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Short, Discontinuous Exposure to Butyrate Effectively Sensitizes Latently EBV-Infected Lymphoma Cells to Nucleoside Analogue Antiviral Agents

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Abstract

Antiviral drugs alone have been unsuccessful in the treatment of Epstein Barr virus (EBV)-associated malignancies because the virus maintains a latent state of replication in these tumors. In recent years, novel therapeutic approaches are being investigated wherein lytic replication of the virus is induced prior to the use of cytotoxic antiviral drugs. The choice of suitable agents to induce lytic replication has been a critical step in this novel approach. We have previously demonstrated that butyrate derivatives induce a lytic pattern of EBV gene expression in patient-derived EBV-positive lymphoblastoid cell lines and, together with nucleoside analog ganciclovir, effectively reduce or eliminate tumor growth in humans. Butyrate has drawbacks as a therapeutic agent, however, as constant intravenous infusion is required to achieve detectable plasma levels of this drug. In this study, we investigated whether discontinuous exposure to butyrate is capable of initiating lytic-phase gene expression and thymidine kinase induction, and sensitizing EBV-positive lymphoma cells to ganciclovir-mediated cell growth arrest and apoptosis. We demonstrate that multiple daily 6 hr exposures of the EBV-positive Burkitt's lymphoma cell line P3HR1 to butyrate induced sustained expression of the EBV lytic-phase protein BMRF. Viral thymidine kinase was also induced by intermittent exposure, although to a lower level than with continuous exposure treatment. However, discontinuous exposure to butyrate in combination with ganciclovir induced a similar level of tumor cell growth inhibition as did continuous treatment, as measured by serial enumeration of viable cells, MTT cell proliferation assays, and measurement of cellular DNA content. We further demonstrated that those cells which survived initial exposure to butyrate plus ganciclovir remained susceptible to further cycles of combination treatment. These findings suggests that continuous infusion of butyrate may not be necessary for maintaining viral thymidine kinase gene expression and sensitization to anti-viral agents in EBV-associated tumors, and that therapeutic regimens which employ more convenient, discontinuous exposure to butyrate may also be effective clinically.

Keywords

Epstein-Barr Virus; Butyrate; Thymidine Kinase; Ganciclovir; Growth inhibition; Apoptosis

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Introduction

Epstein-Barr virus (EBV), a member of γ-herpesvirus family, is a ubiquitous human pathogen. EBV infections in infants and children are usually asymptomatic, whereas infection in adolescent adults causes infectious mononucleosis, characterized by fever, lymphoadenopathy and pharyngitis [1]. EBV has been etiologically linked to a variety of human cancers such as Burkitt's lymphoma (BL) [2], nasopharyngeal carcinoma (NPC) [3], Hodgkin's disease (HD) [4] and more recently with sporadic cases of gastric adenocarcinoma [5,6] and invasive breast carcinoma [7,8]. Nearly 100% of NPC tumors, 90% of BL tumors of African origin, and 40–60% of HD and non-Hodgkin lymphomas contain EBV episomes. Clonality of the EBV genome has been verified in these tumors, suggesting that the tumor arises from a single EBV-infected cell, and that EBV infection is a very early, if not causal, event [9,10]. EBV is also very commonly associated with lymphoproliferative disease in patients with congenital or acquired immunodeficiencies. Examples include X-linked lymphoproliferative syndrome (XLP) [11], human immunodeficiency virus related non-Hodgkin's lymphoma [12] and, most importantly, post-transplantation lymphoproliferative disease (PTLD) [13].

The presence of the EBV genome in these malignancies provides an opportunity for specific virus-directed therapeutic approaches. Like other herpes viruses, EBV infection can exhibit two distinct patterns or states of gene expression. During acute EBV infection, the virus sequentially expresses its entire repertoire of genes, producing a lytic infection. Shortly after initial infection, EBV enters into a latent state, whereupon only select "latent" genes are expressed, thereby evading the host immune surveillance mechanism, and establishing a life-long persistent infection in the host [14]. Nucleoside analogs such as acyclovir (ACV) or ganciclovir (GCV) are often used as antiviral drugs against acute EBV and other herpesvirus infections [15,16]. The virally-encoded thymidine kinase enzyme converts these analogs to their monophosphate forms, which after conversion into the triphosphate form by host kinases, are then incorporated in newly-synthesized DNA, leading to premature termination of DNA synthesis and killing of the infected cell by apoptosis. The EBV thymidine kinase, however, is only expressed during lytic replication of the virus. Because EBV maintains a latent state of replication in all EBV-associated malignancies, nucleoside analog drugs have very limited or no cytopathic effect on the virus-infected cells. Novel therapeutic approaches to target EBV-containing tumor cells, wherein lytic replication of EBV is induced followed by treatment with nucleoside analog, have been proposed [17,18].

We have demonstrated previously that arginine butyrate induces expression of the viral thymidine kinase gene in EBV-positive immunoblastic non-Hodgkin's lymphoma cell lines and lymphoblastic cell lines, and acts synergistically with ganciclovir to inhibit cell proliferation and decrease cell viability [19,20]. Various other agents have also been used to induce lytic replication of the EBV genome in other studies. For example, treatment of EBV positive lymphoblastoid cells or primary central nervous system lymphoma with γ-irradiation has been shown to promote ganciclovir-susceptibility of these cells [21,22]. Other studies successfully used 5-azacytidine, gemcitabine, doxorubicin, and a combination of anti-CD20 monoclonal antibody and dexamethasone to induce lytic-phase gene expression and sensitize EBV-containing tumor cells to ganciclovir or other nucleoside analogues [23–25].

Butyric acid, a short-chain fatty acid, and its derivatives have been experimentally employed in attempts to treat leukemias and other diseases [26–28]. In particular, the arginine salt of butyrate has been used in clinical studies to therapeutically and safely reactivate the expression of the developmentally-silenced gamma globin genes in the therapy of sickle cell disease and thalassemia [29]. Butyrate has been reported to induce the expression of certain EBV lytic proteins from latently EBV-infected cells [30–32], including the thymidine kinase enzyme [19]. The histone deacetylase (HDAC) inhibitory effect of butyrate [33] is required for this

molecular effect [34]. In previously reported clinical studies, we have used systemic administration of arginine butyrate to induce expression of the latent EBV thymidine kinase in the tumors of patients with EBV(+) PLTD or non-Hodgkin's lymphomas, followed by treatment with the antiviral agent ganciclovir [19]. In a recently-completed phase I/II trial of this virus-targeted therapeutic approach, using an intra-patient dose-escalation of arginine butyrate combined with ganciclovir at standard anti-viral doses, we observed complete and partial clinical responses in 10 out of 15 patients with EBV-associated lymphoid malignancies which had been previously refractory to all conventional therapies [35].

Although this arginine butyrate/ganciclovir combination therapy appears to show significant activity towards EBV-associated malignancies, several issues with the protocol may limit its general application. As butyrate is cleared rapidly from the blood by cellular uptake and metabolism [36], and because of the very poor bioavailability of orally-administered butyrate due to near-complete first-pass hepatic clearance, continuously maintaining a sufficient plasma level of butyrate for reactivation of the target gene in the tumors has required a constant intravenous infusion of the drug over several weeks, and thus a prolonged hospitalization.

