Induction of human herpesvirus-8 DNA replication and transcription by butyrate and TPA in BCBL-1 cells

Yimin Yu,1 Jodi B. Black,2 Cynthia S. Goldsmith,2 Philip J. Browning,3 Kapil Bhalla1,4 and Margaret K. Offermann1,4

1 Winship Cancer Center, Emory University, 1365-B Clifton Road NE, Atlanta, GA 30322, USA
2 Centers for Disease Control and Prevention, Atlanta, GA, USA
3 Vanderbilt Cancer Center, Vanderbilt University, Nashville, TN, USA
4 Division of Hematology/Oncology, Department of Medicine, Emory University, Atlanta, GA 30322, USA

Human herpesvirus-8 (HHV-8) is a gammaherpesvirus that is present primarily in a state of low level persistence in primary effusion lymphoma cell lines. Using BCBL-1 cells that harbour HHV-8 but lack Epstein–Barr virus, we demonstrate that sodium butyrate is much more effective than the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) at inducing high levels of class II and III virus transcription and viral DNA replication, but also initiates apoptosis. Apoptosis occurs prior to assembly of virions when high concentrations of butyrate (1–3 mM) are used, whereas reduction of butyrate concentration to 0–3 mM decreases the rate of apoptosis and results in production and secretion of enveloped virions that are visualized at high number by electron microscopy in approximately 20% of BCBL-1 cells. Butyrate induces much higher levels of multiple class II and class III transcripts than does TPA, including v-MIP I, v-IL-6, v-Bcl-2, vGPCR and ORF26. A decrease in concentration of butyrate from 3 to 0–3 mM delays the peak induction of these genes, but peak levels remain higher than peak levels in response to TPA. These studies indicate that the massive apoptosis induced by 3 mM butyrate could be diminished and delayed by reduction of butyrate concentration to 0–3 mM, thereby allowing expression of high levels of lytic-associated genes and production of high yields of HHV-8 virions.

Introduction

Human herpesvirus-8 (HHV-8), also referred to as Kaposi’s sarcoma-associated herpesvirus, is a gammaherpesvirus that was originally identified in Kaposi’s sarcoma (KS) lesions from human immunodeficiency virus (HIV)-infected individuals and has since been found in KS lesions from all known risk groups (Chang et al., 1994, 1996; Chang & Moore, 1996; Offermann, 1996; Rady et al., 1995). HHV-8 is also present primarily in a latent state or in a state of low level persistence in primary effusion lymphoma cells (Arvanitakis et al., 1996; Cesman et al., 1995b; Gaidano et al., 1996). These cells are derived from a rare HIV-associated lymphoma that is characterized by the development of lymphomatous effusions with little lymph node involvement (Cesman et al., 1995a). Although HHV-8 is difficult to culture in cells derived from KS lesions, HHV-8 can be maintained and induced to lytic replication in some cell lines derived from primary effusion lymphomas (Arvanitakis et al., 1996; Gaidano et al., 1996; Miller et al., 1996, 1997; Renne et al., 1996b). Although many primary effusion lymphoma cells are co-infected with Epstein–Barr virus (EBV), some lines, such as BCBL-1 cells, are infected only with HHV-8 (Renne et al., 1996b). The presence of HHV-8 in all reported cases of primary effusion lymphoma suggests that HHV-8 may be essential for the pathogenesis of this rare lymphoma.

In cells derived from primary effusion lymphomas, HHV-8 DNA appears to be predominantly in the nucleus in episomal structures (Renne et al., 1996a). The HHV-8 genome consists of a 140-kb unique coding region flanked by multiple GC-rich 801 bp terminal repeat sequences (Russo et al., 1996). At least 81 open reading frames (ORFs) have been identified in the HHV-8 genome, and most of the viral genes have sequence and positional identity to known gammaherpesvirus genes (Moore et al., 1996a, b; Russo et al., 1996). In addition, some
ORFs encode proteins that are homologous to cellular proteins, including Bcl-2, Flice inhibitory protein (FLIP), interleukin-6 (IL-6), G-protein-coupled receptor (GPCR), cyclin D, interferon-regulatory factor and macrophage inflammatory proteins (MIP) (Moore et al., 1996a; Russo et al., 1996). All of these genes are located in non-conserved interblock regions of the HHV-8 genome (Russo et al., 1996).

