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Mechanism of action of glycyrrhizic acid in inhibition of Epstein-Barr virus replication in vitro

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Abstract

We report here that glycyrrhizic acid (GL), a component of licorice root (*Glycyrrhiza radix*), is active against EBV replication in superinfected Raji cells in a dose-dependent fashion. The IC_{50} values for viral inhibition and cell growth were 0.04 and 4.8 mM, respectively. The selectivity index (ratio of IC_{50} for cell growth to IC_{50} for viral DNA synthesis) was 120. Time of addition experiments suggested that GL interferes with an early step of EBV replication cycle (possibly penetration). GL had no effect on viral adsorption, nor did it inactivate EBV particles. Thus, GL represents a new class of anti-EBV compounds with a mode of action different from that of the nucleoside analogs that inhibit viral DNA polymerase.

Keywords: Glycyrrhizic acid; Epstein-Barr virus; Replication; Antigen synthesis

1. Introduction

Epstein-Barr virus (EBV) is best known as the causative agent of infectious mononucleosis. EBV is classically associated with two human malignancies, endemic Burkitt's lymphoma and nasopharyngeal carcinoma (NPC) (de Thé, 1982). In addition, lymphoma in immunocompromised patients, peripheral T-cell lymphoma, oral hairy leukoplakia, AIDS-related immunoblastic lymphomas, Hodgkin's lymphoma, gastric carcinoma, and AIDS-associated smooth-muscle-tumors (leiomyomas and leiomyosarcomas) are also etiologically linked to EBV (Raab-Traub, 1996). The increasing number of EBV-associated diseases emphasizes the long-term importance either of developing an effective vaccine that can protect against infection or of developing novel antiviral agents that can inhibit virus replication.

In previous years, we have shown that many nucleoside analogs selectively inhibit lytic EBV replication in vitro (Lin et al., 1983, 1984, 1985, 1987, 1988, 1991, 1992; Lin and Machida, 1988; Mar et al., 1995). However, these drugs have no effect in vitro on latent EBV infection, nor do they appear to be of much use in the control of persistent low-level infection in vivo (Pagano, 1995). We started searching for other antiviral agents active against EBV with the specific goal of identifying compounds that might have more prolonged and less cytotoxic effects than nucleoside analogs.

Screening of plant extracts for antiviral action has revealed that a component (glycyrrhizic acid) of licorice roots (*Glycyrrhiza radix*) is active against a variety of viruses including herpes simplex type 1 (HSV-1), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), hepatitis A, B, and C viruses (HAV, HBV, and HCV), human immunodeficiency virus-1 (HIV-1), and influenza virus (Pompei et al., 1979; Baba and Shigeta, 1987; Numazaki et al., 1994; Crance et al., 1994; Sato et al., 1996; Arase et al., 1997; Ito et al., 1987, 1988; Utsunomiya et al., 1997). Licorice extract is an herbal drug that has long been used as a demulcent and elixir in Chinese medicine. In addition to antiviral activities, glycyrrhizic acid (GL) has been extensively studied in relation to various biological effects, such as anti-inflammatory activity (Finney and Somers, 1958) and interferon inducing ability (Shinada et al., 1986; Abe et al., 1982). Clinically, GL has been used to treat patients with chronic active hepatitis (Suzuki et al., 1983).

In view of this broad spectrum of antiviral activity, we decided to test the effects of GL on EBV DNA replication. The results clearly indicate that GL is active against EBV replication and its mode of action is different from that of the nucleoside analogs that inhibit viral DNA polymerase.

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2. Materials and methods

2.1. Cell cultures

A virus-nonproducer cell line (Raji) was maintained between 4×10^5 and 6×10^5 cells/ml in RPMI 1640 medium containing 10% fetal calf serum (FCS) supplemented with 100 IU penicillin per ml and 100 μg streptomycin per ml, as described previously (Lin et al., 1979).

Virus-producer (P3HR-1) cells were grown in the same medium as Raji cells.

