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Effect of Acyclovir [9-(2-Hydroxyethoxymethyl)guanine] on Epstein-Barr Virus DNA Replication

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The effect of acyclovir [9-(2-hydroxyethoxymethyl)guanine] on Epstein-Barr virus (EBV) DNA replication in the lymphoblastoid cell lines P3HR-1 and Raji is reported. Acyclovir at a concentration of 100 μM completely inhibited EBV DNA synthesis in superinfected Raji cells, but did not inhibit DNA synthesis in mock-infected cells. The number of EBV genome equivalents per cell in the virus-producing cell line P3HR-1 was significantly reduced by acyclovir, whereas the number of latent EBV genomes in Raji cells was not affected by the drug. In situ cytohybridization performed on untreated P3HR-1 cultures revealed the presence of relatively large amounts of EBV DNA in 15 to 20% of the cells. After a 100 μM drug treatment, no P3HR-1 cells contained levels of EBV DNA detectable by in situ cytohybridization. Indirect immunofluorescence studies demonstrated that during treatment with 100 μM acyclovir for 7 days, the percentage of P3HR-1 cells expressing viral capsid antigen was reduced. The EBV DNA remaining in P3HR-1 cells after treatment with 100 μM acyclovir (approximately 14 genomes per cell) had the properties of covalently closed circular DNA with an average molecular weight of 108×10^6, as determined by contour length measurements.

Acyclovir [ACV; 9-(2-hydroxyethoxymethyl)guanine] is one of a new class of potent antiviral compounds recently shown to be effective against herpes simplex virus replication (4) and is one of a series of synthetic acyclic nucleoside compounds (28). ACV has a potent inhibitory activity against herpes simplex virus-infected cells but has low toxicity for normal cells. The effect of ACV on another herpesvirus, Epstein-Barr virus (EBV), has not been reported previously.

EBV infects all human populations and causes infectious mononucleosis (5, 8, 13), a disease characterized by infection and transformation of B-lymphocytes (16, 17, 23) and by EBV-determined nuclear antigen expression (27). EBV is also uniquely associated with Burkitt lymphoma and nasopharyngeal carcinoma, two human malignancies. Tissues from such tumor sources carry multiple latent copies of EBV DNA (21, 35-37). Because of its remarkable association with these clinical diseases and its transformation abilities in vitro, EBV is considered by many to be a prime human tumor virus candidate. Until now there has been no drug available which possesses potent antiviral activity without having serious deleterious effects on normal cell replication.

Our studies were designed to determine the effects of ACV on EBV DNA replication in the virus-producing cell line P3HR-1, in the non-virus-producing line Raji, and in latently infected Raji cells after superinfection with P3HR-1 virus. The results presented here are similar to those obtained when phosphonoacetic acid (PAA) was used. Our studies show that in the EBV system ACV is an effective inhibitor of viral DNA replication in productively infected cells but is essentially without effect on the replication of viral DNA in latently infected cells, where cellular control mechanisms apparently regulate EBV DNA synthesis.

MATERIALS AND METHODS

Cell cultures. Two Burkitt lymphoma-derived cell lines, Raji (6, 7, 25) and P3HR-1 (15), were maintained at between 2×10^5 and 10^6 cells per ml by dilution in RPMI 1640 medium containing 10% fetal bovine serum.

ACV. 9-(2-Hydroxyethoxymethyl)guanine, obtained from the Burroughs Wellcome Co., was dissolved in phosphate-buffered saline (130 mM NaCl, 5 mM KCl, 0.01 M sodium phosphate, pH 7.4) at a concentration of 10 mM with the aid of brief sonication, warmed to 37°C, filtered through a cellulose acetate membrane (0.45 μm; Millipore Corp.), and stored at −20°C.

Viral antigen production. Indirect immunofluorescence assays for EBV early antigen (EA) and viral capsid antigen (VCA) (12-14) were performed by the method of Henle and Henle (12); EA^- VCA^+ (Kampala) and EA^+ VCA^+ (Ghana) sera were used in the assays.

Preparation of P3HR-1 virus and viral DNA.

Preparation of virus for superinfection, procedures for superinfection, and purification of DNA from P3HR-1 virus have been described previously (1, 31).