Although HHV-8 is primarily latent in most primary effusion lymphoma cell lines, lytic replication can be induced by either the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) or by butyrate, agents that differ in mechanism of action. Butyrate, but not TPA, preferentially induces replication of HHV-8 over that of EBV, whereas TPA preferentially induces EBV replication in BC-1 cells, a cell line that is dually infected with HHV-8 and EBV (Miller et al., 1997). Despite the greater responsiveness of HHV-8 to butyrate than to TPA in BC-1 cells, a classification system for HHV-8 gene transcription was recently developed based on patterns of responsiveness to TPA in BC-1 cells (Sarid et al., 1998). By this system, class I transcripts are those constitutively expressed and not induced to higher levels by TPA and presumably represent latent transcription; class II transcripts are expressed constitutively but are also induced to higher levels by TPA; class III transcripts are those that are not constitutively expressed but are induced to high levels by TPA, presumably representing genes responsible for lytic replication.

In the current studies, we examine changes in DNA replication, viral gene expression and virus production in response to butyrate and TPA in BCBL-1 cells, a cell line infected with HHV-8 but not with EBV. This cell line is known to undergo lytic replication and production of virions in response to TPA (Renne et al., 1996b). We demonstrate that butyrate is much more effective than TPA at inducing HHV-8 DNA replication and expression of class II and class III genes. Butyrate, but not TPA, also induces apoptosis in a dose-dependent manner. Although apoptosis occurs before virus assembly in cells treated with high concentrations of butyrate, the rate of apoptosis decreases when lower concentrations of butyrate are used, and the enhanced cell survival facilitates secretion of large numbers of enveloped HHV-8 virus.

**Methods**

**Cell culture.** BCBL-1 cells (NIH AIDS Research and Reference Program, Rockville, MD, USA) were cultured in RPMI 1640 supplemented with 10% foetal calf serum, 2 mM l-glutamine, 100 U penicillin and 100 U streptomycin. Cell density was maintained between 5 × 10^5 and 10 × 10^5 cells/mL. At time 0, cells were collected by centrifugation at 1500 rpm for 5 min and suspended in fresh medium at a density of either 2 × 10^6/mL or 5 × 10^6/mL; equal aliquots were then incubated with either standard medium or medium supplemented with TPA (Sigma) or sodium butyrate.

**RNA isolation and Northern blotting.** Total cellular RNA was prepared using RNAzol B (Tel-Test) according to the manufacturer’s recommendation. For Northern blot analysis, RNA (20 µg) was size-fractionated on a 1% agarose-formaldehyde gel in the presence of 1 µg/mL ethidium bromide (Selden, 1987). The RNA was transferred to nitrocellulose and covalently linked by both baking in vacuo for 2 h at 80°C and ultraviolet irradiation using an ultraviolet cross-linker (Stratalinker, Stratagene). Hybridizations were performed at 42°C overnight in 5 × SSC (1 × : 150 mM NaCl, 15 mM sodium citrate), 1% SDS, 5 × Denhardt’s solution, 50% formamide, 10% dextran sulfate and 100 µg/mL sheared denatured salmon sperm DNA. Approximately 1–2 × 10^6 c.p.m./ml labelled probe (spec. act. 10^6 c.p.m./µg DNA) was used in each hybridization. Following hybridization, membranes were washed with a final stringency of 0.2 × SSC in 0.1% SDS at 55°C. The nitrocellulose was stripped using boiling water prior to rehybridization with other probes. To avoid carryover contamination, membranes were checked with a Geiger counter to ensure complete stripping and then rehybridized with probe detecting transcripts with different size. Autoradiography was performed with an intensifying screen at ~70°C. Quantification was done with a scanning densitometer.

