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

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ABSTRACT

The consistent presence of the EBV genome in certain tumors offers the potential for novel EBV-directed therapies. Switching the latent form of EBV infection present in most EBV-positive tumor cells into the cytoplasmic form may be clinically useful because lytic EBV infection leads to host cell destruction, and very few normal cells contain the EBV genome. It would also be therapeutically advantageous to induce expression of EBV-encoded lytic proteins that convert the nucleoside analogues ganciclovir (GCV) and 3'-azido-3'-deoxythymidine (AZT) into their active, cytotoxic forms. In this report, we have explored two different approaches for activating the lytic form of EBV infection in tumors. We show that γ-irradiation at clinically relevant doses induces lytic EBV infection in lymphoblastoid cell lines in vitro as well as in EBV-positive B-cell tumors in SCID mice. In addition, sodium butyrate (given as a single i.p. dose) is effective for activating lytic viral infection in some EBV tumor types in SCID mice. We also examined whether low-dose γ-irradiation treatment of EBV-positive lymphoblastoid cells in vitro promotes GCV or AZT susceptibility. The combination of radiation with either GCV or AZT induced significantly more cell killing in vitro than either radiation or produg treatment alone. Most importantly, we found that the combination of γ-irradiation and GCV was much more effective in treating EBV-positive lymphoblastoid tumors in SCID mice than either agent alone. Thus, GCV or AZT treatment could potentially enhance the therapeutic efficacy of radiation therapy for EBV-positive lymphomas in patients.

INTRODUCTION

EBV is a human herpes virus that infects 90% of individuals (1, 2). Primary EBV infection targets B cells and epithelial cells and causes the infectious mononucleosis syndrome (1, 2). Although the virus persists lifelong in a subset of B cells, it is normally well controlled by the immune system of the host. Nevertheless, EBV has recently been found in an increasing number of tumor types. EBV-associated malignancies that occur in the immunocompetent host include BL (1), nasopharyngeal carcinoma (1), T-cell lymphoma (3), Hodgkin’s disease (1), and gastric carcinoma (4). EBV is also commonly present in B-cell lymphoproliferative tumors (5, 6) and leiomyosarcomas (7, 8) associated with immune deficiencies. Regardless of whether EBV is the actual cause of such malignancies or merely a passenger virus, the almost universal presence of the viral genome in certain tumors (particularly in undifferentiated nasopharyngeal carcinoma (1) and AIDS-associated CNS lymphomas (6)) could serve as a potential target for novel therapeutic strategies.

As is the case for all herpes viruses, EBV can induce both lytic and latent forms of infection (1, 2). The lytic form of infection occurs primarily in epithelial cells and is required for transmission of the virus from cell to cell as well as from host to host. In the lytic form of EBV infection, which commonly results in killing of the host cell, the entire viral genome is expressed, and replication occurs using the virally encoded DNA polymerase and the oriLyt replication origin (1, 2). In the latent forms of infection, which occur primarily in B cells, only a small portion of the viral genome is expressed, and replication of the virus is mediated by an alternate origin of replication (oriP), the host cell DNA polymerase, and the viral EBNA-1 protein (1, 2). Given that the known transforming functions of EBV are encoded by proteins expressed during the latent form of infection (1, 2) and that lytic EBV infection generally results in host cell killing, it is not surprising that EBV-positive tumor cells contain almost exclusively the latent forms of EBV infection (1, 2).

Thus, we (9, 10) and others (11, 12) have suggested that EBV-positive tumor cells could be targeted for destruction if the latent form of EBV infection normally present in tumor cells could be switched into the lytic form of infection. In vitro, high-level expression of either of the two EBV immediate-early proteins, BZLF1 and BRLF1, is sufficient to convert latent EBV infection into the lytic form (13–20). Furthermore, we recently demonstrated using gene delivery strategies that both the BZLF1 and BRLF1 proteins can induce lytic infection in EBV-positive tumors in vivo (9). However, the clinical efficacy of gene delivery strategies for inducing lytic EBV infection in tumors would likely be limited due to inefficient gene delivery. In addition, activation of the lytic form of EBV infection in tumors would result in release of infectious EBV, although it is uncertain that such a release would result in clinical consequences.