Purification of high-molecular-weight EBV DNA from Raji and P3HR-1 cells. Raji cells (8×10^7 cells) and 8×10^7 P3HR-1 cells treated with 100 μM ACV were harvested, and high-molecular-weight EBV DNA was purified as described previously (2), with the following modifications. Covalently closed circular EBV DNA was first localized by isopycnic centrifugation in ethidium bromide-cesium chloride; the DNA was centrifuged to equilibrium in a Sorvall TV-850 rotor at 40,000 rpm and 18°C for 18 h. The DNA banding at approximately 1.59 g/cm^3, which included covalently closed circular EBV DNA (19), was centrifuged again on cesium chloride without ethidium bromide. The viral DNA banding at 1.718 g/cm^3 was dialyzed against 0.1× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M trisodium citrate). During storage at 4°C, approximately 90% of the covalently closed circular DNA spontaneously converted to an open circular form. EBV DNA isolated by this procedure was used for contour length measurements.

Preparation of cellular DNA for EBV genome number determination. Raji and P3HR-1 DNAs from mock-treated and drug-treated cultures were extracted as follows. Approximately 4×10^7 cells were pelleted, washed once, and suspended in phosphate-buffered saline to 5×10^6 cells per ml; 0.5 volume of Sarkosyl solution (3% Sarkosyl, 50 mM Tris, pH 8.6, 15 mM neutralized EDTA) was added, and after 10 min at room temperature, 0.5% pronase was added to a final concentration of 0.1%. After incubation at 37°C for 2 h, each sample was extracted twice at room temperature with water-saturated phenol equilibrated with 0.1 volume of 1 M Tris-hydrochloride (pH 8), followed by two extractions with equal volumes of chloroform-isoamyl alcohol (24:1). DNA and RNA in the aqueous phase were precipitated at −20°C overnight with 2.5 volumes of salt-saturated ethanol. After centrifugation at 10,000 × *g*, the pellet was washed once with cold 95% ethanol, dissolved in 2 ml of 0.1× SSC, and digested with 50 μg of pancreatic RNase per ml for 45 min at 37°C. Sarkosyl and pronase were then added to 1 and 0.1% (final concentrations), respectively; incubation at 37°C was continued for 45 min. After incubation, protein was removed by two phenol extractions, followed by two chloroform-isoamyl alcohol extractions, and the DNA was precipitated with salt-saturated ethanol overnight at −20°C. Precipitates were dissolved in 2 ml of 0.1× SSC; DNA concentrations were estimated by absorbance at 260 nm.

Hybridization. P3HR-1 and Raji cultures treated for 7 days with 100 μM ACV and mock-treated cultures were analyzed by in situ cytohybridization, as described previously (24). Complementary RNA-DNA hybridization, which was used to quantitate EBV DNA and to locate EBV DNA on CsCl gradients, was conducted as previously described (21).

Electron microscopy and contour length measurements. Open circular EBV DNA was spread onto Parlodian-coated grids (200-mesh copper; Pelco) by a modification of the microdiffusion technique (18). In addition to EBV DNA, the spreading solution contained 0.50 M ammonium acetate (pH 7.5) and 60 μg of cytochrome *c* per ml. Simian virus 40 (SV40) form II DNA was included as an internal size marker.

Contour lengths of open circular DNA molecules were determined by projecting electron micrographs onto a Hewlett-Packard 986A digitizer tablet and tracing the DNA images with a cursor electronically coupled to a Hewlett-Packard 9825 calculator.

RESULTS

Effects of ACV on the growth of P3HR-1 and Raji cells. The growth curves of lymphoblastoid cell lines cultured in the presence of various concentrations of ACV are shown in Fig. 1. Cells were pelleted and suspended to 2×10^5 cells per ml in fresh media on day 0. A lag in growth was evident for all cultures after resuspension; however, for both cell lines the period of the lag increased with increasing ACV concentration. By day 7 the cell densities were identical in mock-treated cultures and cultures treated with 100 μM ACV. Cultures treated with higher ACV concentrations did not attain mock-treated cell densities after 7 days.

Superinfection of Raji cells in the presence of ACV. Superinfection of Raji cells with

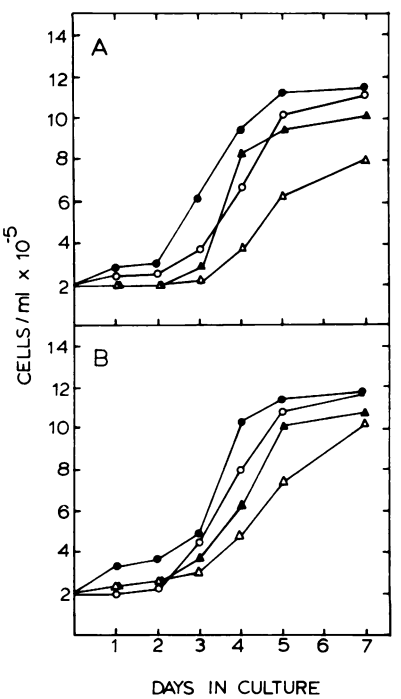

FIG. 1. *Effect of ACV on the growth rate of cultured lymphoblastoid cell lines. The cell lines P3HR-1 (A) and Raji (B) were grown at 37°C without ACV (●) or with ACV at concentrations of 100 μM (○), 500 μM (▲), or 1,000 μM (△). Cells were collected by centrifugation and resuspended in the same volume of fresh medium with or without ACV every 48 h.*