2.2. Chemicals

Glycyrrhizic acid (GL) was purchased from Acros Organics (Pittsburg, PA). The stock solution was prepared by dissolving the drug in phosphate-buffered saline (PBS), pH 7.5 and diluted to the appropriate concentrations in the culture medium. Ganciclovir (GCV) and acyclovir (ACV), provided by Burroughs Wellcome Company (North Carolina), were dissolved in 10 mM Tris–HCl, pH 7.5. The chemical structure of glycyrrhizic acid is shown in Fig. 1.

2.3. Preparation of virus stocks

Viral stocks were prepared from cultures of P3HR-1 cells which had been treated with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) at a concentration of 30 ng/ml (Lin et al., 1979; Lin and Raab-Traub, 1987). Briefly, the P3HR-1 cells were grown in medium containing 10% FCS until a volume of 1 l containing 10^6 cells/ml was reached. The cultures were treated with TPA (30 ng/ml) for 14 days without additional medium. The cells were removed by centrifugation at 1200 × *g* for 10 min; virus was then pelleted from the supernatant fluids by centrifugation at 13,000 × *g* for 90 min in a GS-3 rotor (Ivan Sorvall). The centrifuge bottles were swabbed to remove residual medium and the virus pellets were suspended in 4 ml of RPMI 1640 medium without serum. The viral stocks were clarified to remove cellular debris by filtering through 1.2-, 0.8-, and 0.45-μm-pore-size filters. This 250-fold concentrated viral stock was stored at 4 °C until used.

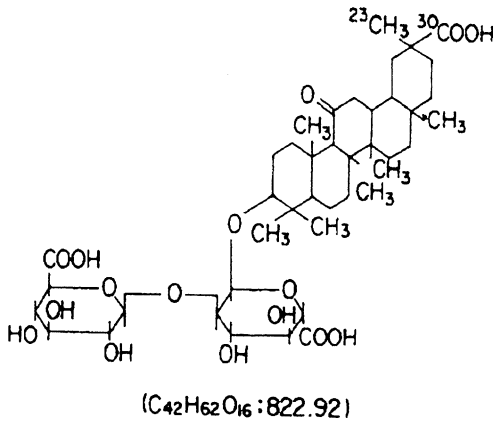

Fig. 1. The chemical structure of glycyrrhizic acid.

To prepare radioactively labeled virus, 50 μCi/ml of [^{3}H]thymidine was added to P3HR-1 cultures during TPA induction. The virus was harvested at 12 days after induction.

2.4. Superinfection of Raji cells

As an assay system we used Raji cells superinfected with P3HR-1 virus (Nonoyama and Pagano, 1972). For superinfection, 10^6 Raji cells in exponential growth were pelleted and suspended in 1 ml of RPMI 1640 medium containing 2% FCS and 10 U of early antigen (EA)-inducing virus. One unit of virus is defined by the amount of virus capable of inducing 10% of infected Raji cells expressing EA. After 1 h adsorption at 37 °C in a CO_2 incubator, the cells were pelleted, and suspended in 1 ml of the medium containing 2% dialyzed serum and drugs (time 0). At 6 h postinfection, 200 μCi of [^{3}H]thymidine was added, and incubation continued until 24 h after infection. Both superinfected and mock-infected Raji cells were harvested and DNA was isolated (Lin et al., 1982). EBV DNA replication was determined by measuring the radioactively labeled DNA after centrifugation on cesium chloride density gradient (Lin et al., 1982).

2.5. Indirect immunofluorescence assay

The synthesis of EBV EA and viral capsid antigen (VCA) was monitored by indirect immunofluorescence assay (Henle and Henle, 1966) at 24 h after infection using sera from NPC patients. Briefly, superinfected Raji cells were smeared on slides and fixed in methanol for 15 min at room temperature. The fixed cells were first reacted with NPC patients' serum, followed by fluorescein-conjugated goat anti-human IgG (H + L) (Santa Cruz Biotechonology, Inc.). After counterstaining with Evans blue, the slides were analyzed under the fluorescent microscope.

