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Prolonged Inhibitory Effect of 9-(1,3-Dihydroxy-2-Propoxymethyl)Guanine Against Replication of Epstein-Barr Virus

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The effects of 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), a new antiviral drug, and acyclovir (ACV) [9-(2-hydroxyethoxymethyl)guanine] on the replication of Epstein-Barr virus (EBV) were compared. Both drugs inhibited EBV DNA replication in P3HR-1 cells and superinfected Raji cells, but neither inhibited replication of the plasmid form of the EBV genome in latently infected Raji cells. However, DHPG had a more prolonged inhibitory effect than ACV. Although the effect of the drugs is prompt, the kinetics of inhibition of EBV replication indicated that a drug exposure of 14 days was needed to reduce the EBV genome copy number to the residual plasmid level (30 copies per cell). The inhibitory effect of ACV was readily reversed within 11 days after removal of the drug, in contrast to the more prolonged effect exerted by DHPG, which persisted for more than 21 days. The 50% inhibitory doses for cell growth of ACV and DHPG were estimated to be 250 and 200 μM, respectively. The viral 50% and 90% effective doses of inhibition were, respectively, 0.3 and 9 μM for ACV and 0.05 and 3 μM for DHPG. The therapeutic indices (50% inhibitory dose/50% effective dose) for ACV and DHPG were 833 and 4,000, respectively. Synthesis of EBV-associated polypeptides was also affected. In superinfected Raji cells, ACV (100 μM) and DHPG (30 μM) inhibited synthesis of polypeptides with molecular weights of 145,000 and 140,000; in addition, synthesis of polypeptides with molecular weights of 110,000 and 85,000 was markedly reduced by DHPG but not by ACV. However, after drug removal, the inhibitory effect of ACV on polypeptide synthesis was abolished in contrast to the more persistent effect of DHPG.

The hallmark of herpes group viruses is their ability to cause latent and persistent as well as acute productive infections. In the past few years, we have shown that acyclovir (ACV) [9-(2-hydroxyethoxymethyl)guanine] (8) has potent effects against Epstein-Barr virus (EBV) infections in vitro when there is active replication of the virus (5, 12, 14). However, ACV has no effect in vitro on latent EBV infection, nor does the drug promise to be of much use in persistent low-level infection with EBV (20). Inasmuch as latent and persistent infections may be key elements of the oncogenic and chronic disease states associated with EBV, we started searching for other anti-EBV agents with the specific goal of identifying compounds whose effects might be more prolonged than those of ACV. We recently reported (17) that three nucleoside analogs, E-5-(2-bromovinyl)-2′-deoxyuridine, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine, and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil, are potent inhibitors of EBV replication in vitro. These three drugs have prolonged effects in suppressing viral replication, even after the drugs are removed from persistently infected cell cultures (17).

Recently, an ACV congener, 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG), was reported to have anti-herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) activity with a marginal activity against EBV and no activity against human cytomegalovirus (CMV) (24). However, we have found that DHPG is active not only against replication of HSV-1 and HSV-2 but also against CMV and EBV (3). In addition, certain variants of HSV which induced altered virally coded thymidine kinase and DNA polymerase and had become resistant to ACV were still as sensitive to DHPG as was the parental virus (3). In view of these results, we decided to extend our findings and characterize the efficacy of DHPG in parallel with ACV in terms of inhibition of replication of EBV in human lymphoblastoid cell lines. The results clearly indicate that DHPG is more potent and has a more prolonged inhibitory effect than ACV.

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MATERIALS AND METHODS

Cell cultures. Virus producer (P3HR-1) and nonproducer (Raji) cells were grown in tissue culture flasks in RMPI 1640 medium as described previously (13). A steady-state mode of growth was established as follows. Cells were seeded at a density of 4×10^5 to 6×10^5 cells per ml and were counted daily until they reached a density of 3×10^6 cells per ml. During this interval, the cells were growing in a strictly exponential fashion (15).

Treatment of cells with drugs. Exponentially growing cells were spun down and suspended in fresh medium containing different concentrations of drugs for an appropriate time as indicated. At the end of drug treatment, cells were harvested, and the number of EBV genome copies per cell was determined. In some experiments, the drug-treated cells were released into drug-free medium and incubated for an appropriate time as specified in the figures.