P3HR-1 virus leads to replication of EBV DNA, to suppression of cellular DNA synthesis, and to fragmentation of cellular DNA (22, 31, 34). Such results are shown in Fig. 2A; superinfection of Raji cells with EBV inhibited host cell DNA synthesis, whereas EBV DNA was synthesized. DNA synthesis was measured as the number of counts incorporated into the DNA banding at 1.718 g/cm^3, the density of viral DNA. Addition of 100 μM ACV to the cultures 1 h after superinfection caused complete suppression of EBV DNA synthesis (Fig. 2A). It is of interest that host cell DNA was not synthesized even in the presence of ACV. Mock-infected cells not treated with ACV and mock-infected cells treated with 100 μM ACV synthesized only cell DNA (Fig. 2B). On Fig. 2B the position of 1.718 g/cm^3 as determined by refractive index is indicated.

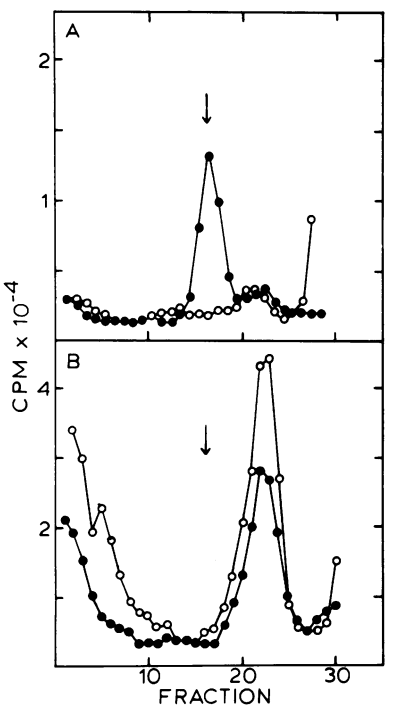

FIG. 2. *Effect of ACV on EBV DNA synthesis in superinfected and mock-infected Raji cells. (A) Neutral CsCl density profile of [^{32}P]DNA synthesized during superinfection without ACV (●) or in the presence of 100 μM ACV (○). (B) Neutral CsCl density profile of [^{32}P]DNA synthesized in untreated mock-infected cells (●) and in mock-infected cells treated with 100 μM ACV (○). The arrows indicate the position of 1.718 g/cm^3 (density increases from right to left). Each profile represents the label incorporated into 10^6 cells. A total of 10^6 Raji cells were pelleted and suspended in 0.3 ml of virus suspension at zero time. After 1 h at 37°C, the cells were pelleted, washed twice, and suspended in phosphate-free medium (minimal essential medium minus phosphate and containing 2% dialyzed fetal calf serum). At 9 h postinfection 200 μCi of ^{32}P was added, and incubation was continued for 24 h.*

The effects of various ACV concentrations on inhibition of EBV DNA synthesis were determined. Raji cells were superinfected in the presence of varying concentrations of ACV, and the percent inhibition of EBV DNA synthesis at each drug concentration was measured. Inhibition values obtained for 0, 0.1, 1, 10, and 100 μM ACV were 0, 20, 9, 70, and 100%, respectively. Percent inhibition values (Fig. 3) were calculated from the following: [1 − (counts per minute incorporated into EBV DNA of 10^6 superinfected Raji cells at a particular drug concentration/counts per minute incorporated into EBV DNA of 10^6 superinfected Raji cells)] × 100. Figure 3 shows that the dose required for 50% inhibition (ED_{50}) of viral DNA synthesis was 7 μM.

Reduction of viral genomes in P3HR-1 cells by ACV. Figure 4 shows the effect of ACV on viral genomes in P3HR-1 and Raji cells cultured for 7 days in the presence of varying drug concentrations. As determined by EBV complementary RNA-DNA membrane hybridization (Table 1), the number of EBV genome equivalents per cell in the producer cell line P3HR-1 decreased with increasing ACV concentration from 133 genomes per cell to 14 EBV genome equivalents per cell in 72 to 96 h at the maximum drug concentration (100 μM). At the same drug concentrations, the numbers of EBV genomes of Raji cells were not affected. The ED_{50} calculated for a 50% reduction in the average genome number for P3HR-1 cells by ACV was 6 μM.