The combination of lytic EBV infection and certain antiviral agents could potentially result in more efficient tumor cell killing. Phosphorylated GCV inhibits the cellular DNA polymerase as well as the viral polymerase, and thus delivery of the herpes simplex virus TK gene, in conjunction with GCV, has been widely used as a method for inducing tumor cell killing (21–23). The lytic (but not latent) form of EBV replication can be inhibited by the antiviral drug GCV (24), suggesting that during lytic EBV replication, one or more viral proteins are expressed that phosphorylate GCV into its active form (25). Indeed, we recently demonstrated that lytic EBV infection (induced by gene delivery methods) results in efficient phosphorylation of GCV and that GCV induces killing of lytically infected host cells, although it suppresses viral replication (9). Although the specific EBV proteins that phosphorylate GCV remain uncertain, it is likely that either the EBV TK protein (26, 27) or the EBV homologue (BGLF4) of cytomegalovirus protein UL97 (28) phosphorylates GCV during lytic EBV infection.

Interestingly, it was recently shown that EBV-encoded TK phosphorylates the nucleoside analogue AZT (27) and that expression of EBV TK in melanoma cells results in greatly enhanced sensitivity to AZT-induced cell killing (27). In fact, the EBV TK/AZT combination was substantially more potent than the EBV TK/GCV combination for...
killing melanoma cells (27). AZT also inhibits the lytic form of EBV replication (29). Thus, AZT, like GCV, could potentially promote effective killing of lytically infected EBV-positive tumor cells while simultaneously preventing the release of infectious virus.

Given the relative inefficiency of current gene delivery methods, we have investigated the possibility of activating the lytic form of EBV infection in vivo using novel methods. Lytic EBV infection in vivo could presumably be induced by activation of either BZLF1 or BLRF1 transcription (which are normally not transcribed during the latent form of viral infection). In vitro, a variety of different treatments, including phorbol esters, sodium butyrate, and engagement of the B-cell receptor with anti-immunoglobulin, can induce the lytic form of EBV infection (1, 2). Here we demonstrate that treatment of EBV-positive tumors in SCID mice with a single dose (100 or 200 mg/kg) of γ-irradiation induces the lytic form of infection in several different tumor types. Similar results were obtained in vitro. Lytic replication was also induced in some EBV-positive tumors in vivo by a single i.p. injection of sodium butyrate. We show that both AZT and GCV substantially enhance γ-irradiation killing of EBV-transformed lymphoblastoid cells in vitro. Finally, we demonstrate that the combination of γ-irradiation and GCV together was much more effective than either agent alone for treating EBV-positive lymphoblastoid tumors in SCID mice. Our data suggest that enhanced therapeutic results could potentially be achieved clinically by combining radiation therapy with either AZT or GCV.

MATERIALS AND METHODS

Cell Lines. Cell lines used in this study included EBV-positive BL cell lines (Jijoye and Akata), two LCLs (LCL-1 and LCL-2) established from normal lymphocytes of two different individuals by infection with EBV strain B95-8, and the gastric carcinoma cell line AGS (available from American Type Culture Collection) and its EBV-positive derivative, AGS/EBV. The latter was obtained by G418 selection of AGS cells that had been infected with a recombinant Akata virus in which a neomycin resistance cassette had been inserted into the nonessential BDLF3 open reading frame (30). The BL and LCL lines were maintained in RPMI 1640 with 10% FBS; the gastric carcinoma line AGS was maintained in Ham’s F-12 medium with 10% FBS, and AGS/EBV was maintained in Ham’s F-12 medium with 10% FBS and 400 μg/ml G418.

Tumors. To establish EBV-positive tumors in vivo, 5 × 10⁷ Jijoye, EBV-positive lymphoblastoid cells, or EBV-positive AGS cells were injected s.c. into both flanks of 4–5-week-old SCID mice. Subsequent experiments (radiation treatment or sodium butyrate treatment) were performed 7–10 days later, when tumors had become palpable. In the treatment experiment, 5 × 10⁷ EBV-positive lymphoblastoid cells (LCL-1 cells) were injected into both flanks of SCID mice in 200 μl of sterile PBS. When the majority of inoculation sites had developed barely palpable tumors (9 days after inoculation), they were treated with either one dose of γ-irradiation (100 cGy) alone, i.p. GCV (100 mg/kg twice a day for 5 days) alone, or a combination of GCV and one dose of γ-irradiation or left untreated. Three perpendicular tumor diameters were measured every few days, and tumor size was estimated by multiplying the three diameters. Mice were euthanized by CO2 inhalation when tumors were measured every few days, and tumor size was estimated by multiplying the three diameters. Mice were euthanized by CO2 inhalation when tumors became extremely large (greater than 1 cm³) or the mice appeared ill. The experiment was terminated on day 44 due to the large size of tumors in some treatment groups. After mice were euthanized, both flanks were surgically explored, and any tumors found were removed and weighed.