2.6. Cell growth and viability

P3HR-1 cells were treated with various concentrations of GL (1.2, 2.4, and 4.8 mM). The cell numbers were counted daily and viability was determined by trypan blue exclusion method.

2.7. Southern blot hybridization

Total cellular DNAs were isolated from control and drug-treated P3HR-1 and Raji cells (Lin et al., 1993), digested with *Bam*HI restriction enzyme, and electrophoresed on 1% agarose gel followed by Southern blotting onto a nitrocellulose sheet. After blotting, the DNA was UV cross-linked by a UV Stratalinker (Stratagene, La Jolla, CA), and baked in a vacuum oven at 80 °C for 30 min. The sheet was hybridized with an EBV-specific *Bam*HI-W fragment probe (Mar et al., 1995; Lin and Raab-Traub, 1987).

3. Results

3.1. Effects of GL on EBV DNA synthesis in superinfected Raji cells

To assess the effects of GL on EBV DNA synthesis, latently infected Raji cells were superinfected with P3HR-1 virus (Nonoyama and Pagano, 1972). The GL dose used was 1.2 mM, which had previously been shown to inhibit both HAV and HIV-1 replication (Crance et al., 1994; Ito et al., 1987). As a positive control, 3 μM of GCV, a nucleoside analogue, which is known to be effective against EBV DNA replication (Lin et al., 1984), was used. A typical result is shown in Fig. 2. In superinfected Raji cells, a large quantity of viral DNA was observed banding at a buoyant density of 1.718 g/cm^3, distinct from the cellular DNA banding at 1.698 g/cm^3 in uninfected cells. Viral DNA replication was greatly reduced in superinfected Raji cells treated with 1.2 mM GL or 3 μM GCV. Thus, GL clearly and effectively inhibited EBV DNA replication in this system.

3.2. Dose-dependent inhibition of EBV antigen expression

To determine the dose-dependent effect of GL, Raji cells were superinfected with P3HR-1 virus and GL was added immediately after EBV adsorption (post treatment). The expression of EA and VCA were monitored by indirect immunofluorescence (IF) 24 h after superinfection. Without GL treatment, approximately 89% of infected cells became positive for viral antigens. In the presence of GL, a dose-dependent inhibition of the expression of viral antigens was observed (Table 1). The concentration of GL required to inhibit EBV antigen expression by 50% (IC_{50}) was approximately 0.38 mM. To examine the effects of pretreatment,

Table 1
Effect of GL treatment on EBV EA/VCA synthesis in superinfected Raji cells

GL concentration (mM)	Percent of IF-positive cells[a]	
	Pretreatment	No pretreatment
0	89.5 ± 3.53	90 ± 2.12
0.006	67.5 ± 2.31	80 ± 1.41
0.06	38.5 ± 1.34	66 ± 2.23
0.3	14.5 ± 2.12	40.5 ± 1.54
0.6	0.9 ± 0.14	15 ± 1.41
1.2	0.4 ± 0.11	2.5 ± 0.56

[a] Prior to superinfection, Raji cells were untreated or pretreated with a sub-effective dose of GL (1 μM). The indicated concentrations of GL were then added immediately after virus adsorption. The synthesis of EA/VCA was monitored by indirect immunofluorescence (IF) assay at 24 h after infection. The expression of EA/VCA was not detectable in Raji cells without superinfection. The results were from two separate experiments with triplicate determinations.