Determination of 50% inhibitory dose (ID_{50}) for cell growth. During drug treatment, cells were counted daily. Cell viability was determined by the trypan blue exclusion method. The number of cells after 4 days of growth was plotted against drug concentration on a semilogarithmic scale. The cell growth ID_{50} was that concentration of drug that inhibited cell growth by 50% after 4 days compared with the no-drug control.

Determination of EBV genome copies per cell. EBV DNA was purified from virus isolated from the supernatant fluids of 12-*O*-tetradencanoylphorbol-13-acetate-induced P3HR-1 cells as previously reported (13, 14). In vitro synthesis of

cRNA and cRNA-DNA hybridization on nitrocellulose filters was carried out as described elsewhere (15, 16).

Determination of viral ED_{50} and ED_{90}. The number of EBV genome copies per cell after drug treatment was plotted against drug concentrations. The viral 50% and 90% effective doses (ED_{50}s and ED_{90}s) of inhibition were those concentrations of drug that inhibited viral DNA replication by 50 and 90%, respectively, compared with the no-drug control.

Superinfection of Raji cells with P3HR-1 virus. Virus used for superinfection of Raji cells was prepared from 12-*O*-tetradecanoylphorbol-13-acetate-induced P3HR-1 culture fluids (13). Virus harvested from 1.5 liters of fluids by centrifugation (13,000 × *g* for 90 min in a GS-3 rotor) was suspended in 6 ml of RPMI 1640 medium containing 2% fetal calf serum and stored at −70°C. For superinfection, 10^6 Raji cells in exponential growth were pelleted and suspended in 1 ml of RPMI 1640 medium containing 2% serum and an appropriate amount of virus (time zero). After 1 h at 37°C in a CO_2 incubator, the cells were pelleted, washed twice with phosphate-free minimal essential medium, and suspended in 1 ml of the same buffer containing 2% dialyzed fetal calf serum and 100 μM ACV or 30 μM DHPG. At 6 h postinfection, 200 μCi of ^{32}P was added, and incubation continued until 24 h after infection. For radiolabeling polypeptides, after viral absorption cells were starved in methionine-free minimal essential medium containing 2% dialyzed fetal calf serum for 5 h; then [^{35}S]methionine (50 μCi/ml) was added. Labeling was continued for 24 h after infection.

Equilibrium density centrifugation. Both superinfected and mock-infected Raji cells were pelleted, and DNA was isolated and processed for analysis in cesium chloride density gradients as described previously (15).

Polylacrylamide gel electrophoresis. The [^{35}S]methionine-labeled proteins were analyzed on slab gels in Laemmli's system (11). The proteins were treated with sample buffer and electrophoresed at 25 mA per slab for 8 h. Radiolabeled proteins in the gels were detected by fluorography (1).

RESULTS

Effects of ACV and DHPG on cell growth. Both Raji and P3HR-1 cells were cultured in the presence of various concentrations of ACV and DHPG. The growth curves of P3HR-1 cells in the presence of the drugs are shown in Fig. 1. Both ACV (Fig. 1A) and DHPG (Fig. 1B) at concentrations of ≤10 μM had slight effects on cell multiplication. At 100 μM ACV and DHPG, there was cell multiplication, but the total cell counts after 4 days of cell growth were 35 and 40%, respectively, below those in control (drug-free) cultures. The viability of cells was maintained at 95% in all concentrations of ACV and DHPG tested. A wide range of drug concentrations was used to determine the concentration that inhibited cell growth by 50% (ID_{50}). The ID_{50} for cell growth was determined by plotting the concentration of drug against the number of cells after 4 days of growth. In these studies, the ID_{50}s for ACV and DHPG were 250 and 200 μM, respectively. Similar values were obtained for Raji cells under the same experimental conditions (data not shown).