The effect of drug removal on the number of EBV genomes was determined. After drug treatment for 7 days, all cultures were suspended in fresh medium lacking ACV. At 14 days after drug removal, the number of EBV genome equivalents per cell in all cultures had returned to the control levels (Fig. 4).

Complementary RNA-DNA cytohybridization in situ of P3HR-1 and Raji cells. The cytohybridization technique, as described above, was used to determine the proportion of cells in mock-treated cultures and in cultures treated with 100 μM ACV which were harboring EBV DNA. After hybridization with EBV-specific complementary RNA, cells from mock- and ACV-treated Raji cultures demonstrated a low background of grains diffusely scattered over all of the cells. Dense accumulations of grains were present over approximately 15 to 20% of the mock-treated P3HR-1 cells, which is in agreement with previously published data (24). In contrast, no ACV-treated P3HR-1 cells demon-

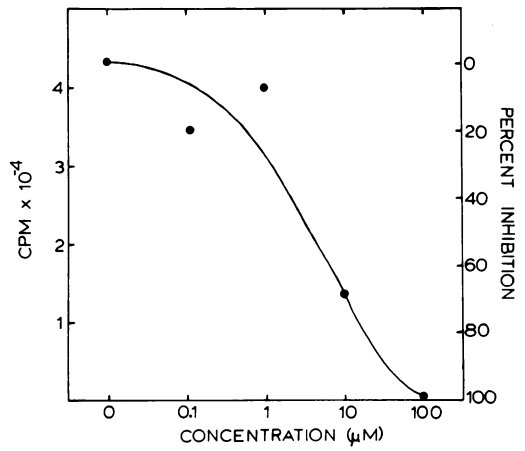

FIG. 3. *Inhibition of EBV DNA synthesis in superinfected Raji cells treated with varying concentrations of ACV. The value obtained for each ACV concentration is the sum of the ^{32}P counts per minute incorporated into EBV DNA (1.718 g/cm^3) when analyzed as described in the legend to Fig. 2. Percent inhibition values were defined as follows: [1 − (counts per minute incorporated into EBV DNA of 10^6 superinfected Raji cells at a particular drug concentration/counts per minute incorporated into EBV DNA of 10^6 superinfected Raji cells)] × 100.*

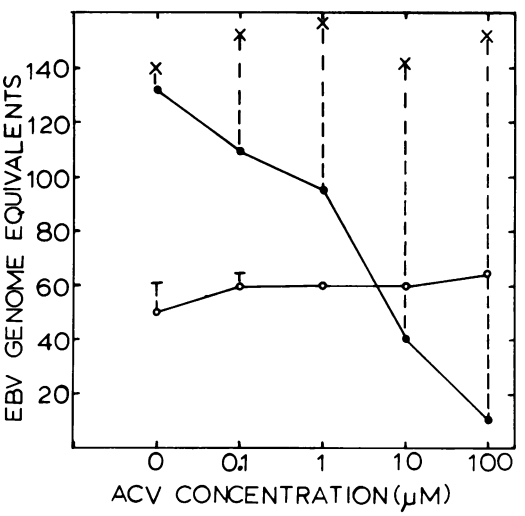

FIG. 4. *Effect of ACV removal on EBV genome equivalents in Raji and P3HR-1 cells. P3HR-1 (●) and Raji (○) cell lines were treated for 7 days with ACV at varying concentrations. On day 7 the number of genomes per cell was determined at each drug concentration. The remaining cells were harvested and suspended in drug-free medium, and numbers of genomes per cell for P3HR-1 (×) and Raji (T) cells were determined on day 22 (14 days after ACV removal from the cultures).*

strated dense accumulation of grains. The scatter of grains was uniform over all cells and the background. Because the sensitivity of in situ cytohybridization is rather low (approximately 60 genomes per cell), these results indicate a very high number of EBV genomes in 15 to 20% of the mock-treated P3HR-1 cells and show that the number of genomes in the ACV-treated P3HR-1 cultures was below the level of detectability.