**32P-labelling of probes.** DNA probes were labelled with 32P using oligolabelling kits (Pharmacia Biotech) according to the manufacturer’s recommendations. For v-cyclin, v-GPCR and v-MIP-I, the probes consisted of the entire coding regions (Russo et al., 1996). For v-Bcl-2, PCR using primers described by Moore et al. (1996a) were used to amplify fragments that were then radiolabelled with the oligolabelling kit. The HHV-8 ORF26 transcript was detected by using a labelled 930 bp amplifier of ORF26 as described (Offermann et al., 1996).

**DNA preparation and Southern blotting.** Approximately 5 × 10^6 cells for each condition were lysed in 3 ml 2× lysis buffer (40 mM Tris–HCl pH 7, 20 mM EDTA, 200 mM NaCl, 1% SDS) supplemented with 100 µg/ml protease K and then incubated at 65°C for 30 min followed by an overnight incubation at 37°C. Cell lysates were extracted twice with phenol. DNA was precipitated in 70% ethanol. DNA pellets were dissolved in 400 ml TE and RNase A was added to a final concentration of 100 µg/ml. Following incubation at 37°C for 1 h, RNase A was removed by phenol extraction. After precipitation with 70% ethanol, DNA pellets were dissolved in TE. DNA samples were digested with restriction enzymes, and DNA was separated by 1% agarose gel and transferred to nitrocellulose membrane. Cross-linking and hybridizations were described as for Northern blot analysis.

**Extracellular HHV-8 virion preparation.** BCBL-1 cells were pelleted by centrifugation at 400 g for 5 min. The cell-free culture medium was collected and centrifuged at 3500 g for 30 min to remove cellular debris, and virus was then pelleted from the medium by centrifugation at 15,000 g for 4 h. Pellets were resuspended in resuspension buffer (40 mM Tris–HCl pH 7, 10 mM NaCl, 6 mM MgCl_2, 10 mM CaCl_2). DNase and RNase (20 U) were added followed by incubation at 37°C for 1 h to eliminate free DNA and RNA. DNase was inactivated by incubating at 65°C for 10 min. An equal volume of lysis buffer supplemented with protease K (100 µg/ml) was added, and the solution was incubated at 65°C for 30 min followed by 37°C overnight. After phenol–chloroform extraction and ethanol precipitation, DNA pellets were dissolved in TE and subjected to restriction digestion and Southern blotting.

For detergent sensitivity, virus pellets were resuspended in TE with or without NP-40 at a final concentration of 1%, and the mixture was incubated at 37°C for 30 min. Proteinase K was added to a final concentration of 0·5 mg/ml and incubated at 37°C for 30 min. MgCl_2 was added to a final concentration of 1 mg/ml. Reactions were incubated at 65°C for 30 min followed by 37°C overnight. DNA was then isolated and analysed as described above.
Determination of cell number and cell viability. Changes in cell number were determined by counting with a haemocytometer at the indicated days. No additions or changes were made to the medium after day 0. Percentage viability was determined by assessing the percentage of cells that were able to exclude 0.1% trypan blue.

Electron microscopy. Thin section electron microscopy was performed as previously described (Goldsmith et al., 1995).

Flow cytometric analysis of apoptotic cells. After incubation with or without butyrate or TPA, $1 \times 10^6$ cells from each condition were fixed and assayed for DNA fragmentation using the APO-Direct kit (Pharmingen) according to the manufacturer’s recommendation. This assay is based on the labelling of DNA fragments found in apoptotic cells using terminal deoxytransferase enzyme with fluorescein isothiocyanate-conjugated dUTP (FITC-dUTP). Flow cytometric analysis was done analysing 10,000 cells using a FACScan (Becton Dickinson).