Determination of viral ED_{50} and ED_{90}. Figure 2 shows the dose-dependent effects of ACV and DHPG on viral genomes in P3HR-1 cells cultured for 14 days in the presence of drugs; EBV genome copies per cell decreased with increasing drug concentrations in both cases but at different rates. The viral ED_{50} and ED_{90} were determined from the semilogarithmic plot of drug concentrations against viral genome copies per cell, assuming the residual genome level (30 copies per cell) achieved by an effective drug concentration (100 μM) as zero and the viral genome level before drug treatment as 100. The ED_{50} and ED_{90} thus obtained were, respectively, 0.3 and 9 μM for ACV and 0.05 and 3 μM for DHPG. On the basis of these data and the cell ID_{50} values, we calculated the therapeutic indices (ID_{50}/ED_{50}) as 833 and 4,000 for ACV and DHPG, respectively.

Concentrations of the drugs 10 times greater than the ED_{90}

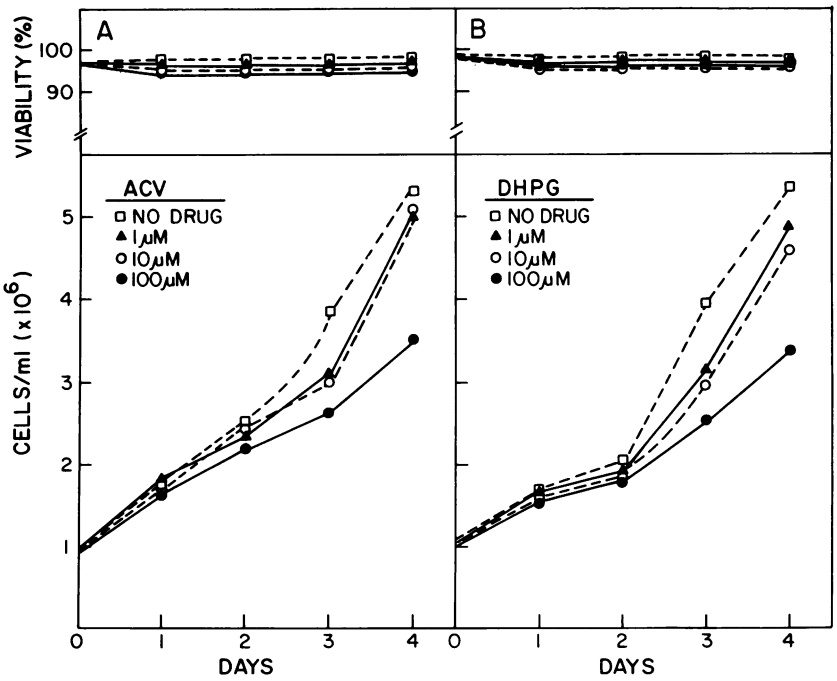

FIG. 1. Effect of ACV and DHPG on the proliferation of P3HR-1 cells in culture.

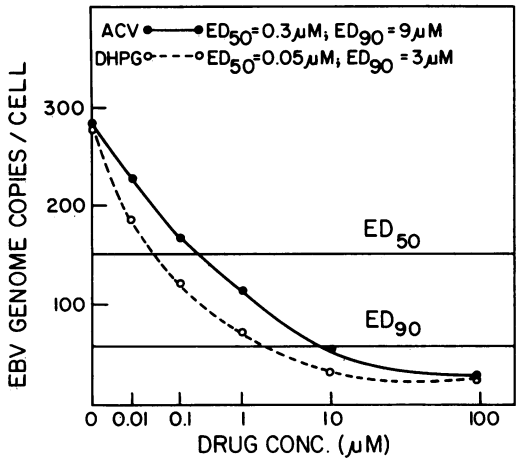

FIG. 2. Determination of viral ED_{50} and ED_{90} of ACV and DHPG. Exponentially growing P3HR-1 cells were seeded at a density of 10^6 per ml and incubated in various concentrations of drugs for 14 days. EBV genome copy numbers per cell determined at each drug concentration were the average of two determinations. ACV: ED_{50} = 0.3 μM, ED_{90} = 9 μM; DHPG: ED_{50} = 0.05 μM, ED_{90} = 3 μM.

did not affect the EBV DNA content of nonproducer Raji cells (data not shown).