Expression of viral antigens in P3HR-1 and Raji cells. Indirect immunofluorescence studies demonstrated that the percentage of P3HR-1 cells expressing VCA was reduced by drug treatment (Fig. 5). Staining with serum which contained both EA and VCA antibodies, however, revealed no change after ACV treatment. This observation must be attributable to the continued expression of EA^+ cells in drug-treated cultures. Most cultures exhibited a decrease in the percentage of VCA^+ cells as early as 24 h; however, some cells continued to express VCA after 7 days, a time when EBV DNA synthesis in P3HR-1 cells was maximally inhibited. The percentage of VCA^+ cells in most drug-treated cultures increased to the level present before drug treatment within 15 days after drug removal. Raji cultures treated for 7 days with ACV concentrations ranging from 1 to 1,000 μM did not exhibit an induction of either EA or VCA.

TABLE 1. *EBV-specific [^{3}H]cRNA hybridized to P3HR-1 and Raji cell DNA*

Cell type	ACV concn (μM)	cpm × 10^{-1} hybridized per 50 μg of DNA[a]	
		Day 7[b]	Day 22[b]
P3HR-1	0	24.8	26.4
	0.1	21.7	28.9
	1.0	18.0	29.9
	10.0	7.7	26.8
	100.0	3.1	28.9
Raji	0	8.0	11.4
	0.1	11.1	12.3
	1.0	11.5	11.4
	10.0	10.4	11.7
	100.0	13.7	12.8

[a] Average hybridization values of duplicate DNA filters, each standardized to 50 μg of DNA per filter. Counts per minute bound by calf thymus (70 cpm) and HEp-2 DNA (100 cpm) were subtracted as background.

[b] Hybridization was conducted as described previously (21) with DNA from P3HR-1 and Raji cells maintained for 7 days in the presence of various ACV concentrations. On day 7 the remaining cells were pelleted, washed, and placed in drug-free medium; genome levels for the same cultures were determined on day 22.

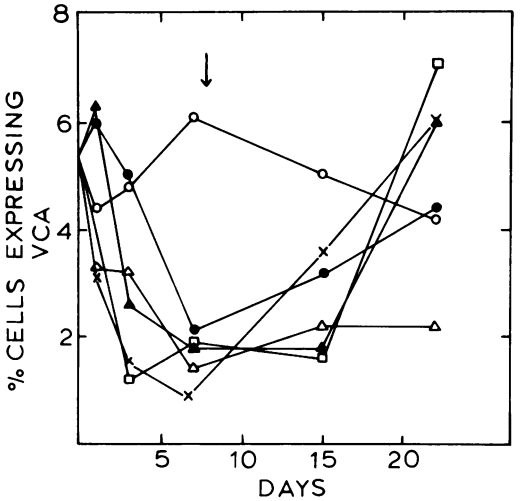

FIG. 5. *Effect of ACV on VCA expression in P3HR-1 cells. Cells were treated for 7 days with ACV at concentrations of 1 μM (●), 10 μM (△), 100 μM (▲), 500 μM (□), and 1,000 μM (×). Mock-treated cells (○) were also assayed for VCA expression. Cells were harvested and suspended in drug-free medium on day 7 (arrow); the cultures were monitored for VCA until day 22.*

Characterization of EBV DNA remaining in P3HR-1 cells after drug treatment. After 10 days, some EBV DNA remained in ACV-treated P3HR-1 cells. The EBV DNA remaining was characterized by isopycnic centrifugation and electron microscopy. Figure 6A shows that a significant quantity of the ACV-resistant viral DNA banded at the position expected for covalently closed circular molecules (1.59 g/cm^3) when analyzed by ethidium bromide-cesium chloride density gradient centrifugation. Some EBV DNA banded between 1.56 and 1.57 g/cm^3, the position of open circular and linear DNAs. The EBV DNA from the peak representing covalently closed circular DNA was isolated and fractionated on a second cesium chloride density gradient without ethidium bromide (Fig. 6B). This DNA rebanded as a single peak at the bouyant density expected for EBV DNA; the DNA from this peak was isolated and visualized by electron microscopy (Fig. 7 and 8). Figure 7 shows a covalently closed circular supercoiled DNA molecule recovered from drug-treated P3HR-1 cells, and Fig. 8 shows a nicked circular DNA molecule isolated from the same preparation.