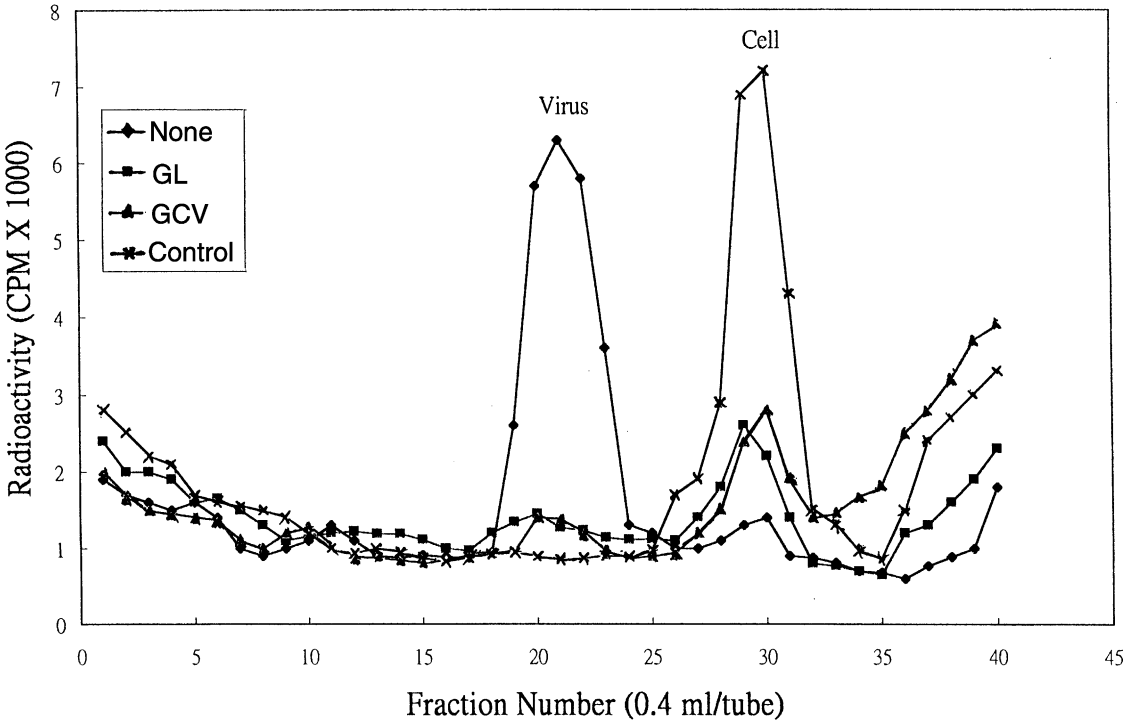

Fig. 2. Inhibition of EBV DNA replication by GL or GCV in superinfected Raji cells. Virus was prepared from TPA-induced P3HR-1 culture fluid (Lin et al., 1979). For superinfection, 10^6 Raji cells in exponential growth were pelleted and suspended in 1 ml of RPMI 1640 medium containing 2% FCS and 10 U of EA-inducing virus. After 1 h adsorption at 37 °C in a CO_2 incubator, the cells were pelleted, washed twice with medium, and suspended in 1 ml of the medium containing 2% dialyzed serum and drugs (time 0). At 6 h postinfection, 200 μCi of [^{3}H]thymidine was added, and incubation continued until 24 h after infection. EBV DNA replication was determined by measuring the radioactively labeled DNA after centrifugation on cesium chloride density gradient (Lin et al., 1982). (◆) No drug, (■) 1.2 mM GL, (▲) 3 μM GCV, and (×) control, no drug and no superinfection.

Table 2
Comparison of relative potency of GL against EBV, VZV, and HIV

Virus	Viral IC_{50} (mM)	Cell IC_{50} (mM)	Therapeutic index (cIC_{50}/vIC_{50})[a]
EBV[b]	0.04	4.8	120
VZV[c]	0.71	21.3	30
HIV[d]	0.15	2.6	17

[a] cIC_{50} and vIC_{50} denote cell IC_{50} and viral IC_{50}, respectively.
[b] The viral IC_{50} value was obtained by pretreatment of Raji cells with sub-effective dose of GL prior to superinfection.
[c] The viral IC_{50} value was determined in embryonic fibroblasts by plaque assay (Baba and Shigeta, 1987).
[d] The viral IC_{50} value was determined in MT-4 cells by plaque formation assay (Ito et al., 1987).