Radiation. Tumor-bearing mice were anesthetized with ketamine (100 mg/kg injected i.p.) before all irradiations. Mice were immobilized in a specially designed lead jig (31), and the tumors were centered in a 2-cm diameter circular field. The tumors were irradiated once with doses ranging from 50–400 cGy in a 46 Cobalt unit at a rate of 150 cGy/min, harvested after different time points, and kept frozen at −80°C. For in vitro radiation experiments, cells in exponential growth phase were irradiated with a single dose ranging from 50–1000 cGy.

RESULTS

AZT- and GCV-induced Killing of EBV-positive B Cells Correlates with the Degree of Lytic Infection. Because GCV is not substantially phosphorylated during the latent form of EBV infection, we anticipated that GCV-induced cell killing would be greatest in cell lines containing the highest level of lytic EBV replication. Although there is little information regarding the effect of AZT on EBV-transformed B-cell lines in vitro, a recent report suggested that EBV-positive B-cell lines are much more susceptible to AZT-induced killing than EBV-negative B cells (32). However, data from this report were limited to a single EBV-positive cell line, P3HR1, which contains a high proportion of lytically infected cells, in distinct contrast to most EBV-positive B-cell lines in vitro and EBV-positive lymphomas in vivo. We therefore compared the effect of AZT and GCV on P3HR1 cells in vitro versus a LCL transformed by the B95-8 strain of EBV. As shown in Fig. 1A, immunoblot analysis confirmed that the P3HR1 cell line, as expected, expresses a much higher level of the early lytic viral protein, BMRF1, than the LCL. In addition, as expected, the lytically infected P3HR1 cell line was much more susceptible to GCV killing (only 40% of cells were alive after 5 days of GCV treatment in comparison with the untreated cells) than was the LCL (72% of cells were alive after 5 days of GCV treatment). Of note, 5 days of AZT treatment induced a similar level of killing as GCV in both cell lines, and AZT killing was also substantially higher in the more lytically infected cell line.

These data suggest that the efficiency of both GCV and AZT cell killing in EBV-positive B cells reflects the number of lytically infected cells and are consistent with a recent report showing that expression of the lytic EBV TK gene confers AZT susceptibility to melanoma cells (27). Furthermore, our results suggest that both AZT and GCV increase the level of cell killing in the P3HR1 cell line relative to the cell killing resulting from lytic EBV infection alone. This suggests that either both drugs have a bystander killing effect in P3HR1 cells i.e., although the activated drugs are only present in the
lytically infected cells, latently infected cells are also killed) or some lytically infected P3HR1 cells normally escape cell killing in the absence of drug. Although little is known regarding the mechanism of cell killing resulting from the AZT/EBV TK combination, GCV has a well-described bystander killing effect in some cell lines (32–35). We did not previously observe a GCV bystander killing effect in lymphoblastoid cells in vitro using the herpes simplex virus 1 TK gene, although some GCV bystander killing was observed in vivo (36).