Analysis of EBV DNA by electron microscopy. Contour lengths and corresponding molecular weights were determined for 22 circular DNA molecules isolated from Raji cells and 21 circular molecules isolated from drug-treated P3HR-1 cells. The contour length of EBV DNA was determined relative to that of SV40 form II DNA. The mean lengths of 10 to 15 SV40 DNA molecules (standard deviation, 1.5%) which were measured within the same frame as the open circular EBV DNA molecules were used to calculate the molecular weight of EBV DNA. The molecular weight of SV40 DNA (3.4×10^6) was calculated for these studies from a precise nucleotide sequence analysis of SV40 DNA (9, 26). The mean values obtained for the molecular weights of EBV DNAs from Raji and ACV-treated P3HR-1 cells were $111 \times 10^6 \pm 1.8 \times 10^6$ and $108 \times 10^6 \pm 2.1 \times 10^6$, respectively. The distribution of molecular weights is shown in Fig. 9.

DISCUSSION

ACV has little cytotoxicity for lymphoblastoid cells at the concentration (100 μM) necessary for complete inhibition of EBV DNA synthesis. A slight delay in cell replication is noted for Raji and P3HR-1 cells immediately after suspension in medium containing 100 μM ACV; however,

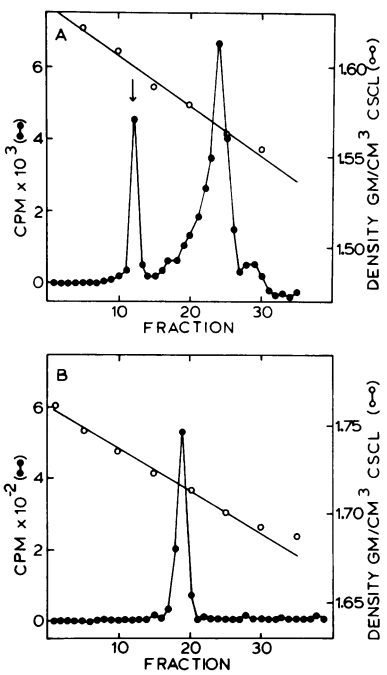

FIG. 6. *Ethidium bromide-cesium chloride (A) and cesium chloride (B) density gradient centrifugations of covalently closed circular EBV DNA remaining in P3HR-1 cells after treatment with 100 μM ACV. EBV DNA was localized by hybridization across the gradients with EBV-specific complementary RNA. The arrow in (A) indicates the position of covalently closed circular DNA which rebands in neutral CsCl (B) with a bouyant density of 1.718 g/cm^3.*

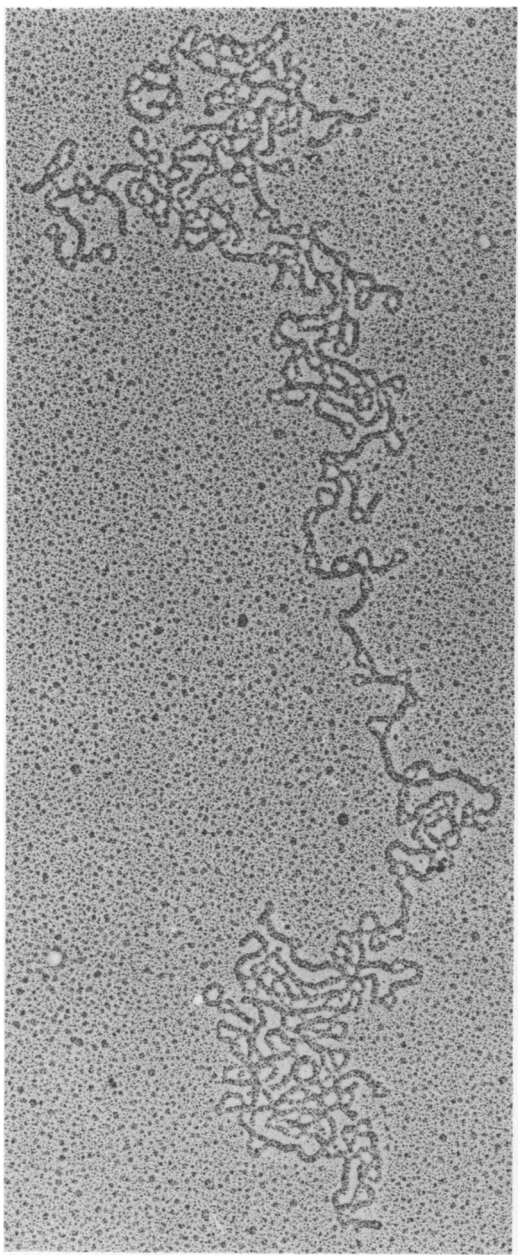

FIG. 7. *Electron micrograph of a covalently closed circular EBV DNA molecule isolated from P3HR-1 cells treated with 100 μM ACV. ×54,000.*

the cell densities return to control levels within 7 days in the continued presence of the drug (Fig. 1). Furthermore, in our laboratory P3HR-1 cells have been grown in medium containing 100 μM ACV for more than 1 year.