Raji cells were first propagated in medium containing a sub-effective dose of GL (1 μM). The pretreated cells were washed before superinfection and then various concentrations (0–1.2 mM) of GL were added immediately after virus adsorption, and EA/VCA-positive cells were assayed 24 h later. As shown in Table 1, pretreatment of Raji cells with the sub-effective dose of GL before virus inoculation produced a marked reduction of viral antigen expression. A dose-dependent inhibition was also observed, and the IC_{50} was estimated to be 0.04 mM, a dose approximately 10-fold less than that obtained without GL pretreatment.

For purpose of comparison, the inhibitory action of GL against EBV and other viruses such as HIV and VZV are summarized in Table 2.

3.3. Mode of GL action

To elucidate the mechanism of GL action, the following experiments were performed. To investigate whether GL interfered in virus adsorption, Raji cells were incubated with [^{3}H]thymidine-labeled virus in the presence or absence of GL. At different times after incubation, aliquots of cells were harvested, washed, and solubilized before counting bound radioactivity. The kinetics of adsorption of the [^{3}H]thymidine-labeled EBV to Raji cells in the presence or absence of GL are shown in Fig. 3. The amount of radioactive EBV associated with Raji cells as a function of time was similar in both cases, and the virus adsorption reached its peak at 60 min after inoculation. Therefore, GL did not inhibit virus adsorption.

Apart from inhibiting the growth of several viruses, GL also produces irreversible inactivation of HSV-1 (Pompei et al., 1979). To test whether GL had virucidal effects, EBV was first incubated at 37 °C with 2.4 mM GL for 4 h, washed, resuspended, and tested for infectivity. No significant reduction in virus infectivity was observed as determined by the IF test (data not shown). These results indicate that the antiviral activity of GL cannot be attributed to a direct inactivation of the virus.

Additional experiments were performed to study the effect of the time of addition or removal of GL on EBV antigen expression. The most effective time to add GL was immediately after virus adsorption. As the time of addition of GL was delayed, the antiviral activity gradually dimin-