γ-Irradiation Induces Lytic EBV Infection in Vitro. A recent report involving several patients suggested that AZT and GCV (in combination with interleukin 2) may be useful for treating EBV-positive CNS lymphomas in AIDS patients (32). However, as discussed above, our in vitro data suggest that effective killing of EBV-positive tumor cells by either GCV or AZT would require the lytic form of EBV infection, whereas most tumor cells contain one of the latent types of infection (1, 2). We therefore investigated potential methods for inducing the lytic form of viral infection in EBV-positive tumors grown in SCID mice. Because the latent form of herpes virus infection can be reactivated into the lytic form by certain forms of radiation (37, 38), and radiation is a common treatment used to treat EBV-positive tumors, we examined the effect of γ-irradiation on EBV gene expression in various EBV-positive cell lines in vitro. Activation of lytic EBV infection was quantitated by immunoblot analysis of two lytic EBV proteins, BZLF1 (an immediate-early protein) and BMRF1 (an early protein), which are not expressed during the latent forms of viral infection. As shown in Fig. 2, a single dose of γ-irradiation efficiently induced the lytic form of EBV infection in two different recently established EBV-positive LCLs. γ-Irradiation also activated the lytic form of EBV infection in the BL cell line Akata and in an EBV-positive gastric carcinoma cell line (AGS/EBV), although the effect in these cell lines was less efficient than that in the lymphoblastoid cells. Of note, the lower doses (200 and 400 cGy) of γ-irradiation used in these experiments are similar to those used to treat patients in vivo. Thus, γ-irradiation reactivates the lytic form of EBV replication in vitro in a variety of different tumor and cell types. Nevertheless, γ-irradiation did not induce lytic EBV infection in all LCLs tested (data not shown), indicating that host cell or viral factors may modulate this effect.
To confirm that γ-irradiation induces a fully lytic form of EBV infection, we used FACS analysis to determine the number of cells expressing a late viral protein (viral capsid antigen) before and after 400 cGy of γ-irradiation (Fig. 2D). In two different LCLs (LCL-1 and LCL-2), γ-irradiation significantly increased the percentage of cells expressing viral capsid antigen. We also performed FACS analysis to determine the number of cells induced into the lytic form of replication by γ-irradiation treatment in vitro. Jijoye (a BL line) or lymphoblastoid cells (LCL-1) were treated with a single dose of γ-irradiation (ranging from 200-1000 cGy) and analyzed for expression of the early EBV lytic protein, BMRF1, 24 h later (Table 1). In the absence of radiation, approximately 3% of the cells in each cell line expressed the lytic BMRF1 protein. In contrast, approximately 12% of the cells expressed BMRF1 24 h after the higher doses of radiation.

**Comparison of Radiation versus Sodium Butyrate for Induction of Lytic EBV Infection in Vitro.** Sodium butyrate is known to be a powerful activator of lytic EBV infection in certain cell lines in vitro and has already been safely administered to patients as an experimental treatment for various illnesses (39, 40). Thus, sodium butyrate might be another potentially useful agent for enhancing lytic EBV infection of tumors in vivo. To further explore the effects of sodium butyrate versus radiation on EBV infection, we examined the ability of sodium butyrate to induce the lytic form of EBV infection in several different cell lines in vitro. As shown in Fig. 3, sodium butyrate activated the lytic form of EBV replication in a variety of different cell lines, including the BL cell lines Jijoye and Akata, a LCL (LCL-1), and an EBV-positive gastric carcinoma line. Radiation was more effective than sodium butyrate in inducing lytic EBV infection in the LCL-1 lymphoblastoid cells, but sodium butyrate was more effective in the Akata BL cell line, and both agents were similarly effective (when used alone) in the gastric carcinoma cell line. In the Akata cell line, the combination of both agents appeared to be more effective in inducing lytic EBV infection in vitro than either agent alone.

These results suggest that both sodium butyrate and radiation could be potentially useful agents for inducing lytic EBV infection in vivo and that the superiority of one agent over the other might depend on the tissue and tumor type.

**Induction of Lytic EBV Infection in Tumors Using Radiation or Sodium Butyrate.** We next examined the ability of radiation and sodium butyrate to activate the lytic form of EBV infection in several different EBV-positive tumor types in vivo. In our initial studies, tumors from a LCL (LCL-1) or the Jijoye BL cell line were derived by implanting 5 × 10^7 cells s.c. into the flanks of SCID mice. Tumors were given a single dose of γ-irradiation after they became palpable (approximately 10 days after the injection of cells) and harvested 24 h or 7 days later. As shown in Fig. 4, a single treatment of γ-irradiation (given at clinically relevant doses) efficiently induced the lytic form of EBV replication in both the LCL-derived tumors and the BL tumor. In general, higher doses of radiation resulted in more efficient activation of lytic infection. Both the EBV IE protein, BZLF1, and the early protein, BMRF1, were clearly induced by radiation treatment.