Our previous *in vitro* studies have demonstrated that even a brief period of exposure of EBV-infected lymphoid cells to butyrate is sufficient to reactivate the viral thymidine kinase gene [19]. If this shorter exposure to butyrate was sufficient to induce viral thymidine kinase to a level which sensitized the cells to antiviral agents, it might then be possible to modify the current clinical protocol to utilize briefer infusion intervals, which would then allow outpatient administration of the therapy. In the present study, therefore, we tested the efficacy of a brief, discontinuous exposure to butyrate on EBV-infected cells, with respect to thymidine kinase gene induction and sensitization ganciclovir-mediated growth inhibition. We demonstrate here that intermittent exposure of the EBV-positive lymphoblastoid cell line P3HR1 to butyrate strongly induces early lytic gene expression in the latent virus. Discontinuous treatment with butyrate also induces thymidine kinase gene expression in these cells. In conjunction with ganciclovir, discontinuous treatment with butyrate reduces the growth of P3HR1 cells to levels comparable to that achieved using continuous treatment. This cytotoxic effect persists even after removal of butyrate and ganciclovir from the cultures, and the growth rate of the cells exposed to the combination of agents was significantly less than those cells which were treated with butyrate alone. Furthermore, we show that the cells surviving one cycle of the combination treatment remained fully susceptible to re-exposure to the same combination of agents. These findings have significant clinical implications, suggesting that patients with EBV-associated tumors might be efficiently treated with ganciclovir and daily, brief periods of infusion with butyrate, thus circumventing the need for prolonged hospitalization.

Materials and Methods

Cell culture and reagents

The EBV-positive lymphoblastoid cell line P3HR1 was obtained from ATCC and maintained in RPMI 1640 with 10% fetal bovine serum, 100 U penicillin/ml and 100 mg streptomycin/ml. ganciclovir (Invivogen, San Diego, CA), sodium butyrate (NaB) and methylthiazolyldiphenyl-tetrazolium bromide [(MTT) Sigma/Aldrich Chemicals (St. Louis, MO)] were purchased commercially.

Immunoblot analysis

Cells were lysed in whole cell lysis buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1.0% sodium deoxycholate, 1.0% Triton X-100 and proteinase inhibitor cocktail (Roche, Indianapolis, IL). Equal amounts of protein samples were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes.

Following blocking with 5% non-fat milk at room temperature for 1 hr, the membrane was incubated with 1:500 dilution of a monoclonal antibody against EBV early antigen diffuse p50/52 (BMRF) (Chemicon International, Temecula, CA) at 4°C overnight. Membranes were then treated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature and bound antibodies were detected by the Western Lightning Chemiluminescent detection reagent (Perkin-Elmer Life Sciences, Wellesley, MA).

RNA Blot analysis

Total cellular RNA was isolated by Trizol extraction method according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). EBV episomal DNA from all RNA preparations were removed by RQ1 RNase-free DNase treatment at a concentration of 0.1 unit/μl. Twenty micrograms of RNA from each sample was separated in 1% agarose gel containing 6.3% formaldehyde and capillary-transferred onto Duralon-UV membrane (Stratagene, La Jolla, CA). Transferred RNA was immobilized onto membrane by UV cross-linking and prehybridized in ExpressHyb hybridization solution (BD Bioscience Clontech, Mountain View, CA) at 68°C for 1 hr followed by hybridization in ExpressHyb solution containing $1x10^6$ cpm/ml ^{32}P-labeled thymidine kinase probe for 1 hr at 68°C. A 300 bp DNA fragment of human thymidine kinase gene was generated by PCR amplification from a cDNA clone (pET-TKB1B, generously provided by Dr. Yang, National Taiwan University, Taiwan) and used as template for probe preparation. PCR primers for this purpose were: forward, 5'-AGATGACGACGGCCTCTACCA-3'; reverse, 5'-CCTCCTTCTGTGCACGAAGT-3'. The PCR amplified fragment was labeled with ^{32}P-dCTP using a random hexamer labeling kit from Invitrogen and purified by Chromaspin-10 column (Clontech).

Quantitative Reverse Transcriptase-PCR

Two micrograms of total RNA was reverse transcribed by Thermoscript reverse transcriptase (Invitrogen) using random hexamer primers, according to the manufacturer's protocol. The cDNA product was then amplified using a SYBR Green PCR master mixture (Applied Biosystems, Foster City, CA) and gene-specific primers (5 μM) in an ABI PRISM 7500 sequence detection system (Applied Biosystems), as recommended by the manufacturer. The following primers were used for real-time PCR amplifications: thymidine kinase, forward, 5'-TCCGGAGCCAGCTTCTCTCC-3'; reverse, 5'-CGTGATTGTTGTTAGACCGG-3' and for ßactin, forward, 5'-TCCCTGGAGAAGAGCTACGA-3'; reverse, 5'-AGCACTGTGTTGGCGTACAG-3'. Relative quantification of gene expression was performed using the comparative threshold (CT) method, as described by the manufacturer. Changes in mRNA expression level were calculated following normalization to ß-actin expression.

Cell viability assay

The trypan blue dye exclusion method was used to enumerate viable cells following various treatments. The MTT cell proliferation assay was also used to monitor viability of treated cells. One-tenth volume of 0.5% MTT solution in PBS was added to the cells and incubated at 37° C for 30 min. Cells were then harvested and the formazan precipitate in the cells was solubilized by DMSO. The absorbance of the resulting solution was determined in an automated microtiter plate reader at 650/570 nm wavelengths.

Flow cytometric analysis of apoptosis

Cells were fixed in cold ethanol (35% in DMEM) for 15 min and stained with 50 μg/ml propidium iodide in phosphate-buffered saline containing 1 mg RNase A/ml for 30 min at 4° C in the dark. Percentages of cells in the different phases of the cycle were determined by flow cytometric analysis of propidium iodide-stained nuclei using CellQuest Pro software FACScan

(BD Biosciences, San Jose, CA). Cells present in the sub-G_1 fraction (sub-diploid with respect to DNA content) were considered to be apoptotic.

Results

Brief exposure of EBV-positive lymphoma cells to butyrate induces a lytic pattern of viral gene expression

To investigate the efficacy of short, intermittent exposure to butyrate on the killing of EBV-containing lymphoma cells by antiviral agents, we first tested whether discontinuous treatment was sufficient to induce certain lytic-phase genes. We tested a well-characterized EBV positive Burkitt's lymphoma cell line P3HR1 in this study. The P3HR1 virus is lytic replication-competent, although it cannot transform normal B lymphocytes because of partial deletion of the EBV nuclear antigens EBNA-LP and EBNA2 genes [14]. P3HR1 cells were treated with varying concentrations of butyrate (1.0 mM, 2.5 mM, or 5.0 mM) for a 6 hr period, whereupon butyrate was removed from the cultures by washing the cells, and replacing normal growth medium. The cell cultures were subjected to this 6 hr exposure for three consecutive days at the same time of the day, and were then harvested for preparation of a whole-cell lysate. In parallel, separate cultures of P3HR1 cells were maintained continuously in presence of similar concentrations of butyrate for the same 3-day period before preparing whole-cell lysates. The presence of EBV protein early antigen diffuse (EAD or BMRF), which is expressed during the lytic stage of replication, was analyzed in these samples by immunoblotting. Continuous exposure of P3HR1 cells to butyrate induced the BMRF protein robustly, in a dose-dependent manner (Figure 1). Discontinuous exposure to butyrate also induced BMRF protein in a similar fashion, albeit at lower levels. The level of BMRF expression induced by discontinuous treatment with butyrate at 5.0 mM was similar to that induced by continuous treatment with butyrate at 2.5 mM. This result demonstrated that multiple 6 hr discontinuous periods of treatment with butyrate were sufficient to induce EBV lytic-phase gene products in EBV-positive lymphoma cells.