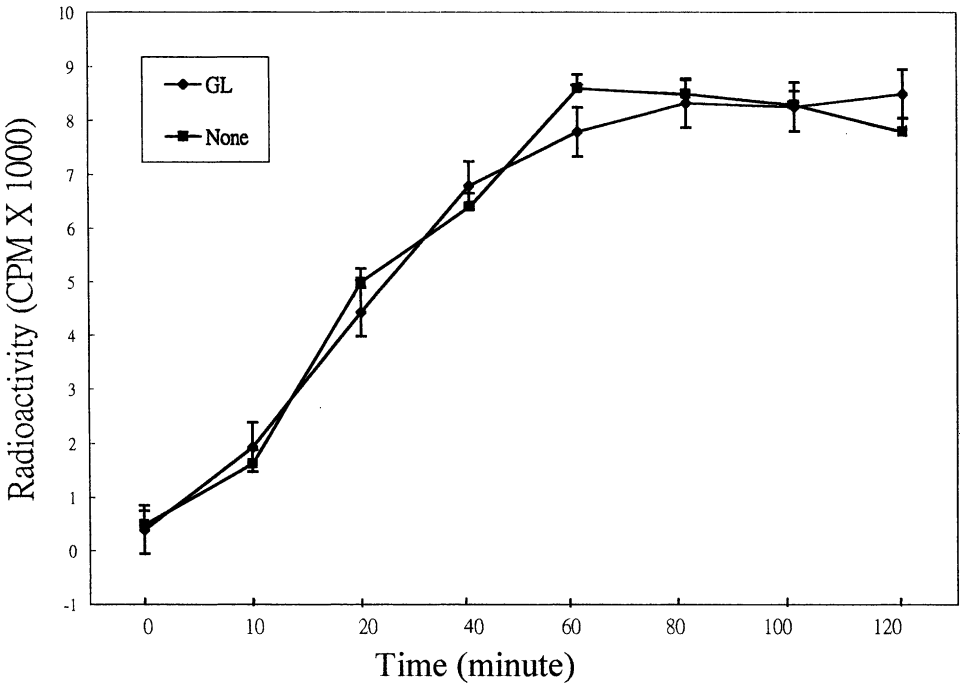

Fig. 3. Effect of GL on the adsorption of EBV to Raji cells. EBV was radiolabeled by adding 50 μCi/ml of [^{3}H]thymidine in P3HR-1 cultures during TPA induction. The virus was harvested at 12 days after induction (Lin et al., 1979). Raji cells (10^5) were incubated at 4 °C with [^{3}H]thymidine-labeled EBV (20,000 cpm) in the presence or absence of GL (2.4 mM). At the indicated times, the cells were washed four times with ice-cold PBS and lysed in 0.5 ml of 2% sodium dodecylsulfate to solubilize the cells for radioactive counting.

Table 3
Effect of time of addition and removal of GL on the synthesis of EBV EA/VCA in superinfected Raji cells

GL addition time (h)[a]	Percent of IF-positive cells[b]
0	0.7 ± 0.07
1	9.5 ± 1.41
2	30.5 ± 2.12
4	79 ± 2.82
6	86.5 ± 2.41
Control[c]	90 ± 2.47
GL removal[d]	1.5 ± 0.13

[a] GL (1.2 mM) was added immediately (0 h) or at the indicated times after virus adsorption. The percent of IF-positive cells was determined at 24 h after viral inoculation.

[b] The results were from two separate experiments with triplicate determinations.

[c] Superinfected Raji cells without GL treatment.

[d] GL was added immediately (0 h) after virus adsorption and incubated for 5 h. The cells were washed and incubated in drug-free medium until 24 h post-infection.

ished (Table 3). The inhibitory effect of GL on EBV antigen synthesis was completely lost when the drug was added at 6 h post infection or thereafter. When GL was added at the time of EBV inoculation and removed 5 h after adsorption, its antiviral activity was not reversible. These results, taken together, indicate that one possible mode of action of GL is to selectively block the penetration of EBV into Raji cells, a process that occurs within the first 5 h after virus adsorption.

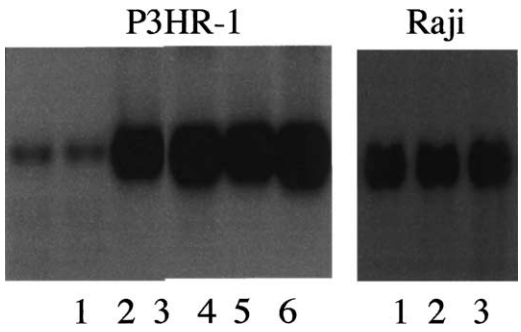

Fig. 4. Effects of GL and nucleoside analogs on EBV DNA replication. Both P3HR-1 and Raji cells were treated with two doses (0.6 or 2.4 mM) of GL for 14 days. As positive controls, cells were similarly treated with 3 μM GCV or 9 μM ACV for 14 days (Lin et al., 1984). *Bam*HI restriction enzyme digested genomic DNA was separated by agarose gel electrophoresis. The effects of the drugs on EBV DNA were determined by Southern blot hybridization using a *Bam*HI-W fragment as a probe. Left panel (P3HR-1): lane 1, 3 μM GCV; lane 2, 9 μM ACV; lane 3, no drug; lane 4, 0.6 mM GL; lane 5, 2.4 mM GL; lane 6, no drug. Right panel (Raji): lane 1, 0.6 mM GL; lane 2, 2.4 mM GL; lane 3, no drug.