ACV completely inhibits EBV DNA synthesis in superinfected Raji cells and reduces the average number of EBV genomes per cell in P3HR-1 cultures to a maximum level of 14 in 72 to 96 h. In contrast, EBV DNA synthesis in Raji cells is not affected by ACV; the number of genomes per cell remains unchanged for at least 2 weeks in its presence. It has been reported previously that the number of latent EBV genome equivalents in P3HR-1 cells is approximately 11 after cells are treated with cycloheximide (33) or PAA (34). The close agreement between these results and ours implies that ACV inhibits only the productive replication of EBV.

In situ cytohybridization performed on P3HR-1 cells reveals the presence of significant levels of EBV DNA in 15 to 20% of the population. After drug treatment, no cells contained levels of EBV DNA detectable by the technique of in situ cytohybridization. This is consistent with our data, which demonstrate that approximately 14 EBV genomes per cell remain in P3HR-1 cultures treated with 100 μM ACV, a level well below the limits of detectability of cytohybridization. EA synthesis continues normally despite the fact that most cultures exhibit a decrease in the percentage of VCA^+ cells within 24 h after drug addition.

These results are comparable to those obtained by treating Raji, P3HR-1, and superinfected Raji cells with PAA (29, 32–34), although the effective dose of PAA was sevenfold greater than the concentration of ACV used in these studies.

As ACV and PAA inhibit only the productive replication of EBV, they may have similar mechanisms of action, even though these two antiviral compounds are structurally dissimilar. It will be interesting to determine whether ACV or its phosphorylated derivatives inhibit EBV DNA replication by binding to a virus-specific DNA polymerase, as has been suggested for the mechanism of action of PAA in the herpes simplex virus (HSV) system (20), or whether it inhibits EBV DNA synthesis by chain termination. ACV, an acyclic nucleoside analog of guanosine, is phosphorylated in HSV-infected cells to mono-, di-, and triphosphates (4). ACV is also phosphorylated in superinfected Raji cells, but the level of phosphorylation is considerably lower than that observed in HSV-infected cells (unpublished data). The formation of ACV monophosphate in HSV-infected cells is a result of virus-induced thymidine kinase activity in these cells (4). A similar enzyme which phosphorylates ACV in the EBV system has not been found (unpublished data).

Different DNA polymerases may replicate EBV DNA in productively infected and latently infected cells. ACV has no inhibitory effect on the incorporation of label into DNA in Raji cells

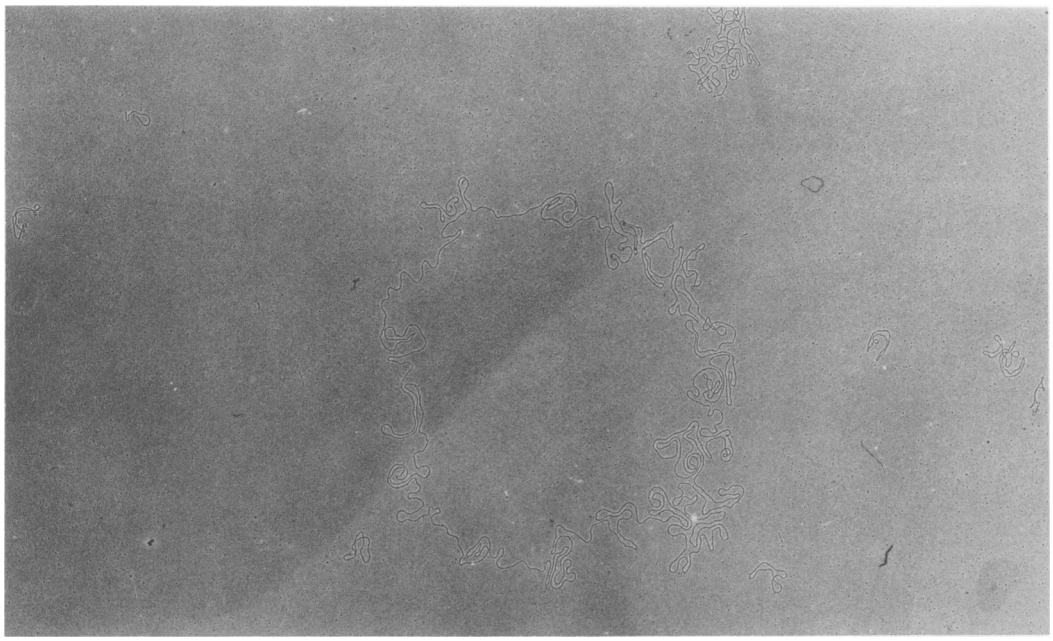

FIG. 8. *Electron micrograph of an open circular EBV DNA molecule after spontaneous conversion from covalently closed circular form. ×15,000. The open circular form of SV40 was included as an internal size reference.*