Effects of ACV and DHPG on superinfected Raji cells. To establish that the inhibitory effect of DHPG on EBV DNA replication is a general phenomenon and is not limited to one kind of cell system, we tested the drugs in a superinfected Raji cell system. Superinfection of Raji cells with P3HR-1 virus results in shutdown of host functions and stimulation of viral DNA synthesis (18). A large quantity of EBV DNA was synthesized after superinfection of Raji cells (Fig. 3A) as measured by the radioactivity incorporated into DNA banding at 1.718 g/cm^3, the density of viral DNA. In contrast, viral DNA replication was greatly reduced in the presence of 100 μM ACV (Fig. 3B) and 30 μM DHPG (Fig. 3C).

Kinetics of inhibition of EBV genome replication and reversibility. Our previous work (3) and the present study (Fig. 2) indicated that 30 μM DHPG was equivalent to 100 μM ACV (10-fold ED_{90}) in inhibition of EBV DNA synthesis. We next compared the inhibitory effects of ACV (100 μM) and DHPG (30 μM) in P3HR-1 cells. We determined the kinetics of reduction of EBV genome replication by growing the cells in drug-containing medium. At various intervals, samples of cells were taken and viral genome copies per cell were determined. EBV genome numbers were rapidly reduced from 390 to 60 copies per cell after 7 days of exposure to ACV (Fig. 4A). By 14 days, the viral genome copies per cell were reduced to 30 and stably maintained at this low level for 42 days under continuous exposure to the drug. However, the inhibitory effect of ACV was readily reversed upon removal of the drug (Fig. 4B). Viral genome replication returned to the original level by 11 days after release of the cells into drug-free medium.

Identical experiments were carried out for DHPG (Fig. 5). Again, 14 days were needed to reduce the viral genome number to the minimum residual level (30 copies per cell). However, upon removal of the drug, the viral genome copies per cell remained at low levels for 14 days, becoming gradually restored and reaching 23% of the control value by 21 days. Eventually, the genome numbers returned to control levels of untreated cells after 42 days (data not shown).

Differential effects of ACV and DHPG on synthesis of EBV-associated polypeptides. We compared the effects of ACV and DHPG on the synthesis of virus-associated polypeptides. Figure 6 shows the results of a fluorogram made by exposing an electropherogram of ^{35}S-labeled polypeptides synthesized in superinfected Raji cells in the presence and absence of drugs. Superinfection of Raji cells (lane D), as compared with mock-infected cells (lane C), resulted in the synthesis of at least seven new polypeptides of molecular weights 145,000 (145K), 140K, 135K, 110K, 85K, 55K, and 32K detected 24 h postinfection in a continuous labeling experiment. In the presence of 30 μM DHPG (lane E) and 100 μM ACV (lane A), synthesis of polypeptides of molecular weights 145K and 140K were significantly inhibited in both cases. In addition, synthesis of polypeptides 110K and 85K was markedly reduced by DHPG but not by ACV. When ACV was removed 6 h posttreatment, synthesis of the 145K and 140K polypeptides resumed (lane B). In contrast, the inhibitory effect of DHPG on polypeptides of 145K, 140K, 110K, and 85K persisted even after drug removal (lane F).

DISCUSSION

DHPG has several features that make it a potentially valuable and unique antiviral drug: broad spectrum of effect against all of the human herpesviruses including CMV, heightened activity against HSV, and activity against some ACV-resistant HSV mutants. In this study, we have clearly shown that DHPG has a more prolonged inhibitory effect against EBV than does its parental compound, ACV, yet the drug is nontoxic with a very high therapeutic index in cell culture. As with ACV, the EBV plasmids in the nonvirus-producing cell line (Raji), as well as in the nonproductive fraction of the P3HR-1 cells, are not affected by this drug. Since there is no plaque assay for EBV, determinations of ED_{50} have to be done differently than those for HSV. Precise estimates can be made by measurements of genome numbers through nucleic acid hybridization techniques.

