JOURNAL OF VIROLOGY, Feb. 1987, p. 607–610
0022-538X/87/020607-04$02.00/0

Vol. 61, No. 2

Differential Effects of Acyclovir and 9-(1,3-Dihydroxy-2-Propoxymethyl)Guanine on Herpes Simplex Virus and Epstein-Barr Virus in a Dually Infected Human Lymphoblastoid Cell Line

CHARLES M. VAN DER HORST[1,2]†* JUNG-CHUNG LIN,[2,3] NANCY RAAB-TRAUB,[2,4] M. CAROLYN SMITH,[2] AND JOSEPH S. PAGANO[1,2,4]

Departments of Medicine,[1] Biochemistry,[3] and Microbiology and Immunology,[4] and Lineberger Cancer Research Center,[2] School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

Received 13 June 1986/Accepted 22 October 1986

We investigated the effects of acyclovir and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) on a lymphoblastoid cell line dually infected with Epstein-Barr virus and herpes simplex virus (HSV) type 1. The numbers of Epstein-Barr virus genomes were reduced during 70 days of treatment with either drug. Both drugs suppressed HSV replication in a dose-related manner. In the continued presence of the drugs, HSV developed resistance, rapidly to acyclovir and much more slowly to 30 μM DHPG. Analysis of HSV glycoprotein C production and viral DNA showed that treatment with 100 μM DHPG eliminated HSV production, curing the cell line of HSV persistent infection.

Treatment of both herpes simplex virus (HSV) and Epstein-Barr virus (EBV) infections in humans with 9-(2-hydroxyethoxymethyl)guanine (acyclovir; ACV) is problematic since viral replication is only suppressed when the drug is present (5, 18); neither virus can be eradicated by treatment with ACV.

We studied the effects of ACV and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) on a human lymphoblastoid cell line dually infected with HSV type 1 and EBV (19, 21) during 70 days of therapy and up to 360 days after release from the drug. When initially characterized, less than 1% of the cells in this line were found, by infectious-center assay, to produce HSV, although 80% of the cells had HSV antigens (19). This cell system represents true HSV persistent infection, with levels of HSV in the supernatant fluid ranging from 10^6 to 10^7 PFU/10^6 cells, documented for years without spontaneous curing of either HSV or EBV infection. The disparity between the number of cells producing HSV and the number of cells with HSV antigens suggests that, analogous to clinical HSV infection, there are cells which are productive of HSV and cells in which the virus is persistent.

To determine the effect of the drugs on cell growth kinetics and virus production, equal numbers of viable cells were suspended in 30 μM ACV, 30 μM DHPG, 100 μM ACV, or 100 μM DHPG or were not treated. As shown in Fig. 1, 100 μm DHPG was toxic, with the number of viable cells determined by trypan blue exclusion dropping 10-fold below the number of viable untreated control cells by day 24 of treatment. With cells exposed to 30 or 100 μM ACV or 30 μM DHPG, their numbers increased fivefold over the number of control cells in the same period, presumably because of protection from HSV cytopathic effects.

The amount of infectious virus released into the supernatant fluid, as determined by plaque assay with Vero cells and an agarose overlay, fluctuated as a function of cell density (Fig. 2). Calculating HSV PFU per cell permitted comparison between different flasks. Within 10 days of treatment with 30 or 100 μM ACV, the numbers of HSV PFU were reduced 450- and 1,780-fold, respectively. DHPG at 30 μM had maximal effect at 14 days, with a 317,000-fold reduction of the HSV titer. This effect was confirmed by the reduction of the number of cells expressing glycoprotein C, a late viral gene product associated with HSV replication, from 22 to less than 10% of the cells by 14 days. Continued treatment of the cells with 30 or 100 μM ACV resulted in the generation of apparently drug-resistant HSV by day 24, as evidenced by the return of HSV titers to the control levels (Fig. 2). In contrast, it took 70 days for drug-resistant HSV to develop in cells treated with 30 μM DHPG.