Results

Induction of HHV-8 DNA replication by TPA and butyrate

A range of concentrations of TPA and butyrate were examined for their ability to induce HHV-8 DNA replication in BCBL-1 cells. When examined at 2 days, TPA at concentrations ranging from 20 to 200 ng/ml led to twofold induction of intracellular HHV-8 DNA compared to control (Fig. 1 A). The increase in response to 0-3 mM butyrate was comparable to the increase that resulted from TPA, whereas 3 mM butyrate led to a greater than sixfold increase. When examined at 5 days, the increases in intracellular viral DNA in response to TPA (20–200 ng/ml) and butyrate (0-3 mM) were at least seven- and ninefold greater, respectively, in comparison to control cells (Fig. 1 B). Cells incubated with 3 mM butyrate suffered massive cell death, precluding analysis of intracellular HHV-8 DNA under this condition.

We next compared the ability of butyrate and TPA to induce virus production and secretion. Cell-free culture medium from control BCBL-1 cells and from cells incubated with TPA or butyrate for 5 days were assessed for the presence of viral DNA. Comparable amounts of DNase-resistant HHV-8 DNA were present in medium from BCBL-1 cells incubated with TPA at either 20 or 200 ng/ml, whereas incubation with 0-3 mM butyrate led to higher amounts of DNase-resistant HHV-8 DNA than resulted from incubation with TPA at any concentration (Fig. 1 C). In contrast, massive cell death occurred in response to incubation with 3 mM butyrate, and conditioned medium from these cells contained only trace DNase-resistant HHV-8 DNA. The HHV-8 DNA that was induced by 0-3 mM butyrate was protected from DNase digestion by both protein and lipid. The DNA remained resistant to DNase in the presence of proteinase K unless it was also treated with NP-40 (Fig. 1 D); this is indicative of enveloped virus. The production of HHV-8 virions by BCBL-1 cells in response to incubation with 0-3 mM butyrate for 5 days was confirmed by electron microscopy. Viral nucleocapsids accumulated within a dense granular material in cell nuclei (Fig. 2 A). Also present in some cells were nuclear inclusions of viral core-like material, marginated chromatin and a duplication of the nuclear membrane. Nucleocapsids passed through the perinuclear space into the cytoplasm and appeared to acquire their envelope by budding upon the membranes of the Golgi apparatus or the plasma membrane. Mature particles contained a layer of tegument between the nucleocapsid and the envelope (Fig. 2 B). Virus particles were detected in approximately 20% of cells (data not shown).

Toxicity in response to butyrate and TPA in BCBL-1 cells

Although butyrate enhanced HHV-8 DNA replication in a dose-dependent manner, it was extremely toxic to cells when used at high concentrations. Analysis of changes in cell number and viability were undertaken to characterize these effects. Cell number increased linearly for control cells between days 1 and
Fig. 2. Electron microscopy of HHV-8 virions in BCBL-1 cells incubated with 0–3 mM butyrate for 5 days. (A) HHV-8 nucleocapsids (filled arrows), viral core-like material (open arrows) and duplicated nuclear membranes (curved arrows) are present in the nucleus of this infected cell. Extracellular particles surround the cell (arrowheads). Bar, 1 µm. (B) Extracellular HHV-8 particles consist of the nucleocapsid (filled arrow), surrounded by the tegument (curved arrow) and viral envelope (arrowhead). Note that the tegument apparently adheres to the viral envelope in some particles, and that the envelope is sometimes ‘tailed’. Bar, 100 nm.

4, whereas butyrate at either 3 mM or 1.5 mM completely prevented the increase in cell number that occurred in control cells (data not shown). Both TPA (20 ng/ml) and butyrate at 0.3 mM decreased the rate of increase in cell number of BCBL-1 cells, so that after 4 days of culture, cell number was 60% lower in cells incubated with either agent compared to control BCBL-1 cells. Despite the comparable increase in cell number, incubation with butyrate, but not with TPA, compromised cell
HHV-8 induction by TPA and butyrate