The EBV Thymidine Kinase gene is induced following discontinuous butyrate treatment

We next evaluated whether discontinuous exposure to butyrate was sufficient to induce EBV thymidine kinase gene expression. First, the kinetics of thymidine kinase expression in P3HR1 cells following exposure to two different concentrations of butyrate was examined. RNA blot analysis of total cellular RNA from these cells demonstrated that thymidine kinase gene expression was easily detected within 24 hr in cells treated with butyrate at concentrations of either 1.0 mM or 2.5 mM (Figure 2A). On occasion, the expression of thymidine kinase RNA could be detected within as early as 6 hr of exposure to butyrate (data not shown). To analyze thymidine kinase expression in cells treated discontinuously with butyrate, a real-time quantitative RT-PCR assay was employed, as this method of analysis is more quantitative at lower levels of gene expression. An oligo-dT primer was used to synthesize cDNA from all RNA samples, which was then amplified by PCR for detection of the thymidine kinase transcript, using specific primers. ß-actin RNA levels in these samples was measured from the same first round cDNA synthesis product and used to normalize thymidine kinase transcript expression levels. Discontinuous exposure to butyrate (at concentrations of 1.0 mM, 2.5 mM and 5.0 mM) induced thymidine kinase gene expression by 2.1-, 2.2- and 3.9-fold, respectively (Figure 2B). At each concentration, however, the level of expression induced by continuous exposure was higher than that induced by discontinuous treatment.

Discontinuous butyrate exposure efficiently sensitizes P3HR1 cells to ganciclovir-mediated cell growth inhibition

We next determined whether the thymidine kinase expression induced by discontinuous exposure to butyrate would sensitize P3HR1 cells to killing mediated by the nucleoside

analogue ganciclovir. Sensitivity of P3HR1 cells to ganciclovir was tested using various concentrations of ganciclovir over six consecutive days and enumerating the viable cells remaining in cultures. Increasing concentrations of ganciclovir produced proportional growth-inhibitory effects on P3HR1 cells (Figure 3A). On average, a 6-day exposure of P3HR1 cells to 40 μM ganciclovir reduced the cell growth to about 70–80%, compared to untreated cells, consistent with the findings of our earlier studies [20]. To assay sensitization of the lymphoma cells to ganciclovir by discontinuous exposure to butyrate, cells were plated at a low density and treated with butyrate (1.0 mM, 2.5 mM or 5.0 mM) plus ganciclovir (60 μM). Following a 6 hr incubation, media containing butyrate was removed and replaced with fresh media containing only ganciclovir. As control, cells were also treated in parallel with butyrate alone (without ganciclovir). The 6 hr exposure to butyrate and 18 hr wash-out was repeated on each of the following two days. At the end of day 3, butyrate was removed from all wells and replaced with fresh medium with or without ganciclovir (as appropriate), and the cultures were maintained for three more days. Untreated cells and cells treated with ganciclovir alone served as controls. Cells from these experiments were then subjected to three different assays to evaluate and compare the effect of ganciclovir on the growth of cells treated continuously or discontinuously with butyrate.

Analysis of cell growth and viability by the trypan blue dye exclusion method demonstrated that continuous treatment with butyrate as a single agent produced a robust growth-inhibitory effect, causing as much as 92% relative suppression of growth at 5 mM concentrations of butyrate (33% at 1.0 mM and 84% at 2.5 mM) (Figure 3B). The addition of ganciclovir further reduced cell growth (60% with 1.0 mM, 66% with 2.5 mM and 90% with 5.0 mM). Discontinuous exposure to butyrate as a single agent produced substantially less growth inhibition (10% at 1.0 mM, 33% at 2.5 mM, and 55% at 5.0 mM), but in combination with ganciclovir the growth inhibition produced was comparable to that achieved by continuous treatment with butyrate (56% at 1.0 mM, 61% at 2.5 mM and 85% at 5.0 mM). These results show that discontinuous treatment with butyrate produces less suppression of cell growth than continuous treatment, but when combined with ganciclovir is quite effective in suppressing growth. Comparable results were obtained when the cultures were analyzed using an MTT cell viability assay (Figure 3C). Assessment of cell proliferation by formazan dye formation also demonstrated that tumor cell growth was repressed by continuous exposure to butyrate, and that the addition of ganciclovir strongly enhanced this growth suppression. Discontinuous treatment with butyrate as a single agent did not reduce cell numbers to the extent that continuous treatment did, but the anti-proliferative activity was significantly enhanced by combination with ganciclovir.

Cell cultures exposed to varying concentrations and durations of butyrate, with or without ganciclovir, were also analyzed for the presence of hypodiploid (sub-G1 fraction, apoptotic) cells by flow cytometry after staining the DNA with propidium iodide. Continuous exposure to butyrate induced apoptosis of the cells in a dose-dependent manner and this cytotoxic activity was enhanced by combination with ganciclovir (Figure 3D). In contrast, discontinuous exposure to butyrate did not induce any significant increase in the apoptotic fraction at any of the butyrate concentrations studied. When ganciclovir was added, there was no significant increase in apoptosis as measured by this assay with butyrate concentrations of 1.0 and 2.5 mM despite profound suppression of proliferation, but the apoptotic fraction increased to 31% with butyrate concentrations of 5.0 mM plus ganciclovir.

Exposure to the combination of ganciclovir and butyrate alters cellular morphology

Cellular morphological changes consistent with a cytotoxic effect were next assessed. P3HR1 cells were treated with varying concentrations of butyrate, continuously or discontinuously, in presence of ganciclovir for 3 days, at which time butyrate was removed and the cells were

cultured in ganciclovir alone for 3 more days. Cells were then washed and maintained in normal growth media for 6 more days and their morphology under phase contrast microscopy was recorded. The combination of butyrate and ganciclovir exerted a significant cytopathic effect on P3HR1 cells even 6 days after both of the drugs had been removed (Figure 4). Cells with large vacuoles, ragged membranes, and lacking distinct intracellular organelles were prominent and frequent. The magnitude of these cytopathic effects was butyrate dose-dependent. Discontinuous combination treatment induced similar morphological changes, although with somewhat less frequency than found after continuous exposure. In contrast, cells treated with ganciclovir alone appeared morphologically unchanged. (Long-term maintenance of these cells [and also EBV-negative lymphoid cell lines] in presence of ganciclovir is toxic to the cells and a reduction in cell proliferation is apparent after continuous exposure to ganciclovir for two weeks or more [data not shown]).

Tumor cells surviving exposure to butyrate plus ganciclovir remain susceptible to additional cycles of treatment

As a fraction of P3HR1 cells invariably survived the first exposure to the butyrate plus ganciclovir combination and resumed a normal proliferation rate, it was important to determine whether these cells represented a resistant sub-population, or instead would be susceptible to further rounds of similar treatment. To address this issue, P3HR1 cells surviving a prior exposure to ganciclovir (60 μM) plus either a 72 hr continuous exposure to butyrate (2.5 mM), or three discontinuous 6 hr exposures to butyrate, were allowed to expand in normal growth medium, and were then subjected to new round of continuous or discontinuous treatment with butyrate in presence of ganciclovir. We tested the effect of butyrate only at a concentration of 2.5 mM in these assays. Discontinuous combination treatment reduced the growth of these previously-treated cells by 67% (Figure 5), and this effect was similar to the level of growth inhibition observed with naïve (previously-untreated) cells (compare with Figure 3B).