Because expression of either EBV IE protein (BZLF1 or BRLF1) is sufficient to activate the entire cascade of lytic EBV infection, γ-irradiation likely induces the lytic form of EBV infection by increasing the level of BZLF1 (or BRLF1) transcription. Of note, enhanced lytic EBV infection in Jijoye tumors was still observable 7 days after a single dose of radiation, implying that whatever the mechanism, it is long-acting.

We next examined the ability of a single dose of i.p. sodium butyrate to induce lytic EBV replication in lymphoblastoid cell and gastric carcinoma tumors in SCID mice (Fig. 5). Sodium butyrate alone was effective for inducing lytic infection in the EBV-positive gastric carcinoma cell line AGS-EBV (Fig. 5A). However, sodium butyrate alone did not reproducibly induce lytic EBV infection in vivo in Jijoye BL tumors (Fig. 5B), in contrast to its in vitro effect.

Finally, we compared the ability of sodium butyrate alone, γ-irradiation alone, or the combination of both treatments to induce the lytic form of EBV infection in lymphoblastoid tumors derived from LCL-1 cells. The combined treatment of SCID mice with sodium butyrate and γ-irradiation together was not significantly more effective than γ-irradiation alone in inducing lytic EBV in the LCL-1-derived tumors.

### Table 1 Disruption of EBV latency by radiation in EBV-positive B cells

<table>
<thead>
<tr>
<th>Radiation dose</th>
<th>LCL-1</th>
<th>Jijoye</th>
</tr>
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<tbody>
<tr>
<td>0 cGy</td>
<td>2.9%</td>
<td>2.9%</td>
</tr>
<tr>
<td>200 cGy</td>
<td>6.6%</td>
<td>9.3%</td>
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<tr>
<td>400 cGy</td>
<td>10.2%</td>
<td>8.6%</td>
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<tr>
<td>600 cGy</td>
<td>12.5%</td>
<td>11.0%</td>
</tr>
<tr>
<td>1000 cGy</td>
<td>12.9%</td>
<td>12.4%</td>
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Fig. 3. Induction of lytic EBV infection in vitro by γ-irradiation versus sodium butyrate. EBV-positive lymphoblastoid cells (LCL-1: A), BL cells (Akata (C) and Jijoye (D)), BL cells (Akata (B); BL cells (Jijoye (A)) and sodium butyrate (2.5 mM) were treated with a single dose of γ-irradiation (400 cGy) and analyzed for expression of the early EBV lytic protein, BMRF1, 24 h later (Table 1). In the absence of radiation, approximately 3% of the cells in each cell line expressed the lytic BMRF1 protein. In contrast, approximately 12% of the cells expressed BMRF1 24 h after the higher doses of radiation. The same blots were subsequently reprobed with an actin-specific antibody.
AZT and GCV Enhance γ-Irradiation Killing of EBV-transformed B Cells in Vitro. The finding that γ-irradiation induces lytic EBV infection in lymphoblastoid cells suggests that it could enhance the killing of these cells by GCV or AZT. To examine this possibility, LCL-1 lymphoblastoid cells were incubated with AZT or GCV, with or without prior irradiation at 200 cGy, and 9 days later, the number of surviving cells was determined by trypan blue staining. As shown in Fig. 6, cells treated with either 9 days of GCV alone, AZT alone, or a single dose (200 cGy) of γ-irradiation each had approximately 50% viable cells in comparison to the untreated cells. In contrast, cells receiving the combination of either GCV and irradiation or AZT and irradiation had significantly fewer viable cells (14–15% in comparison with untreated cells). Hence, the combination of γ-irradiation with AZT or GCV enhances cell killing in vitro in EBV-positive lymphoblastoid cells.

GCV Enhances the Effectiveness of γ-Irradiation Treatment of EBV-positive Lymphoblastoid Tumors in SCID Mice. To determine whether the combination of γ-irradiation and GCV enhances tumor regression of EBV-positive lymphoblastoid tumors in vivo, 16 SCID mice were inoculated with 5 × 10⁷ LCL-1 cells s.c. in both flanks. Nine days after inoculation (when most injection sites had barely palpable tumors), eight of the mice were irradiated over each injection site with a single dose of 100 cGy of γ-irradiation. Four of the eight mice in each treatment group (irradiated versus not irradiated) were also treated with i.p. GCV (100 mg/kg twice a day for 5 days) starting on the day after irradiation.