The viral ED_{50} and ED_{90} were 0.3 and 9 μM for ACV, respectively, and 0.05 and 3 μM for DHPG (Fig. 2). Our previous estimate of the ED_{50} of ACV was 6 μM (5). This value represents a 20-fold underestimation compared with the present results (0.3 μM). This discrepancy is probably due to (i) treatment with drug continuously for 14 days

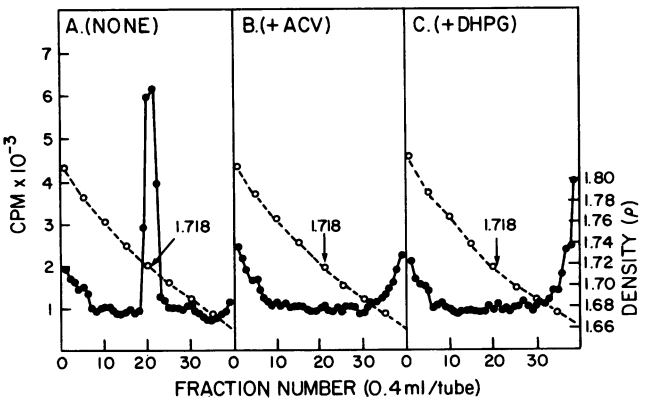

FIG. 3. Inhibition of EBV DNA replication by ACV (100 μM) and DHPG (30 μM) in superinfected Raji cells. EBV DNA replication was determined by measuring the incorporation of ^{32}P into viral DNA and analyzing the DNA by cesium chloride density gradient centrifugation.

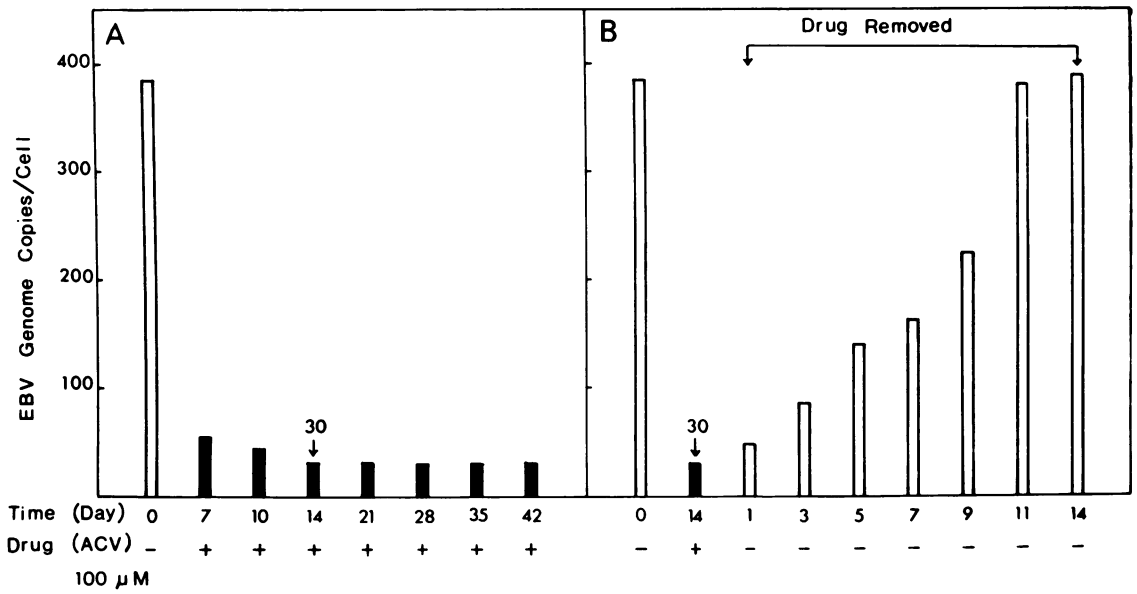

FIG. 4. Kinetics of inhibition and reversibility of EBV DNA replication in P3HR-1 cells treated with ACV.

instead of 7 days as done by Colby et al. (5); and (ii) taking as the zero point the residual level of 30 genome copies per cell. Depending upon the cells used, the ED_{50} values of ACV for HSV-1 (as determined by plaque reduction assay) were 0.1 μM in Vero cells (23) and 0.7 μM in HeLa cells (6). Our new results put the level of sensitivity of EBV to ACV in the same range as that of HSV. The ED_{50} of DHPG for EBV estimated from the data in our previous report (3) was approximately 1 μM. This value was obtained by treating the P3HR-1 cells with various concentrations of DHPG for only 5 days, which is not enough time for linear genomes already synthesized before the addition of drug to become undetectable.