A single, brief exposure to butyrate is sufficient to sensitize tumor cells to ganciclovir

As the experiments above established that multiple discontinuous exposures to butyrate sensitized P3HR1 cells to ganciclovir-mediated growth inhibition and/or apoptosis, we next determined the relationship between numbers of discontinuous exposures to butyrate and sensitization to ganciclovir. P3HR1 cells were exposed to butyrate (2.5 mM) in one, two, or three 6 hr periods, over one, two or three days, respectively, in presence of ganciclovir (60 μM), and then allowed to grow in presence of ganciclovir alone until day six. Ganciclovir was subsequently removed from the media of the cells, and kinetics of cell growth were monitored. One or two 6 hr exposures to butyrate alone did not affect the growth rate, although three daily 6 hr exposures reduced cumulative cell number by 23% (Figure 6A). The addition of ganciclovir to butyrate, however, reduced proliferation by 57%, 63% and 61% for cells exposed to butyrate once, twice, or three times, respectively. Thus, a single 6 hr exposure to butyrate sensitized cells to ganciclovir almost as effectively as three daily 6 hr exposures. Analysis of growth kinetics of these cells for a period of eleven days, even after removal of ganciclovir and butyrate from the cultures, demonstrated that the growth of cells in cultures exposed to discontinuous combination treatment remained significantly retarded compared to cells in parallel cultures which were exposed to butyrate alone (Figure 6B). The growth of cells exposed to three 6 hr cycles of butyrate, in combination with ganciclovir, was only marginally slower than cells exposed to a single 6 hr cycle of butyrate.

Discussion

EBV has been etiologically linked to a number of human tumors, such as Burkitt's lymphoma, Hodgkin's disease, non-Hodgkin lymphoma, nasopharyngeal carcinoma, and other sporadic cancers of gastrointestinal tract and breast [37]. Persistent expression of certain of the EBV

gene products is believed to be necessary for tumor progression. In recent years, several virus-directed therapeutic approaches have been proposed for treatment of EBV-associated tumors. The key features of these approaches are intentional induction of lytic phase of the virus, with the anticipation that lytic release of the virus would destroy the host tumor cell. Our approach has been mechanistically different, in that induction of full lytic phase has not been the goal. Rather, induction of at least one early lytic gene, viral thymidine kinase, followed by administration of one of the nucleoside analog pro-drugs targeting viral thymidine kinase (such as ganciclovir), has been the strategy. The method of activation of lytic phase viral gene expression is a critical step in either of these approaches. Previously we have successfully used arginine butyrate and other butyrate derivatives to induce lytic phase EBV gene expression in EBV lymphoblastoid cell lines (LCL) and in patients [17,19,20]. Butyrate as a viral gene inducer is preferable to other modalities such as radiation or chemotherapeutic drugs, which have been reported to induce at least some lytic phase gene expression, because it has been safely administered to a number of patients with malignant and non-malignant chronic genetic diseases, and is not myelosuppressive or immunosuppressive. Because of the very short half-life of butyrate *in vivo*, however, the first trials of this agent in combination with ganciclovir for the treatment of EBV malignancies utilized a continuous infusion of the drug for several weeks [17]. In this study, we investigated whether a shorter duration of exposure of EBV-positive lymphoma cells to butyrate would be sufficient to induce early lytic phase gene expression and sensitization to ganciclovir-mediated cytotoxicity. We demonstrated herein that multiple brief exposures to butyrate efficiently sensitize EBV-positive tumor cells to ganciclovir.

The switch between latent and lytic phases during EBV infection is primarily regulated by the two immediate-early proteins BZLF1 and BRLF1 [38,39]. These two proteins are efficient transcriptional activators and their concerted action results in production of other lytic phase proteins and ultimately virus particles. In latently-infected cells, the promoters for these two genes are usually hyper-methylated and the surrounding area is hypoacetylated, resulting in repression of transcription of these genes [40-42]. The histone deacetylase activity of butyrate and its derivatives alters the epigenetic state of the episomal EBV genome and facilitates transcription of these genes and subsequent entry into lytic phase [33,42].

Our studies demonstrate that exposure to butyrate induces expression of the EBV immediate-early antigen-diffuse protein (also known as BMRF) in the Burkitt's lymphoma cell line P3HR1, which maintains typical EBV type-I latency. We have previously demonstrated that arginine butyrate induces lytic-phase gene expression in multiple EBV-positive lymphoblastoid and lymphoma cell lines, and enhances expression of the early lytic phase protein thymidine kinase [20]. Our present findings thus corroborate our earlier findings, and extend them by demonstrating that butyrate can be used to induce EBV lytic-phase gene expression in both latency type-I and type-III EBV infections. Further, our data demonstrate that continuous exposure to butyrate is not necessary for sustained BMRF expression. Similar requirements and kinetics were also observed with regard to thymidine kinase induction by butyrate. Although the level of thymidine kinase transcript expression was several-fold higher in response to continuous exposure to butyrate, a significant and sustained increase in thymidine kinase expression levels was observed with discontinuous exposure. Furthermore, this lower level of thymidine kinase expression was quite efficient in suppressing cell growth in the presence of ganciclovir. It is noteworthy that the relative reduction in cell growth produced by the continuous treatment protocol was similar to that resulting from the discontinuous treatment protocol. The apoptosis assay employed (generation of cells with a hypodiploid DNA content) indicated that continuous combination treatment induced apoptosis in a significant fraction of the cells, whereas the discontinuous exposure did not, except at the higher concentrations of butyrate. Yet, both continuous and discontinuous exposures produced profound morphological changes in the cells suggesting irreversible damage. These results

suggest that the growth inhibition due to combination treatment may not necessarily depend upon induction of a classical apoptotic process, at least as assayed by the generation of cells with a hypodiploid DNA content.

In the studies presented here, we have shown that butyrate exerts a strong, dose-dependent, and reversible growth-inhibitory effect on P3HR1 cells. This is consistent with our previous report, in which we demonstrated that butyrate induces G_1-phase growth arrest in fibroblasts and other cell types [43]. This growth-inhibitory effect was significantly more pronounced when cells were exposed to butyrate continuously, in comparison to discontinuous exposure. Although the addition of ganciclovir had enhanced the growth-inhibitory effect, continuous exposure of cells to 2.5 mM butyrate alone inhibited cell growth by 85%. In contrast, discontinuous exposure at the same 2.5 mM concentration of butyrate reduced P3HR1 cell growth by only 32%. These findings may therefore suggest that continuous exposure to high doses of butyrate in a clinical protocol might be more likely to evoke undesired adverse effects than would discontinuous exposure. Indeed, in our recent phase I clinical trial with arginine butyrate in EBV-associated lymphoid malignancies, we observed that escalation of the arginine butyrate dose was associated with an increase in adverse events [35]. Our present finding that a discontinuous treatment regimen has little or no effect on cultured cells in absence of ganciclovir suggests that discontinuous exposure to butyrate may decrease toxicities to normal tissues.

Because a fraction of cells inevitably survived a single round of exposure to the combination of butyrate and ganciclovir, and would re-grow when the drugs were removed, it was of interest to determine whether these surviving cells remained susceptible to the treatment regimen or instead represented escape mutants. We demonstrated that these surviving cells remained equally susceptible to further rounds of combination treatment. We also demonstrated that even a single 6 hr discontinuous combination treatment had a significant anti-proliferative effect on EBV-infected lymphoma cells.