Induction of HHV-8-encoded mRNAs by TPA and butyrate

The kinetics and magnitude of induction of HHV-8-encoded genes by TPA and butyrate were compared. When concentrations of TPA and butyrate that were associated with peak responses were used, peak levels of the class II transcripts v-MIP I, v-IL-6 and the class III transcripts v-GPCR and v-Bcl-2 were at least fourfold higher in response to butyrate than in response to TPA (Fig. 4A). Furthermore, peak levels occurred by 24 h in response to butyrate but did not occur until 48 h in response to TPA. The class I transcript, v-cyclin, was detectable in unstimulated cells and also showed a minor transient increase in response to both TPA and butyrate. The kinetics and magnitude of the induction of v-cyclin mRNA differed dramatically from the class II and III transcripts, with induction peaking by 4 h in response to both TPA and butyrate and declining more rapidly in response to butyrate than to TPA. Induction of ORF26, a class III transcript expressed late in the lytic phase, occurred much later than any of the other transcripts examined. Elevated levels were detectable after 36 h incubation with either butyrate or TPA, and the levels were more than tenfold higher in response to butyrate compared to TPA. Only the most abundant transcript for each probe is shown in Fig. 4(A). Less abundant, higher molecular mass transcripts consistent with known polycistronic patterns of gene expression were also detected with probes for v-cyclin, v-MIP I, v-GPCR, v-Bcl-2 and ORF26, several of which are shown in Fig. 4(C). In general, changes in the higher molecular mass forms were similar to changes in the major transcripts shown in Fig. 4; however, detailed analysis of these was not undertaken to examine possible changes in mRNA processing. The cellular gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control and showed a decrease in expression in response to butyrate but not to TPA.
Fig. 4. Induction of HHV-8 mRNA expression by TPA or sodium butyrate. BCBL-1 cells were incubated under control conditions or with TPA or butyrate for the indicated times. Total cellular RNA (20 µg) was size-fractionated and analysed by Northern blot analysis as described in Methods. The blot was serially probed for the indicated genes. The dominant band for each of the genes indicated is shown in (A) with its estimated molecular mass. The time-course of induction using concentrations of TPA (20 ng/ml) and butyrate (3 mM) associated with peak responses is shown in (A). Dose-response analysis at 24 h is shown in (B). An analysis at late time-points is shown (C) in response to a low concentration of butyrate (0.3 mM) compared to the concentration of TPA that leads to peak induction (20 ng/ml).

The kinetics of induction of class II and III transcription, but not the magnitude of response, were dramatically altered when the concentration of butyrate was reduced from 3 mM to 0.3 mM. Neither v-GPCR nor v-Bcl-2 was induced by 0.3 mM butyrate at 24 h, whereas both of these mRNAs were induced to high levels of expression by 3 mM butyrate at the same time-point (Fig. 4B). Although no induction by 0.3 mM butyrate was seen at 24 h, high levels of v-GPCR and v-Bcl-2 were seen in response to incubation with 0.3 mM butyrate for 4 and 5 days (Fig. 4C). In contrast to butyrate, higher levels of TPA decreased the induction of v-GPCR (Fig. 4B). Furthermore, examination up to 5 days showed only a marginal induction of v-Bcl-2 by TPA at time-points when high levels of v-Bcl-2 were induced by both 3 mM and 0.3 mM butyrate (Fig. 4A and C, respectively). The late lytic gene ORF26 was also more effectively induced by butyrate than by TPA at all concentrations examined (Fig. 4A, C). The induction of ORF26 by butyrate occurred later in response to 0.3 mM compared to 3 mM, with high levels of induction in response to 0.3 mM butyrate not occurring until day 4 (Fig. 4C).

Discussion

In these studies, we demonstrate that butyrate at concentrations ranging from 0.3 to 3.0 mM induced much higher levels of transcription of HHV-8 class II and class III genes and greater amounts of DNA replication in BCBL-1 cells than did TPA at concentrations ranging from 20 to 200 ng/ml. Butyrate at 3 mM also induced massive apoptosis that occurred prior to the production of virions. A tenfold reduction in butyrate concentration to 0.3 mM delayed both apoptosis and the expression of HHV-8 class II and class III transcripts. Despite this delay, peak levels of class II and class III transcripts remained considerably higher and were associated with greater viral DNA production than occurred in response to TPA. Furthermore, the decrease in the rate and magnitude of apoptosis that occurred as a consequence of the reduction of butyrate concentration was associated with the secretion of mature, enveloped virus.