Production of resistant HSV was confirmed by increases in the 50% effective dose (ED_{50}), calculated from a plot relating percent surviving plaques in a plaque reduction assay to the log of drug concentrations. HSV was cross

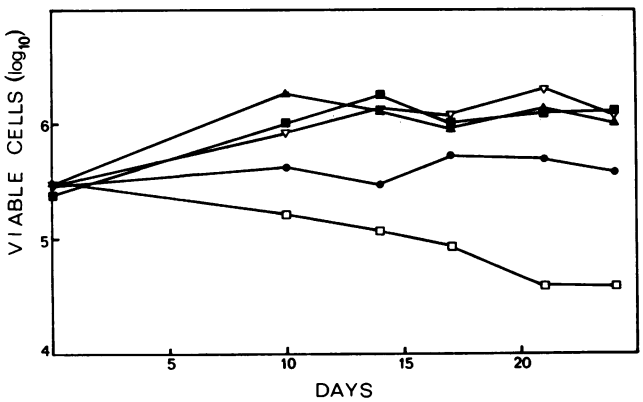

FIG. 1. Growth kinetics of dually infected cells treated with antiviral drugs. Symbols: ●, untreated cells; ▲, 30 μM ACV-treated cells; ▽, 100 μM ACV-treated cells; ■, 30 μM DHPG-treated cells; □, 100 μM DHPG-treated cells.

* Corresponding author.

† Present address: Division of Infectious Diseases, Veterans Administration Medical Center, Duke University Medical Center, Durham, NC 27705.

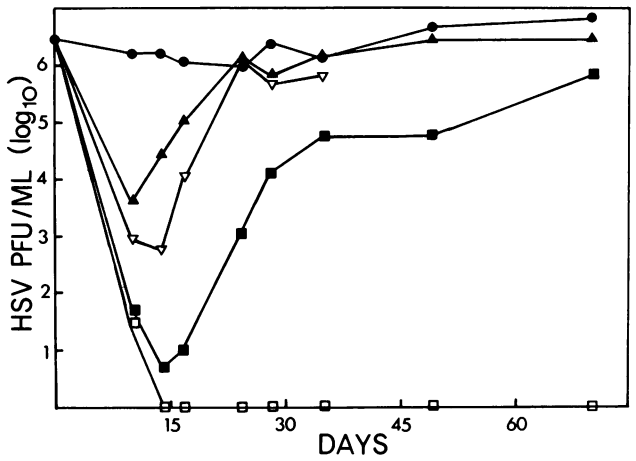

FIG. 2. HSV released by dually infected cells during drug treatment. Titers were normalized to 10^6 cells. Symbols: ●, untreated cells; ▲, 30 μM ACV-treated cells; ▽, 100 μM ACV-treated cells; ■, 30 μM DHPG-treated cells; □, 100 μM DHPG-treated cells.

resistant to DHPG and ACV, as is clearly shown in Fig. 3. The ED_{50} for HSV recovered from 30 μM ACV-treated cells increased from 3.5 to 250 μM when assayed in the presence of ACV and from 1.5 to 220 μM when assayed in the presence of DHPG. For virus from 100 μM ACV-treated cells, the ED_{50} rose to 100 μM in the presence of ACV and 150 μM in the presence of DHPG. The ED_{50} for HSV from cells exposed to 30 μM DHPG rose to 85 μM in the presence of ACV and 500 μM in the presence of DHPG. Development of resistant HSV was further confirmed by plaque autoradiography (16), which revealed that most virus produced reduced levels of thymidine kinase (TK) (data not shown).

Despite the increase in infectious virus released into the supernatant fluids, the percentage of cells producing glycoprotein C remained less than 10% for the 70 days of drug therapy, suggesting that fewer cells were producing more virus. Upon release from the drug, the percentage of cells making antigen gradually rose to control levels (data not shown).

Previous studies showed that resistant HSV develops on repeated passage in cell monolayers in the presence initially of up to 27 μM ACV through selection for resistance mediated by an altered TK or TK-negative state (4, 10, 20, 22). In contrast, the HSV in our persistently infected cell line was able to develop resistance when exposed to the much higher dose of 100 μM ACV. A persistently infected cell population could continue to generate mutants in the presence of the drug. Cross-resistance of HSV to DHPG and ACV (3) has occurred when the mechanism of resistance involved an altered TK or TK-negative condition, but cross-resistance was not an invariable consequence. The differences in the rapidity of development of drug resistance may indicate differences in the metabolism of DHPG and ACV (12). ACV triphosphate is a chain terminator (8), whereas DHPG triphosphate is incorporated internally into replicating HSV DNA (2). ACV triphosphate is believed to form an irreversible complex with HSV DNA polymerase (11).