In summary, the data presented here strongly suggests that a combination treatment regimen employing intermittent exposure to butyrate and continuous (daily) exposure to ganciclovir may display significant activity in treating EBV-positive lymphomas. On the basis of these data, we have initiated such a brief, discontinuous treatment protocol for EBV-positive lymphomas and lymphoid malignancies.

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References

1. Straus SE, Cohen JI, Tosato G, Meier J. NIH conference. Epstein-Barr virus infections: biology, pathogenesis, and management. Ann Intern Med 1993;118:45–58. [PubMed: 8380053]
2. Epstein M, Achong B, Barr Y. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet 1964;1:702–703. [PubMed: 14107961]
3. Liebowitz D. Nasopharyngeal carcinoma: the Epstein-Barr virus association. Semin Oncol 1994;21:376–381. [PubMed: 8209269]
4. Deacon EM, Pallesen G, Niedobitek G, et al. Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. J Exp Med 1993;177:339–349. [PubMed: 8381153]
5. Ott G, Kirchner T, Muller-Hermelink HK. Monoclonal Epstein-Barr virus genomes but lack of EBV-related protein expression in different types of gastric carcinoma. Histopathology 1994;25:323–329. [PubMed: 7835837]
6. Yuen ST, Chung LP, Leung SY, et al. In situ detection of Epstein-Barr virus in gastric and colorectal adenocarcinomas. Am J Surg Pathol 1994;18:1158–1163. [PubMed: 7943537]

7. Bonnet M, Guinebretiere JM, Kremmer E, et al. Detection of Epstein-Barr virus in invasive breast cancers. J Natl Cancer Inst 1999;91:1376–1381. [PubMed: 10451442]
8. Brink AA, van Den Brule AJ, van Diest P, Meijer CJ. Re: detection of Epstein-Barr virus in invasive breast cancers. J Natl Cancer Inst 2000;92:655–656. [PubMed: 10772685]
9. Pathmanathan R, Prasad U, Sadler R, et al. Clonal proliferations of cells infected with Epstein-Barr virus in preinvasive lesions related to nasopharyngeal carcinoma. N Engl J Med 1995;333:693–698. [PubMed: 7637746]
10. Weiss LM, Movahed LA, Warnke RA, Sklar J. Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease. N Engl J Med 1989;320:502–506. [PubMed: 2536894]
11. Morra M, Howie D, Grande MS, et al. X-linked lymphoproliferative disease: a progressive immunodeficiency. Annu Rev Immunol 2001;19:657–682. [PubMed: 11244050]
12. Shibata D, Weiss LM, Hernandez AM, et al. Epstein-Barr virus-associated non-Hodgkin's lymphoma in patients infected with the human immunodeficiency virus. Blood 1993;81:2102–2109. [PubMed: 8386027]
13. Hopwood P, Crawford DH. The role of EBV in post-transplant malignancies: a review. J Clin Pathol 2000;53:248–254. [PubMed: 10823119]
14. Kieff, E. Epstein-Barr virus and its replication. In: Fields, BN.; Knipe, DM.; Howley, PM., editors. Fundamental Virology. Lippincott-Raven Publishers; Philadelphia, PA: 1996. p. 1109-1162.
15. Crumpacker CS. Ganciclovir. N Engl J Med 1996;335:721–729. [PubMed: 8786764]
16. Resnick L, Herbst JS, Ablashi DV, et al. Regression of oral hairy leukoplakia after orally administered acyclovir therapy. JAMA 1988;259:384–388. [PubMed: 2826830]
17. Faller DV, Mentzer SJ, Perrine SP. 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 2001;13:360–367. [PubMed: 11555713]
18. Israel BF, Kenney SC. Virally targeted therapies for EBV-associated malignancies. Oncogene 2003;22:5122–5130. [PubMed: 12910249]
19. Mentzer SJ, Fingeroth J, Reilly JJ, et al. Arginine butyrate-induced susceptibility to ganciclovir in an Epstein-Barr-virus-associated lymphoma. Blood Cells Mol Dis 1998;24:114–123. [PubMed: 9628848]
20. Mentzer SJ, Perrine SP, Faller DV. Epstein--Barr virus post-transplant lymphoproliferative disease and virus-specific therapy: pharmacological re-activation of viral target genes with arginine butyrate. Transpl Infect Dis 2001;3:177–185. [PubMed: 11493400]
21. Roychowdhury S, Peng R, Baiocchi RA, et al. Experimental treatment of Epstein-Barr virus-associated primary central nervous system lymphoma. Cancer Res 2003;63:965–971. [PubMed: 12615710]
22. Westphal EM, Blackstock W, Feng W, et al. 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 2000;60:5781–5788. [PubMed: 11059774]
23. Daibata M, Bandobashi K, Kuroda M, et al. 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. J Virol 2005;79:5875–5879. [PubMed: 15827204]
24. Feng WH, Hong G, Delecluse HJ, Kenney SC. Lytic induction therapy for Epstein-Barr virus-positive B-cell lymphomas. J Virol 2004;78:1893–1902. [PubMed: 14747554]
25. Moore SM, Cannon JS, Tanhehco YC, et al. Induction of Epstein-Barr virus kinases to sensitize tumor cells to nucleoside analogues. Antimicrob Agents Chemother 2001;45:2082–2091. [PubMed: 11408227]
26. Miller AA, Kurschel E, Osieka R, Schmidt CG. Clinical pharmacology of sodium butyrate in patients with acute leukemia. Eur J Cancer Clin Oncol 1987;23:1283–1287. [PubMed: 3678322]
27. Novogrodsky A, Dvir A, Ravid A, et al. Effect of polar organic compounds on leukemic cells. Butyrate-induced partial remission of acute myelogenous leukemia in a child. Cancer 1983;51:9–14. [PubMed: 6571794]