3.4. GL has no effects on EBV DNA in P3HR-1 and Raji cells

The effects of GL on EBV DNA replication were also tested in a virus-producing cell line (P3HR-1) and a latently infected cell line Raji. To test the effect of GL in these cell lines, exponentially growing P3HR-1 and Raji cells were treated with 0.6 or 2.4 mM of GL for 14 days. As posi-

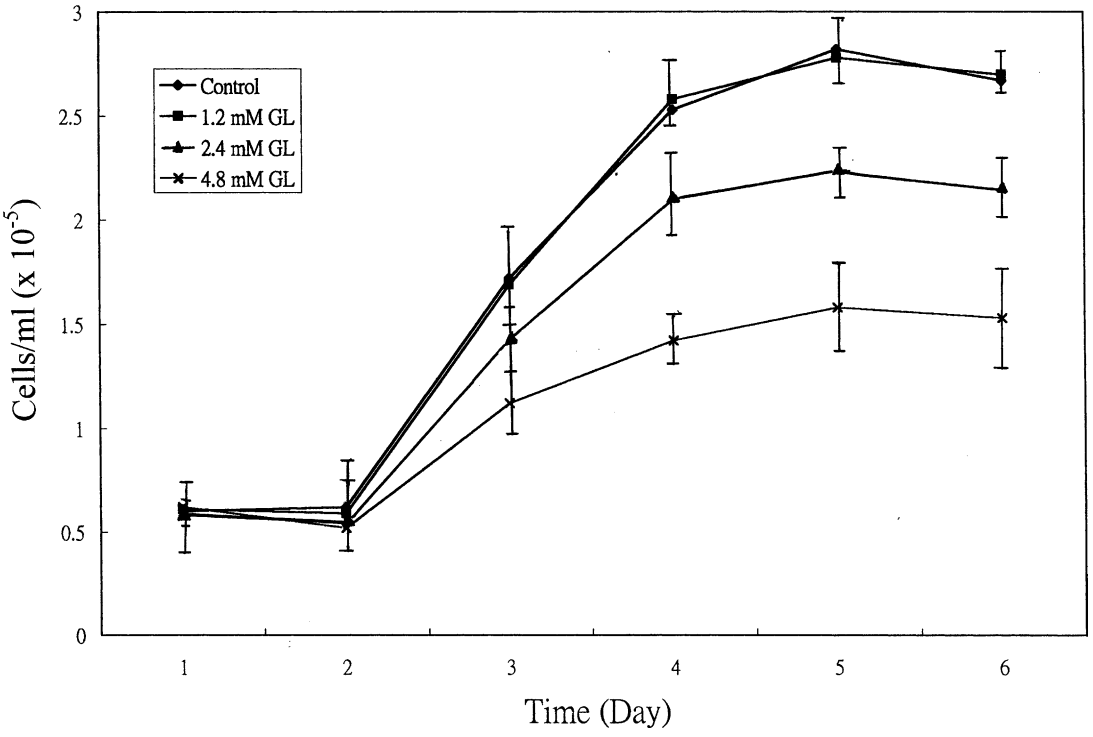

Fig. 5. Effects of GL on cell proliferation and viability. P3HR-1 cells were treated with 1.2, 2.4 and 4.8 mM of GL and cell numbers and viability were monitored daily for 5 days. The viability was maintained at 95% for all doses tested.

tive controls, cells were similarly treated with an effective dose of GCV (3 μM) or ACV (9 μM) (Lin et al., 1984). To examine the effect of GL on EBV DNA, genomic DNA was prepared and digested with *Bam*HI restriction enzyme, electrophoresed on an 1% agarose gel, and Southern blotted onto a nitrocellulose sheet. The sheet was hybridized with an EBV-specific *Bam*HI-W fragment probe (Mar et al., 1995). Fig. 4 shows that GL treatment had no apparent effect on EBV DNA load in P3HR-1 and Raji cells, in contrast to the markedly inhibitory effects exerted by GCV and ACV, whose molecular target is the virus-encoded DNA polymerase (Pagano, 1991).