No infectious virus was detected in 100 μM DHPG-treated cells beginning at 14 days and continuing throughout treatment and for 360 days after release of the cells from the drug (Fig. 2), nor was glycoprotein C found in the cells. Induction of HSV production was attempted in the cells which had been exposed to 100 μM DHPG by using 12-*O*-tetradecanoyl-phorbol-13-acetate (30 ng/ml) or 5-bromo-2-deoxyuridine (50 mg/ml). Despite an increase of EBV capsid antigen from 1 to 70%, no HSV was released into the supernatant fluid, nor was HSV glycoprotein C made.

In an effort to determine whether HSV sequences were retained in the cell line which had been exposed to 100 μM DHPG, DNA was isolated from cells 2 and 6 months after release from the drug. We were able to detect less than one copy of the HSV genome per cell in our control reconstructions. However, probes made with HSV *Eco*RI fragments F, A, L, M, O, and G and *Bam*HI fragment Q, representing 42% of the HSV genome, failed to disclose residual HSV DNA in cells 2 and 6 months after release from DHPG at our limits of sensitivity (Fig. 4). All probes had a specific activity of 2×10^7 to 9.9×10^8 cpm/μg. Since HSV was continuously produced by the cell line for years before treatment with 100 μM DHPG, it is likely that this result was directly due to drug exposure. One possible explanation for this cure is that cells infected with HSV are more susceptible to the toxic effects of DHPG. Another possibility is that, unlike EBV, perhaps all of the HSV present in the cells is replicated by viral DNA polymerase, and treatment with 100 μM DHPG was able to eradicate the HSV infection.

During drug treatment, the effects of DHPG and ACV on EBV genomes in dually infected cells were determined by

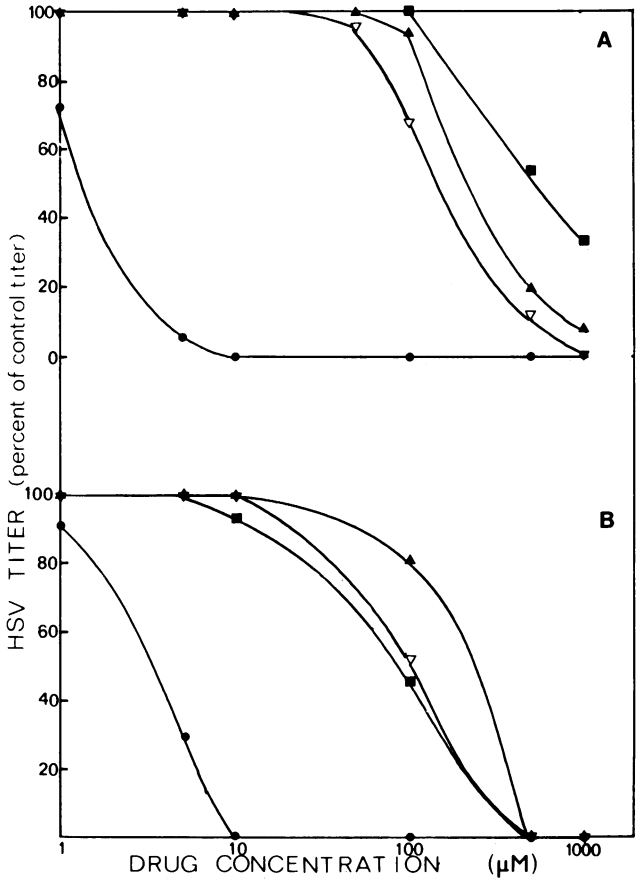

FIG. 3. Determination of ED_{50} for resistant HSV isolated from dually infected cells during drug treatment. Supernatant fluid collected on day 35 was diluted to 1,250 HSV PFU/ml and titrated against dilutions of DHPG (A) or ACV (B) or no drug. Source of virus: ●, untreated cells; ▲, 30 μM ACV-treated cells; ▽, 100 μM ACV-treated cells; ■, 30 μM DHPG-treated cells.