28. Ferrante RJ, Kubilus JK, Lee J, et al. Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. J Neurosci 2003;23:9418–9427. [PubMed: 14561870]
29. Pace BS, White GL, Dover GJ, et al. Short-chain fatty acid derivatives induce fetal globin expression and erythropoiesis in vivo. Blood 2002;100:4640–4648. [PubMed: 12393583]
30. Anisimova E, Prachova K, Roubal J, Vonka V. Effects of n-butyrate and phorbol ester (TPA) on induction of Epstein-Barr virus antigens and cell differentiation. Arch Virol 1984;81:223–237. [PubMed: 6089703]
31. Contreras-Salazar B, Ehlin-Henriksson B, Klein G, Masucci MG. Up regulation of the Epstein-Barr virus (EBV)-encoded membrane protein LMP in the Burkitt's lymphoma line Daudi after exposure to n-butyrate and after EBV superinfection. J Virol 1990;64:5441–5447. [PubMed: 2170681]
32. Saemundsen AK, Kallin B, Klein G. Effect of n-butyrate on cellular and viral DNA synthesis in cells latently infected with Epstein-Barr virus. Virology 1980;107:557–561. [PubMed: 6256952]
33. Sealy L, Chalkley R. The effect of sodium butyrate on histone modification. Cell 1978;14:115–121. [PubMed: 667928]
34. Park JH, Faller DV. Epstein-Barr virus latent membrane protein-1 induction by histone deacetylase inhibitors mediates induction of intercellular adhesion molecule-1 expression and homotypic aggregation. Virology 2002;303:345–363. [PubMed: 12490396]
35. Perrine SP, Hermine O, Small T, et al. A Phase I/II Study of Arginine Butyrate and Ganciclovir in Patients with Epstein-Barr Virus-Associated Lymphoid Malignancies. Blood. 2006in press
36. Egorin MJ, Yuan ZM, Sentz DL, et al. Plasma pharmacokinetics of butyrate after intravenous administration of sodium butyrate or oral administration of tributyrin or sodium butyrate to mice and rats. Cancer Chemother Pharmacol 1999;43:445–453. [PubMed: 10321503]
37. Cohen JI. Epstein-Barr virus infection. N Engl J Med 2000;343:481–492. [PubMed: 10944566]
38. Countryman J, Jenson H, Seibl R, et al. Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. J Virol 1987;61:3672–3679. [PubMed: 2824806]
39. Chevallier-Greco A, Manet E, Chavrier P, et al. Both Epstein-Barr virus (EBV)-encoded transacting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. EMBO J 1986;5:3243–3249. [PubMed: 3028777]
40. Jenkins PJ, Binne UK, Farrell PJ. Histone acetylation and reactivation of Epstein-Barr virus from latency. J Virol 2000;74:710–720. [PubMed: 10623733]
41. Falk KI, Ernberg I. Demethylation of the Epstein-barr virus origin of lytic replication and of the immediate early gene BZLF1 is DNA replication independent. Brief report. Arch Virol 1999;144:2219–2227. [PubMed: 10603176]
42. Gruffat H, Manet E, Sergeant A. MEF2-mediated recruitment of class II HDAC at the EBV immediate early gene BZLF1 links latency and chromatin remodeling. EMBO Rep 2002;3:141–146. [PubMed: 11818339]
43. Vaziri C, Stice L, Faller DV. Butyrate-induced G1 arrest results from p21-independent disruption of retinoblastoma protein-mediated signals. Cell Growth Differ 1998;9:465–474. [PubMed: 9663465]

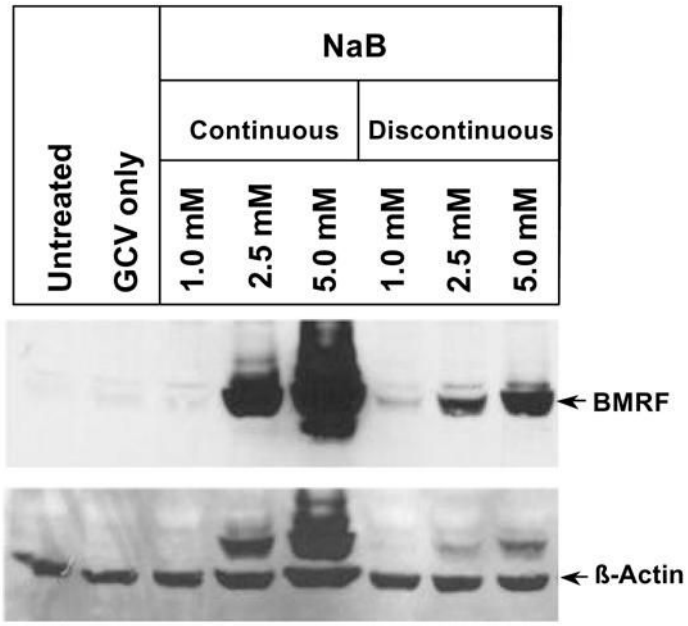

Figure 1.

Discontinuous butyrate exposure induces expression of the EBV early lytic phase protein BMRF. P3HR1 cells were treated with 1.0 mM, 2.5 mM or 5.0 mM butyrate for a 6 hr period daily for three days (discontinuous) and whole cell lysates from these cells were tested for EBV early antigen diffuse protein BMRF by immunoblotting. Untreated cultures, cultures treated with GCV alone and cultures exposed to the same concentrations of butyrate as above but continuously for three days, were used as controls. The same membrane was stripped and immunoblotted for ßactin protein to demonstrate equal loading of protein in the gel.

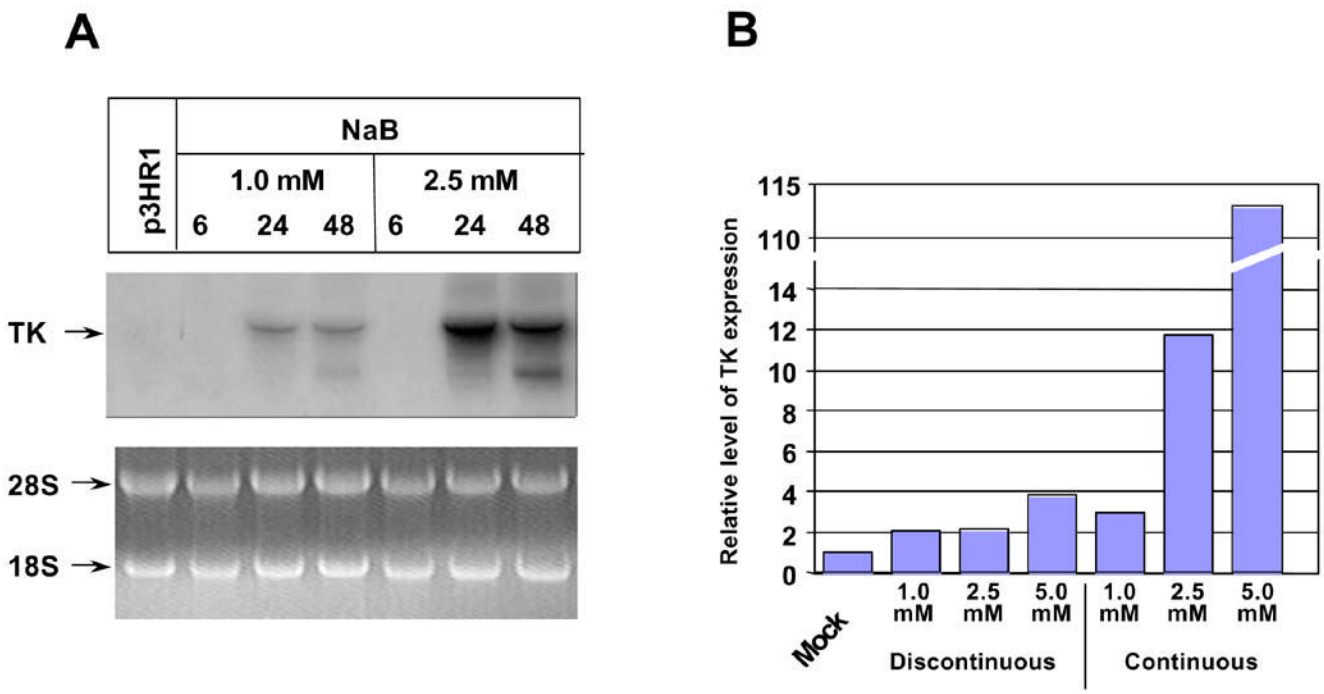

Figure 2.

Discontinuous butyrate exposure induces expression of viral thymidine kinase. **A**. Total cellular RNA from P3HR1 cells treated with 1.0 mM or 2.5 mM butyrate for the indicated time was analyzed by RNA blot analysis, using a ^{32}P-labeled thymidine kinase cDNA probe. The ethidium bromide stained gel before transfer is shown in the lower panel to demonstrate equal loading. Positions of the thymidine kinase mRNA (TK), 28S and 18S ribosomal RNAs are indicated. **B**. Real-time PCR analysis of thymidine kinase gene expression in P3HR1 cells treated discontinuously or continuously with butyrate at the indicated concentrations for three days. ßactin expression levels in the same cDNA amplification products were determined and used to normalize thymidine kinase expression level in each individual sample.