3.5. *Cytotoxicity test*

The effects of GL on cell viability and proliferation were also determined (Fig. 5). The proliferation and viability of P3HR-1 and Raji cells were not affected by drug concentrations as high as 1.2 mM. At 2.4 mM GL, the total cell count after 5 days of cell growth was 20% below that of the control culture. However, at 4.8 mM GL, the total cell numbers were approximately 50% of the control culture. The viability of cells was maintained at 95%, indicating that GL was cytostatic but not cytotoxic at this dose. Thus, the IC_{50} for inhibition of cell growth was 4.8 mM.

4. Discussion

Evaluation of antiviral agents effective against EBV has been hampered by the lack of a permissive cell system for the replication of this virus. Superinfection of Raji cells with P3HR-1 virus results in shut down of host cell DNA synthesis and stimulation of viral DNA replication and lytic antigen synthesis and production of virus (Nonoyama and Pagano, 1972). It has been shown that the percentage of EA- and VCA-positive cells is directly proportional to copy number of EBV genomes (Pagano, 1991). Thus, superinfected Raji cells were used to evaluate the effect of GL on EBV replication. Superinfection of Raji cells with EBV has a somewhat variable effect on cellular DNA synthesis (see Fig. 1), and no conclusions about the effects of the drugs on cellular DNA synthesis can be reached for this system (Pagano, 1991).

Our results indicate that GL is highly selective in its antiviral action in that it inhibits EBV replication at a concentration (0.04 mM), far below the cytostatic concentration (4.8 mM). The selectivity index (ratio of IC_{50} for cell growth to IC_{50} for viral DNA synthesis under pretreatment conditions) was thus 120, which is two times higher than the selectivity index of AZT (67) (Lin et al., 1988), but two times lower than that of acyclovir (250) (Lin et al., 1984).

GL exerts a dose-dependent inhibitory effect on both EBV DNA replication and virus-antigen synthesis. The marked reduction of IC_{50} value obtained in pretreatment of cells with sub-effective dose of GL has clinical implication regarding to increasing GL potency. In order to elucidate the mechanism of inhibition, the effect of GL was studied under different conditions. We showed that the antiviral effect of GL could not be attributed to a direct inactivation of the virus, nor did it inhibit virus adsorption. Its mode of action appears to be at the step of virus penetration, an early stage of the EBV replicative cycle, the exact nature of which remains to be elucidated. Our results are in contrast to previous reports showing that GL has an inactivating effect on HSV-1 and VZV particles (Pompei et al., 1979; Baba and Shigeta, 1987). However, no such effect could be demonstrated with HIV infection (Ito et al., 1987). This difference in the mode of action may be related to the nature of each individual virus.

P3HR-1 cells spontaneously and continuously replicate large numbers of linear EBV genomes, synthesize EA and VCA, and make virus particles. The spontaneous induction of virus replication fluctuates cyclically between 3 and 10% of the cells under our culture conditions (Lin et al., 1979). In the latently infected nonvirus-producer Raji cells, the episomal form of EBV genome is stably maintained at a constant copy number (Pagano, 1991). Likewise, in the virus-producer line P3HR-1, the majority of latently infected cells also maintains EBV episomes (Lin et al., 1984). Interestingly, GL has no effects on EBV DNA in P3HR-1 and Raji cells. The lack of effect of GL in P3HR-1 cells, which are already infected and producing virus, is consistent with this proposed mechanism, i.e. inhibition of virus penetration.

The molecular target of all nucleoside analogs is the virus-encoded DNA polymerase (Pagano, 1991). Therefore, the mode of action of GL is different from that of nucleoside analogs. The therapeutic and prophylactic effects of GL on chronic active viral hepatitis (Fujisawa et al., 1980) and the inhibitory effect on EBV replication observed in the present study together with its relative lack of toxicity at the cellular level all suggest that GL should be further evaluated for its efficacy in the treatment of active EBV infections.

Acknowledgements

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