Periodic immunoblotting of total TK143b cell protein with a polyclonal rabbit antiserum from a rabbit immunized with an EBV TK peptide confirmed that the EBV TK was being expressed in TK143b cell clones over time (data not shown). Our sensitization results using two stable (cellular TK^2) clones were parallel to those of a transient (cellular TK^2) transfection system that overexpressed EBV TK. However, we were unable to determine transfection efficiencies, and sensitization was modest (50% at 50 μM GCV for 4 days when EBV TK and EBVmutTK were compared). Our HPLC analysis of a cell clone that constitutively expressed the EBV TK and of a transiently transfected cell line overexpressing EBV TK compared with an empty vector control suggested that the EBV TK does phosphorylate GCV. Our experiments further raised the possibility that, as suggested by Gustafson et al. (19), another EBV kinase, the EBV PT, might phosphorylate and sensitize cells to GCV. Though overall activity was low, together these experiments suggest that the presence of viral enzymes could contribute to phosphorylation of and sensitization of cells to GCV.

![Image](http://aac.asm.org/)
required? The susceptibility of EBV-associated disease in vivo to the combination of an EBV-inducing agent and GCV has been reported (36; H. Oettle, F. Wilborn, C. A. Schmidt, and W. Siegert, Letter, Blood 82: 2257–2258, 1993), supporting the hypothesis that EBV-associated malignancies can be targeted with an inducing agent in combination with a prodrug. Although the levels of GCV required for killing in combination with 5-azacytidine in vitro are higher than those achieved with standard doses of these drugs in vivo, those standard doses are based on regimens that involve chronic administration. 5-Azacytidine-induced EBV$^{+}$ cells were sensitive to GCV when treated with concentrations of GCV at 25 to 50 $\mu$M for 4 days. After 24 h, the plates were centrifuged to pellet the cells, the medium was removed, and cells were resuspended in medium containing GCV (a), PCV (b), BVdU (c), S-BVdU (d), or AZT (e). Plates were incubated for an additional 7 days. Viability was determined by the MTT assay. The values of cells treated with drug are presented as the fractions of cells surviving relative to untreated controls (100% viable). Experiments for panels a to d were performed at the same time, while the experiment for panel e was performed at a later date. Each experiment was repeated three times, with each MTT absorbance value representing replicates of six wells. Each bar represents the average of three experiments, with error bars representing the standard errors of the means.

A variety of lytic inducers have been studied in the laboratory. These include phorbol esters, anti-immunoglobulin antibodies, butyrate, and DNA methyltransferase inhibitors (3, 30, 50, 56). For the studies described here, we chose to study a DNA methyltransferase inhibitor because there is a large amount of clinical experience with this agent in other settings (7, 9, 29). 5-Azacytidine has been used clinically in the treatment of leukemia, myelodysplasia, and hemoglobinopathies for more than two decades at doses that achieve concentration ranges similar to the concentrations studied. In a recent clinical trial, 5-azacytidine administered at a dose of 75 mg/m² per day for 5 to 7 days was associated with demethylation of EBV genomes in an EBV$^{+}$ AIDS lymphoma and in nasopharyngeal carcinoma as assessed by biopsy within 72 h after treatment (Ambinder, unpublished data). This suggests that administra-
FIG. 7—Continued.
tion of agents that impact on patterns of gene expression in vitro may also impact on viral gene regulation in vivo.

To implement this therapy in vivo, a large percentage of EBV+ tumor cells need to be induced. As shown in Fig. 1, approximately 60% of Rael cells were induced by 5-azacytidine in vitro may also impact on viral gene regulation in vivo.

Similarly, the ability of 5-azacytidine to induce lytic EBV infection is not limited to one particular cell line. The Burkitt’s-derived Akata cell line and the nasopharyngeal carcinoma C666 cell line also show lytic activation following treatment with this agent (unpublished data). Greater levels of induction are likely to be required in order to target tumors. Other inducing agents or a combination of inducing agents, such as the combination of a methyltransferase inhibitor and a histone deacetylase inhibitor, may be able to induce levels of EBV TK closer to 100%.

Further investigation to optimize induction strategies for specific EBV-associated tumors is underway.

The principle that EBV-infected cells can be selectively killed by the combination of an inducer of lytic cycle viral gene expression with an antiviral nucleoside analogue merits further evaluation. In contrast to gene therapy approaches for treating EBV+ tumors, the approach we suggest eliminates the requirement for the introduction of foreign genes. The demonstration that the EBV TK and PT can be pharmacologically induced in EBV+ tumor cells and that these induced cells are sensitive to antiviral nucleoside analogues suggests a pharmacologic approach which specifically targets EBV-associated tumor cells.

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