The results of this experiment are depicted in Fig. 7. In this animal model system, tumor take rate is not 100% because some of the early palpable tumors spontaneously regress. When the experiment was terminated at day 44 (due to the large size of tumors in some animals), each injection site was explored, and any tumors found were dissected and weighed. Definite tumors were present in five of the eight injection sites in the animals that received no treatment, in six of eight injection sites in the animals treated with GCV alone, and in four of eight injection sites in the animals treated with γ-irradiation alone. Of the four mice treated with the combination of GCV and γ-irradiation, one mouse was found dead (of an unknown cause) shortly after the start of treatment (day 4). However, in the three remaining mice, none of the six injection sites developed a definite tumor. As shown in Fig. 7, the average tumor weight at the termination of the experiment was similar in the control group, the radiation alone group, and the GCV alone group, but it was much smaller in the GCV plus radiation treatment group.

These results suggest that the combination of GCV and γ-irradiation together is much more effective than either agent alone for inducing tumor regression of EBV-positive lymphomas in SCID mice. However, the death of one of the four mice receiving the GCV and γ-irradiation combination suggests that there may also be some added toxicity to this combined regimen.

DISCUSSION

The consistent presence of the EBV genome in certain tumor types suggests novel therapeutic strategies for targeting such tumors for destruction. Although several investigators have suggested the possibility of converting the latent (transforming) form of EBV infection normally present in tumor cells into the lytic (cytotoxic) form, to our knowledge, this conversion has never been successfully accomplished in vivo except by gene delivery techniques (9). Although we have been able to induce lytic infection in vivo using adenovirus vectors expressing either of the two EBV immediate-early proteins (BZLF1 and BRLF1) (9), gene delivery methods for treating EBV-positive malignancies will undoubtedly require substantial time for develop-
ment and testing before they are available on a widespread basis. In contrast, we describe here a simple yet previously unrecognized way to activate lytic EBV infection in tumors. We show that γ-irradiation at clinically relevant doses efficiently induces lytic EBV infection in lymphoblastoid tumors in vivo. We demonstrate that γ-irradiation treatment of EBV-positive lymphoblastoid cells in vitro enhances cell killing by prodrugs (GCV and AZT) known to be activated during the lytic form of EBV infection. Furthermore, in SCID mice, we show that the combination of GCV and γ-irradiation induces much more efficient tumor regression of an EBV-positive lymphoma than either treatment alone.

Interestingly, many EBV-positive tumors are initially very susceptible to radiation therapy. Our finding that γ-irradiation activates the lytic form of EBV infection suggests that in addition to the usual mechanisms for radiation-induced cell killing, EBV-positive tumor cells may be killed by the additional mechanism of cytolysis. Although we have not defined here the exact pathway by which γ-irradiation induces lytic EBV infection, it is likely to occur through activation of EBV IE gene transcription (BZLF1 or BRLF1 or both). Cells that are latently infected with EBV do not express the IE genes, yet it is known that high-level expression of either IE protein is sufficient to induce the lytic form of viral infection. Thus, γ-irradiation may activate cellular transcription factor(s) that induce either BZLF1 or BRLF1 transcription. In this regard, it is interesting to note that one of the key cellular transcription factors known to be activated by radiation is EGR-1 (41) and that we have previously shown that EGR-1 binding sites in the BRLF1 promoter are required for its activation by another known EBV-inducing agent, phorbol ester (42).

Although γ-irradiation appeared to be a more effective agent than a single dose of sodium butyrate in this study for activating lytic EBV infection in lymphoblastoid tumors, it is possible that a longer course of treatment with sodium butyrate would be more effective. Thus, sodium butyrate and radiation therapy might both be clinically useful for activating lytic EBV infection in tumors. Interestingly, some of the EBV-positive cell lines (such as Akata) in which γ-irradiation was relatively ineffective for inducing lytic EBV infection were derived from patient tumors. Such tumors could potentially have been exposed previously to radiation therapy while still in the patient and therefore selected for cells that lost the ability to induce lytic EBV infection in response to radiation. In any event, it would be difficult at this point to predict for any particular tumor whether sodium butyrate or radiation therapy would be more likely to activate the lytic form of viral replication. Because we found that the combination of both agents, although not generally synergistic, was at least was not antagonistic in vivo, the use of both agents together might offer the greatest likelihood for inducing lytic EBV infection in tumors in the absence of any specific documentation of tumor susceptibility in vivo.