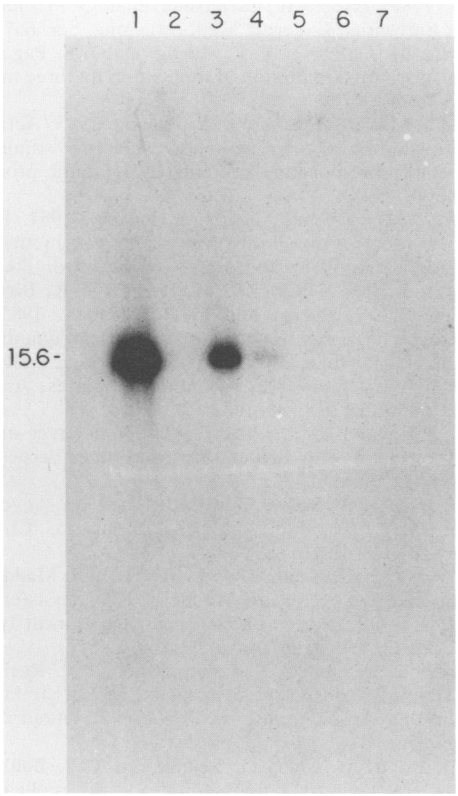

FIG. 4. Analysis of HSV DNA in cell lines. DNA was isolated from cells which had been exposed to 100 μM DHPG 2 and 6 months after release from the drug (lanes 6 and 7). About 4 μg of DNA was digested with *Eco*RI, electrophoresed in a 0.7% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled HSV DNA *Eco*RI fragment G at 1.4×10^8 cpm/μg. Lanes 1, 3, and 4, Hybridization to 100, 10, and 1 copy of HSV DNA, respectively; lanes 2 and 5, empty.

xcRNA-DNA hybridization with an EBV-specific probe. Copies of the EBV genome fluctuated from 125 to 305 per cell over time in the untreated control cells. A previous study indicated that variation was not unusual for P3HR-1 cells, the parent line of our dually infected cells, monitored over the course of 4 months and at different phases of cell growth (14). Similar to results of our earlier work (13, 15), the numbers of genomes were suppressed with drug treatment and remained so with continuous presence of the drug for 70 days. The number of EBV genomes dropped to 25 or 50 copies per cell by day 14 when exposed to 100 or 30 μM DHPG, respectively. ACV at levels of 30 and 100 μM similarly reduced the number of copies of the EBV genome (data not shown).

After centrifugation of the control cells and suspension in fresh medium, the number of EBV genomes fell from 120 to 75, as determined 28 days after resuspension; this effect was probably due to logarithmic growth of the cells, which reduces virus production (14). In the drug-free medium, the number of EBV genomes then rapidly increased to 270 copies per cell 66 days after resuspension. However, in cells treated with 30 μM DHPG or ACV or 100 μM DHPG, the number of EBV genome copies increased but did not rise to control levels, remaining at 70 to 170 copies 66 days after resuspension in drug-free medium.

ACV and DHPG both act only on actively replicating EBV. EBV plasmids in the nonproductive fraction of P3HR-1 cells are not affected, thus accounting for the residual copy numbers (15). One of the interesting features of drug treatment of cell lines persistently infected with EBV is that the speed of recovery of virus production after removal of the drug differs from drug to drug (13, 15), probably because of differences in metabolism of the drugs (12). The present results show a similar behavior, with much slower recovery of EBV replication after removal from DHPG or ACV (data not shown). In a previous study with P3HR-1 cells (15), the number of EBV genomes eventually reached control levels when the cells were released from ACV or DHPG.