The inhibition of EBV DNA replication in P3HR-1 cells at concentrations that do not inhibit cell proliferation suggests that a virus-specific function is affected. Inhibition of growth of the host cell requires 833- and 4,000-fold (ID_{50}/ED_{50}) greater concentrations of ACV and DHPG, respectively, than does the inhibition of viral multiplication. These results and our previous report clearly show that DHPG could effectively inhibit the replication of HSV-1, HSV-2, CMV, and EBV (3) at noncytotoxic concentrations. These observations are in contrast to a recent report by Smith et al. (24) which indicated a lack of activity against EBV and CMV at the noncytotoxic concentration. The potent anti-EBV and anti-CMV activity of DHPG may have been overlooked by these investigators since they examined the effect of DHPG on the expression of virus-specific antigens which were expressed despite inhibition of viral DNA synthesis. Moreover, the assays (immunofluorescence and radioimmunoassay) that they employed are generally less sensitive and less quantitative than cRNA-DNA hybridization.

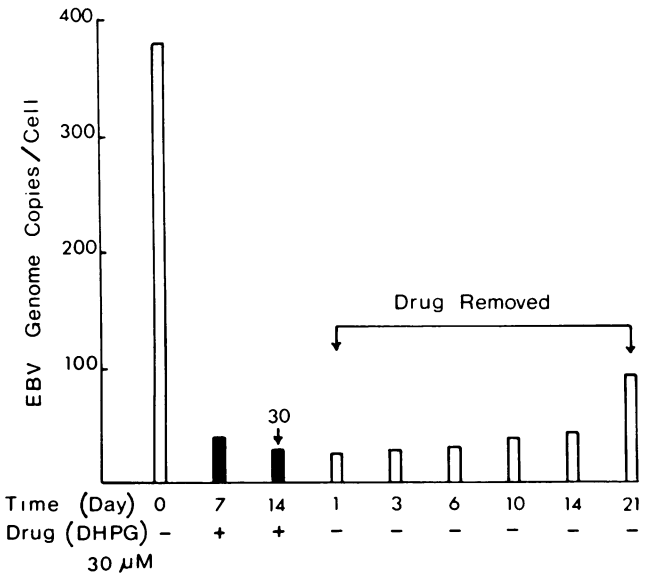

FIG. 5. Kinetics of inhibition and reversibility of EBV DNA replication in P3HR-1 cells treated with DHPG.

Studies on the activity of DHPG against drug-resistant HSV-1 mutants, both at the thymidine kinase (TK) and DNA polymerase loci, clearly indicate that the virus-induced TK plays an important role in the action of DHPG against HSV-1 (3, 24). We have shown previously that an HSV-1 mutant (MDK strain) which could not induce TK in infected cells was resistant to DHPG (3). However, HSV mutants (B3, Tr7, S1) that induced an altered TK with no apparent change of induced DNA polymerase in infected cells were still sensitive to DHPG, despite the fact that those mutants were resistant to ACV (3). Thus, mutation to resistance to ACV due to alteration of viral TK does not necessarily result in resistance to DHPG. These results suggest that DHPG is less fastidious in its TK requirements than is ACV.

There is some evidence for and against the existence of an EBV-specific TK (2, 4, 7, 19, 20). However, there has been no unequivocal demonstration of a novel enzyme activity induced by EBV that might represent a virally coded TK. It remains to be established whether the inhibition of EBV replication by ACV and DHPG follows a mechanism similar to that reported in HSV (8).