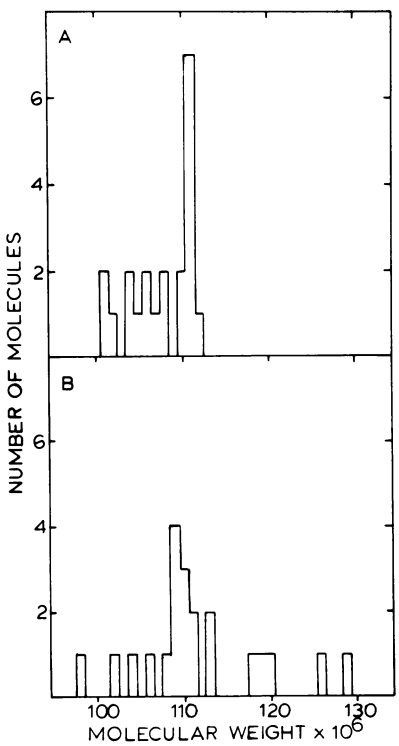

FIG. 9. *Size distribution of circular EBV DNA molecules isolated from P3HR-1 (A) and Raji (B) cell lines.*

(Fig. 2B). Perhaps EBV DNA synthesis in Raji cells is resistant to ACV because DNA replication is dependent on host rather than viral DNA polymerases.

After superinfection, host DNA synthesis in ACV-treated Raji cells is inhibited. Inhibition occurs even though ACV blocks the replication of EBV DNA (Fig. 2A). This result suggests that the inhibition of host DNA synthesis during superinfection is an early event which precedes the synthesis of viral DNA. This hypothesis is consistent with previous findings which show that both events occur at about the same time (22). Another possibility is that ACV inhibits host DNA synthesis in superinfected Raji cells.

The ED_{50} for the replication of EBV DNA in superinfected Raji cells was 7 μM. This value agrees well with 6 μM, the concentration required for a 50% reduction in the average genome number of P3HR-1 cells. ED_{50} values for HSV type 1 (based on a 50% reduction in infectious titer, as determined by plaque assay) are 0.1 μM in Vero cells (4) and 0.7 μM in HeLa cells (3). The variation in dose response observed for ACV in the HSV and EBV systems could be attributed to the different cell systems and methods used to measure the ED_{50}. In support of this idea is the recent report that the ED_{50} for HSV DNA replication in infected Vero cells is approximately 1 μM (10), a value slightly lower than that observed by us for EBV DNA repli-

cation (determined by the same method).

A significant quantity of the EBV DNA remaining in P3HR-1 cells after drug treatment is covalently closed circular DNA. This possibly represents the nucleosomal EBV DNA recently found in P3HR-1 cells (30). The viral episomes have an average molecular weight of 108×10^6, as determined by contour length measurements. This is close to the molecular weight (111×10^6) which we obtained for the episomal DNA of the non-virus-producing cell line Raji. A previous estimate for the molecular weight of EBV DNA from Raji cells was 106×10^6 (19). However, this measurement was determined by prior selection of DNA from glycerol gradients. We may have detected the larger forms of molecules in Raji cells because our isolation procedure did not preselect covalently closed EBV DNA molecules on the basis of size. The presence of covalently closed circular EBV DNA with an average molecular weight of 100×10^6 in PAA-treated P3HR-1 cells has been reported recently (11). Our molecular weight values are based on SV40 DNA as the internal size reference. The values reported previously for Raji and P3HR-1 circular viral DNAs were determined by using PM2 DNA as the size reference.

In conclusion, ACV inhibits the productive replication of EBV DNA but has no apparent effect on the latent EBV genomes in cultured lymphoblastoid cell lines. Consistent with this fact, it has been possible to demonstrate the presence of ACV-resistant covalently closed circular EBV DNA in the P3HR-1 cell line. Because of its low cytotoxicity, ACV is a useful tool for the elucidation of cellular and viral processes related to EBV replication and to viral DNA persistence in latently infected cells. Its potential as a clinically useful antiherpetic agent is currently under assessment.

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