It is useful to speculate on the mechanism of resistance of HSV in the presence of continued suppression of EBV. Both ACV and DHPG must be phosphorylated to act on viral-encoded DNA polymerase. Previous work showed that ACV and DHPG are monophosphorylated by HSV TK (8, 9). There is increasing evidence of an EBV-encoded TK. There are rudimentary HSV TK sequences in the EBV genome (1). Three groups recently reported detection of an EBV-induced TK (1a, 7, 23). In our system, HSV develops resistance by mutating to an altered TK or a TK-negative state. However, DHPG and ACV remain active against EBV in the same cells in which they are presumably less phosphorylated and inactive against HSV. Thus, the EBV DNA polymerase must be much more sensitive than the HSV DNA polymerase to the low level of ACV triphosphate made by host enzymes (6, 17). Perhaps the best explanation is that EBV produced in P3HR-1 cells arises from a clone of latently infected cells. In latent cells, EBV is in an episomal form which does not use virally encoded polymerase to replicate and thus is not affected by the antiviral drugs (15). The HSV produced comes from a mixed population of virus from persistently infected cells, from which resistant virus can be selected by drug exposure.

We have demonstrated that the use of the dually infected line is a convenient and predictable method by which drug-resistant HSV mutants can be generated. Since simultaneous active infection with two or three different herpesviruses is not unusual in immunocompromised patients, this dually infected cell system is a useful in vitro model for the study of antiviral chemotherapy. This cell system also offers a chance to study the interaction of HSV and EBV during drug treatment. Finally, unlike EBV, HSV was eradicated by treatment with a high dose (100 μM) of DHPG. Thus, the dually infected cell line is potentially useful to screen antiviral agents aimed not merely at suppression of viral replication but at eradication of persistent infection.

We thank J. L. Martin of the Burroughs Wellcome Company for assistance with the plaque autoradiography and S. Bachenheimer for HSV cloned fragments. We also thank B. Leonard and V. McNeil for preparing the manuscript. We are grateful to W. G. Robey for the dually infected cell line, the Burroughs Wellcome Company for the antiviral drugs, and the Syva Company for the monoclonal antibody to HSV glycoprotein C.

This work was supported by Public Health Service grants 5-PO1-CA-19014 and 5T32AI0-7151-05 from the National Cancer Institute and the National Institute of Allergy and Infectious Diseases, respectively.

LITERATURE CITED

1. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA

sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.

1a. **Chen, S. T., J. E. Estes, E.-S. Huang, and J. S. Pagano.** 1978. Epstein-Barr virus-associated thymidine kinase. J. Virol. **26**:203–208.

2. **Cheng, Y.-C., S. P. Grill, G. E. Dutschman, K. Nakayama, and K. F. Bastow.** 1983. Metabolism of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, a new anti-herpes virus compound, in herpes simplex virus-infected cells. J. Biol. Chem. **258**: 12460–12464.

3. **Cheng, Y.-C., E.-S. Huang, J.-C. Lin, E.-C. Mar, J. S. Pagano, G. D. Dutschman, and S. P. Grill.** 1983. Unique spectrum of activity of 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine against herpes viruses *in vitro* and its mode of action against herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **80**:2767–2770.

4. **Coen, D. M., and P. A. Schaffer.** 1980. Two distinct loci confer resistance to acycloguanosine in herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **77**:2265–2269.

5. **Crumpacker, C. S., L. E. Schnipper, S. I. Marlowe, P. N. Kowalsky, B. J. Hershey, and M. L. Levin.** 1982. Resistance to antiviral drugs of herpes simplex virus isolated from a patient treated with acyclovir. N. Engl. J. Med. **306**:343–346.

6. **Datta, A. K., B. M. Colby, J. E. Shaw, and J. S. Pagano.** 1980. Acyclovir inhibition of Epstein-Barr virus replication. Proc. Natl. Acad. Sci. USA **77**:5163–5166.

7. **de Turenne-Tessier, M., T. Ooka, G. de The, and J. Daillie.** 1986. Characterization of an Epstein-Barr virus-induced thymidine kinase. J. Virol. **57**:1105–1112.

8. **Elion, G. B., P. A. Furman, J. A. Fyfe, P. de Miranda, L. Beauchamp, and H. J. Schaeffer.** 1977. Selectivity of action of an antiherpetic agent 9-(2-dihydroxyethoxymethyl)guanine. Proc. Natl. Acad. Sci. USA **74**:5716–5720.