It is somewhat paradoxical that butyrate was the agent that induced both the highest levels of v-Bcl-2 and the greatest amount of apoptosis. Although v-Bcl-2 has been shown to suppress Bax-mediated toxicity in both human and yeast cells (Cheng et al., 1997; Sarid et al., 1997), the large increases in v-Bcl-2 mRNA in response to 3 mM butyrate occurred after the apoptotic pathway was well under way. A decrease in butyrate concentration to 0.3 mM dramatically reduced the rate of apoptosis, but it also delayed the induction of v-Bcl-2. Thus, much of the enhanced cell survival and virion production associated with the lower concentration of butyrate occurred prior to the induction of v-Bcl-2. Furthermore, cells incubated with TPA did not have any evidence of apoptosis until day 4, even though v-Bcl-2 mRNA levels were barely detectable. It is thus unclear whether the induced v-Bcl-2 had any role in the inhibition of apoptosis and production of virions in these cells. Our results with BCBL-1 cells are consistent with those seen in BC-1 cells, which also undergo more HHV-8 DNA replication in response to butyrate than in response to TPA (Miller et al.,...
1997). The majority of BC-1 cells incubated with 3 mM butyrate were reported to lyse prior to production of encapsidated HHV-8 virus, but it remains to be determined whether a dose response to butyrate occurs in BC-1 cells that is similar to the one we report in BCBL-1 cells.

The transient induction of v-cyclin that occurred in response to TPA and butyrate was unexpected. Previous reports on v-cyclin have reported that it is insensitive to induction with TPA when examined at 48 h; therefore, it is classified as a class I gene (Sarid et al., 1998). The induction of v-cyclin that we observed occurred early, and v-cyclin mRNA levels were at or lower than baseline by 12 h and 24 h in response to butyrate and TPA, respectively. Furthermore, the magnitude of the induction of this class I gene was low compared to the magnitude of induction of the class II and class III genes. Thus, our data are consistent with the classification of v-cyclin as a class I transcript. The 2.0 kb transcript seen in Fig. 4(A) with the v-cyclin probe is bicistronic and encodes both v-cyclin (ORF72) and v-FLIP (ORF71) (Rainbow et al., 1997). This transcript originates upstream of ORF73 (LANA) from the same promoter used for ORF73 and splices out the complete ORF73. Our Northern blots lacked the sensitivity to determine whether butyrate or TPA altered splice patterns for these transcripts, but no obvious changes in the transcripts hybridizing with the v-cyclin probes were detected to suggest alternative splicing. The v-cyclin was the only HHV-8 gene examined that showed less response to butyrate than to TPA, consistent with butyrate being a more effective inducer of lytic replication. It was nonetheless surprising that its levels did not decline when cells began to express class II and III transcripts.

These studies demonstrate that the toxicity associated with the use of butyrate in BCBL-1 cells can be largely overcome by a reduction in concentration to one that decreases the rate of apoptosis while maintaining the ability of butyrate to induce higher levels of class II and class III transcription than result from using TPA. The lower concentrations of butyrate delay the peak induction of class II and III transcripts but are nonetheless associated with higher peak levels than occur in response to TPA. These studies thus define conditions that overcome many of the difficulties associated with induction of lytic replication by butyrate and offer an alternative to TPA that may help for production of HHV-8 virions and for studies on changes from latent to lytic replication of HHV-8.

We would like to thank Dr Philip Pellet for helpful discussions and for critical review of the manuscript. This work was supported in part by the Winship Cancer Center of Emory University and by NIH grants RO1 CA67382 and P30AR42687.

References


associated herpesvirus (human herpesvirus 8) is encoded by orf73 and is a component of the latency-associated nuclear antigen. *Journal of Virology* 71, 5915–5921.


Received 19 June 1998; Accepted 2 September 1998