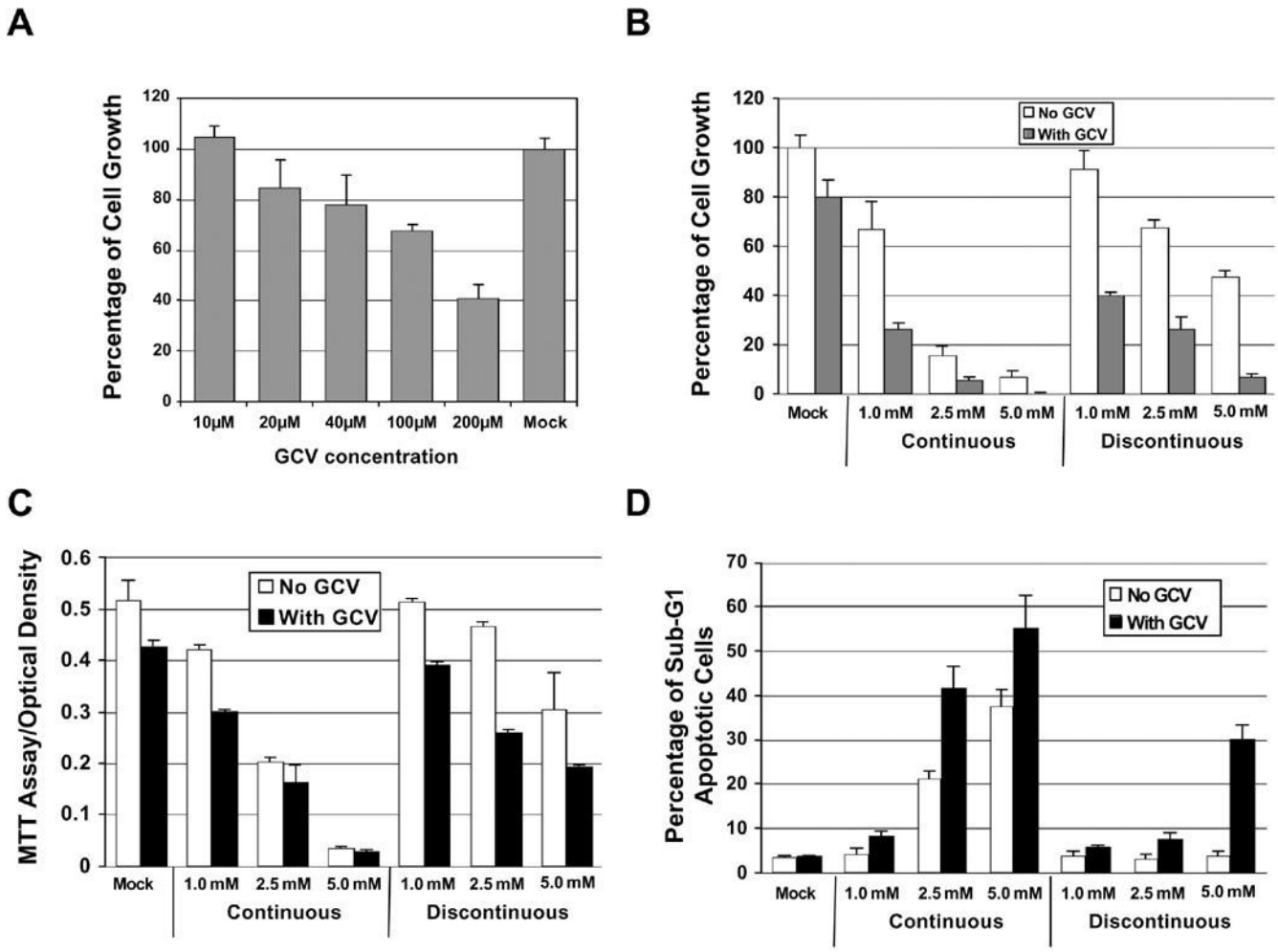

Figure 3.

Ganciclovir reduces growth of EBV-positive lymphoid cells treated discontinuously with butyrate. **A.** Dose-response tolerance analysis of P3HR1 cells to ganciclovir (GCV). Survival of P3HR1 cells exposed to ganciclovir for six consecutive days was measured by trypan blue dye-exclusion method. **B.** Survival of the P3HR1 cells treated discontinuously or continuously with butyrate at the concentrations indicated, in presence of 60 μM ganciclovir, was monitored by serial enumeration of trypan blue dye-excluding cells. **C.** Cell proliferation of P3HR1 cells treated as in panel B was assayed by colorimetric measurement of formazan dye (MTT assay). D. Apoptosis analysis of P3HR1 cells treated as in panel B by flow-cytometric analysis of sub-G_1 DNA content of the propidium iodide-stained cells. Error bars represent standard deviations from three independent experiments.

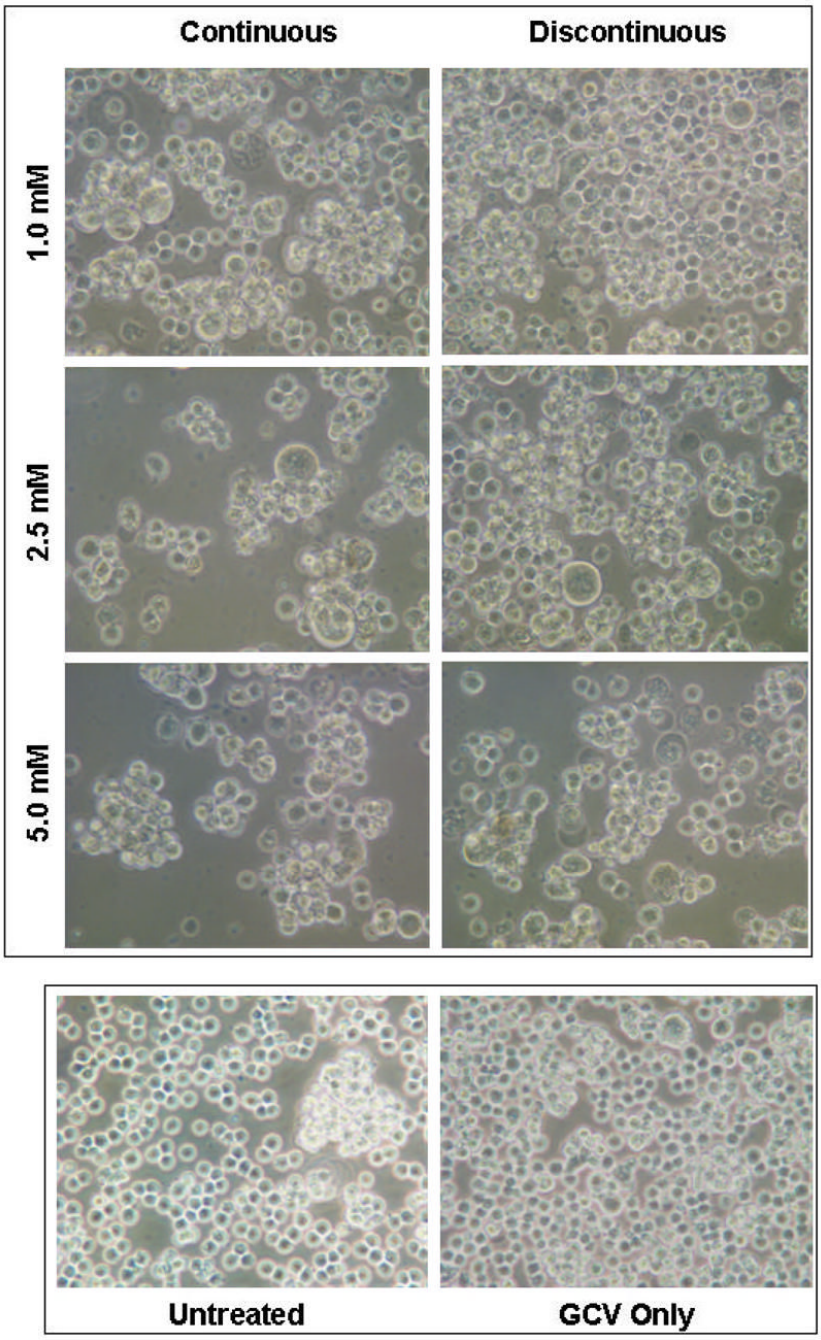

Figure 4.