A previous report suggested that AZT preferentially kills EBV-positive versus EBV-negative B-cell lines in vitro (32). Our data here, as well as the recent report showing that the EBV TK gene alone is sufficient to confer AZT susceptibility to melanoma cells (27), suggest that AZT alone will not preferentially kill latently infected EBV-positive tumor cells. The susceptibility of EBV-positive cell lines to AZT killing closely paralleled their susceptibility to GCV killing, which is known to require the lytic form of EBV infection. Because the great majority of cells in EBV-positive tumors contain only the latent form of EBV infection, it is unlikely that AZT alone (or GCV alone) would effectively treat most EBV-positive lymphomas. Nevertheless, in combination with radiation, AZT appears to be at least as effective as GCV in inducing killing of EBV-positive lymphoblastoid cells in vitro and may be less toxic in vivo.

To our knowledge, this is the first report of sodium butyrate being used to successfully induce lytic EBV infection in a mouse tumor model in vivo. However, a lung transplant recipient with an EBV-positive immunoblastic lymphoma was recently given i.v. arginine butyrate and GCV for 15 days, based on the rationale that activation of lytic EBV infection would promote susceptibility to GCV killing (39). Unfortunately, any therapeutic effect of the GCV/butyrate com-

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**Fig. 6. Effect of AZT, GCV, and γ-irradiation on lymphoblastoid cell survival.** An EBV-positive LCL (LCL-1) was treated with AZT alone (10 μg/ml), GCV alone (10 μg/ml), a single dose of γ-irradiation (200 cGy), a combination of GCV and radiation, or a combination of AZT and radiation. Cell viability (normalized to untreated control cells) was determined 9 days later by trypan blue exclusion.

**Fig. 7.** The combination of γ-irradiation and GCV induces regression of EBV-positive lymphoblastoid tumors in SCID mice. EBV-positive lymphoblastoid cells (5 × 10⁶ LCL-1 cells) were injected into both flanks of 16 SCID mice. When the majority of inoculation sites had developed barely palpable tumors (9 days after inoculation), they were treated with either one dose of γ-irradiation (100 cGy) alone (four mice; RT), i.e., GCV alone (100 mg/kg twice a day for 5 days; four mice; GCV), a combination of GCV and one dose of γ-irradiation (four mice; RT + GCV) or left untreated (four mice; Control). One of the four mice in the RT + GCV treatment group died of an unknown cause 4 days into the treatment. The average weight of the tumors in each group when the experiment was terminated at day 44 is shown (bars, SE).
Combination was obscured in this patient due to the concomitant administration of cytotoxic chemotherapy and a fatal Aspergillus infection (39). Nevertheless, no toxicity due to the arginine butyrate/GCV regimen was observed (39). In addition, another group recently reported a small number of AIDS patients with CNS lymphomas who clinically improved after receiving the combination of AZT, GCV, and interleukin 2 (32). Of note, several of these patients also received radiation therapy (32). Thus, the therapeutic effect of AZT and GCV for EBV-positive CNS lymphomas in this report could at least partially reflect the utility of combining radiation therapy with GCV and AZT.

Our finding that the combination of GCV and γ-irradiation is much more effective than either agent alone in inducing regression of lymphoblastoid tumors in SCID mice suggests that this combination may likewise be synergistic in the treatment of EBV-positive lymphomas in patients. There may be two different mechanisms by which the combination of γ-irradiation and GCV induces synergistic killing of EBV-positive tumor cells, as depicted in Fig. 8. One mechanism for synergistic killing of EBV-positive tumor cells by the GCV/γ-irradiation combination thus appears to be a potentially promising approach for improving the therapeutic outcome in EBV-positive tumors (such as CNS lymphomas in AIDS patients), which often respond initially to γ-irradiation alone but then commonly relapse. Nevertheless, the fact that one of four mice receiving the combination of both agents (versus none of the mice in the other treatment groups) died on day 4 of the treatment protocol suggests that adding GCV to γ-irradiation may also cause enhanced toxicity.

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Activation of Lytic Epstein-Barr Virus (EBV) Infection by Radiation and Sodium Butyrate \textit{in Vitro} and \textit{in Vivo}: A Potential Method for Treating EBV-positive Malignancies


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