9. **Field, A. K., M. E. Davies, C. DeWitt, H. C. Perry, R. Liou, J. Germershausen, J. D. Karkas, W. T. Ashton, D. B. R. Johnston, and R. L. Tolman.** 1983. 9-{[2-hydroxy-1-(hydroxymethyl) ethoxy]methyl}guanine: a selective inhibitor of herpes group virus replication. Proc. Natl. Acad. Sci. USA **80**:4139–4143.

10. **Field, H. J., and G. Darby.** 1980. Pathogenicity in mice of strains of herpes simplex virus which are resistant to acyclovir in vitro and in vivo. Antimicrob. Agents Chemother. **17**:209–216.

11. **Furman, P. A., M. H. St. Clair, and T. Spector.** 1984. Acyclovir triphosphate is a suicide inactivator of the herpes simplex virus DNA polymerase. J. Biol. Chem. **259**:9575–9579.

12. **Lin, J. C., D. J. Nelson, C. U. Lambe, E. I. Choi, and J. S. Pagano.** 1985. Effects of nucleoside analogs in inhibition of Epstein-Barr virus. Excerpta Med. Int. Cong. Ser. **667**:225–227.

13. **Lin, J.-C., M. C. Smith, Y. C. Cheng, and J. S. Pagano.** 1983. Epstein-Barr virus: inhibition of replication by three new drugs. Science **221**:578–579.

14. **Lin, J.-C., M. C. Smith, and J. S. Pagano.** 1983. Activation of latent Epstein-Barr virus genomes: selective stimulation of synthesis of chromosomal proteins by a tumor promoter. J. Virol. **45**:985–991.

15. **Lin, J.-C., M. C. Smith, and J. S. Pagano.** 1984. Prolonged inhibitory effect of 9-(1,3-dihydroxy-2-propoxymethyl)guanine against replication of Epstein-Barr virus. J. Virol. **50**:50–55.

16. **Martin, J. L., M. N. Ellis, P. M. Keller, K. K. Biron, S. N. Lehrman, D. W. Barry, and P. A. Furman.** 1985. Plaque autoradiography assay for the detection and quantitation of thymidine kinase-deficient and thymidine kinase-altered mutants of herpes simplex virus in clinical isolates. Antimicrob. Agents Chemother. **28**:181–187.

17. **Pagano, J. S., and A. K. Datta.** 1982. Perspectives on interactions of acyclovir with Epstein-Barr and other herpes viruses. Am. J. Med. **73(1a)**:18–26.

18. **Pagano, J. S., J. W. Sixbey, and J.-C. Lin.** 1983. Acyclovir and Epstein-Barr virus infection. J. Antimicrob. Chemother. **12**(Suppl. B):113–121.

19. **Robey, W. G., B. J. Graham, C. L. Harris, J. J. Madden, G. R. Pearson, and G. F. Vande Woude.** 1976. Persistent herpes simplex virus infections established in two Burkitt lymphoma derived cell lines. J. Gen. Virol. **32**:51–62.

20. **Schnipper, L. E., and C. S. Crumpacker.** 1980. Resistance of herpes simplex virus to acycloguanosine: role of viral thymidine kinase and DNA polymerase loci. Proc. Natl. Acad. Sci. USA **77**:2270–2273.

21. **Shaw, J. E., B. M. Colby, J. Leong, and T. J. Bolling.** 1982. Effect of acyclovir on herpes simplex virus replication in a persistently infected human lymphoblastoid cell line (P3HR-1). Am. J. Med. **73(1a)**:72–76.

22. **Smith, K. O., W. L. Kennell, R. H. Poirier, and F. T. Lynd.** 1980. In vitro and in vivo resistance of herpes simplex virus to 9-(2-hydroxyethoxymethyl)guanine (acycloguanosine). Antimicrob. Agents Chemother. **17**:144–150.

23. **Stinchcombe, T., and W. Clough.** 1985. Epstein-Barr virus induces a unique pyrimidine deoxynucleoside kinase activity in superinfected and virus-producer B cell lines. Biochemistry **24**:2027–2033.