Butyrate exposure in presence of ganciclovir affects the morphological integrity of EBV-positive lymphoid cells. P3HR1 cells were treated intermittently or continuously with butyrate at the indicated concentrations for three days in presence of 60 μM ganciclovir and three additional days in presence ganciclovir alone before the cellular morphology was analyzed by phase-contrast microscopy of living cell cultures.

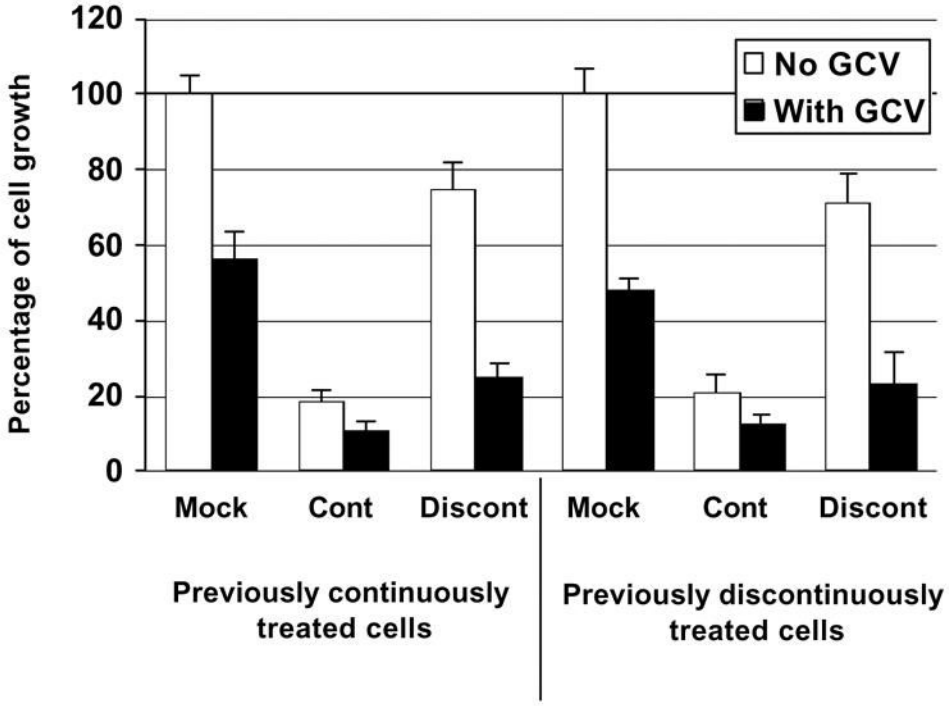

Figure 5.

EBV-positive lymphoid cells surviving an earlier cycle of butyrate/ganciclovir combination treatment remain susceptible to further rounds of treatment. P3HR1 cells surviving an initial round of continuous or intermittent butyrate treatment (2.5 mM) in presence of 60 μM ganciclovir were allowed to expand in normal growth medium, and then challenged with another round of combination treatment with 2.5 mM butyrate and 60 μM ganciclovir as in Figure 3, panel B. Cell survival was analyzed by enumeration of trypan blue dye-excluding cells. Error bars represent standard deviations from three independent experiments.

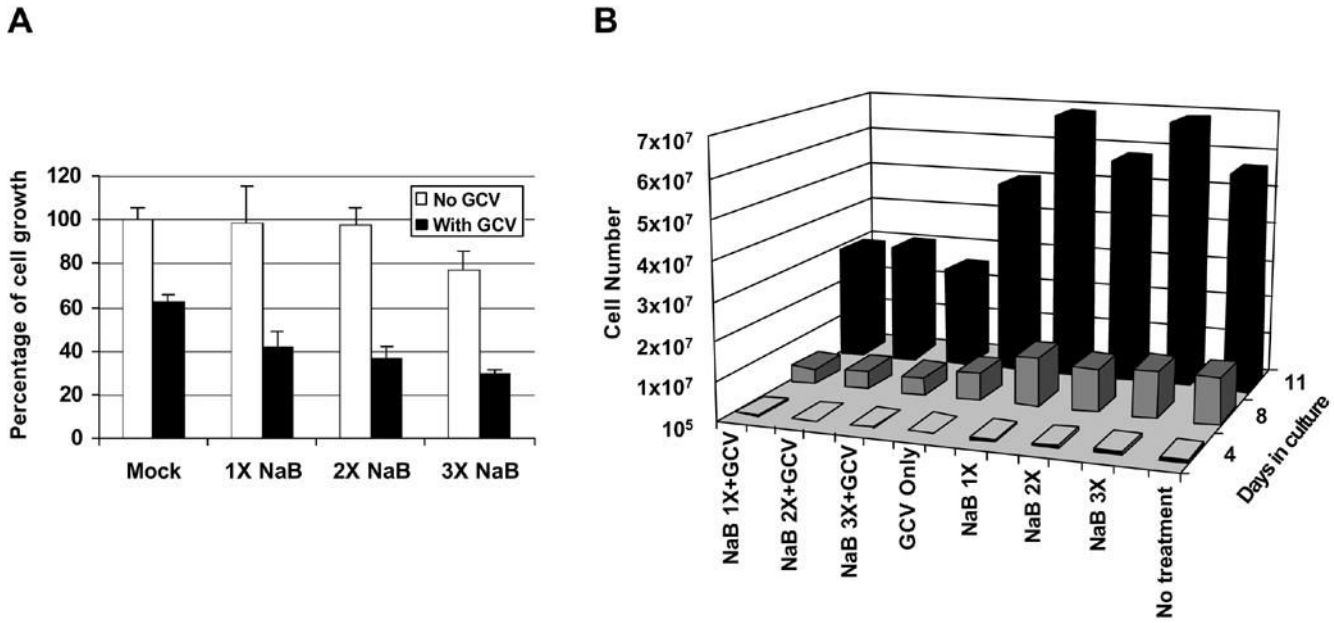

Figure 6.

Comparison of efficiency of sensitization of lymphoid cells to ganciclovir by single or multiple short exposures to butyrate. **A.** P3HR1 cells were treated with 2.5 mM butyrate for one, two or three six hour periods over one, two or three days in presence of 60 μM ganciclovir as in Figure 3, panel B, and cell survival were analyzed by serial enumeration of trypan blue dye-excluding cells. **B.** One hundred thousand cells from each treatment group were then replated into fresh growth media without ganciclovir or butyrate, and their growth kinetics were monitored for next eleven days.