Consistent with the prolonged inhibition of EBV DNA synthesis by DHPG is the demonstration of the more sustained and differential effects of DHPG on EBV-associated

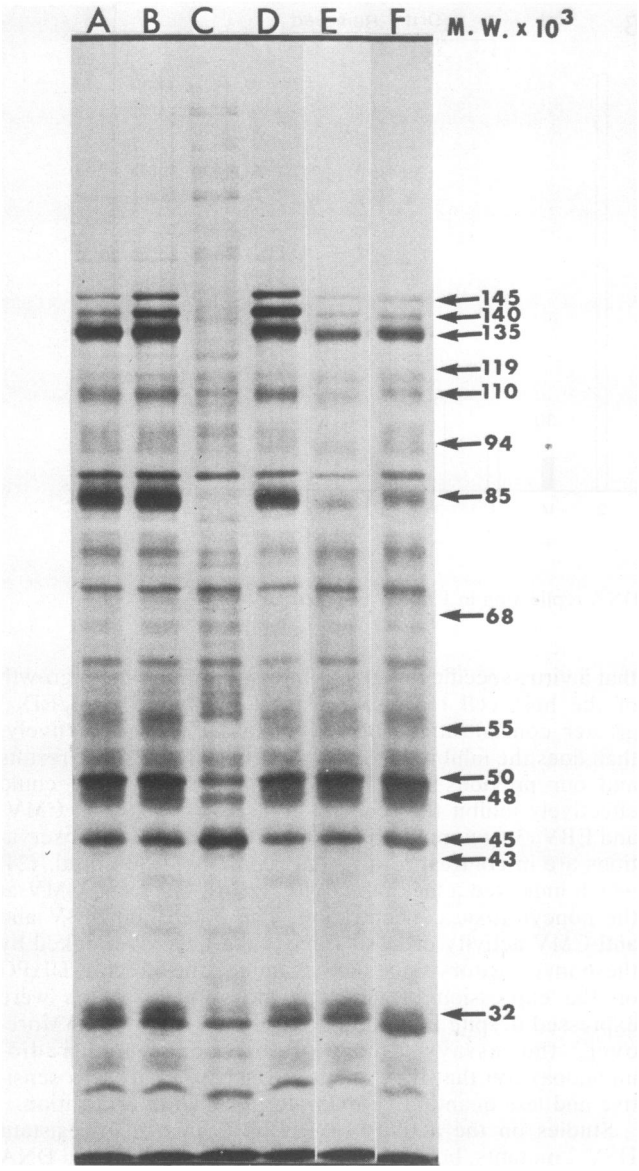

FIG. 6. Differential effects of ACV and DHPG on synthesis of EBV-associated polypeptides. Lanes: A, polypeptides synthesized in superinfected Raji cells, in the continuous presence of ACV (100 μM); B, same as A except that ACV was removed at 6 h posttreatment; C and D, respectively, mock-infected and superinfected Raji cells in the absence of drugs; E, polypeptides synthesized in superinfected Raji cells in the continuous presence of DHPG (30 μM); F, same as E except that DHPG was removed at 6 h posttreatment.

polypeptides. Although both DHPG and ACV inhibit synthesis of at least two EBV-induced polypeptides (molecular weights 145K and 140K), the inhibitory effect of ACV was abolished, in contrast to the persistent effect of DHPG after drug removal. Of interest is the additional effect on the synthesis of polypeptides with molecular weights of 110K and 85K exerted by DHPG but not by ACV. These findings are similar to our previous work on differential effects of DNA inhibitors on viral polypeptide synthesis (9). As is the case for other antiviral drugs, the effect of DHPG on viral polypeptides is most likely due to inhibition of EBV DNA synthesis. Thus, the greater persistent effect of DHPG on the synthesis of virus progeny DNA that can serve as template for mRNA may be responsible for the decrease in synthesis of these polypeptides (22). The differential reduction in EBV-induced polypeptide synthesis, although a secondary effect of the drug, could play a role in the inhibition of virus replication, as suggested in HSV systems (10, 21).

Despite the structural similarities of ACV and DHPG, DHPG appears to have a more potent and prolonged inhibitory effect against EBV than does ACV. Whether these effects are due to the differences in drug metabolism (phosphorylation) or to relative effects in incorporation of the drugs into DNA and chain termination remains to be determined. The greater antiviral activity in comparison with existing antiherpes compounds, the very low cytotoxicity, the higher solubility in water (24), and the more persistent effect make DHPG a promising candidate for clinical trial if the drug is nontoxic in animal studies.

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