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Effects of the Nucleoside Analog 2′-Nor-2′-Deoxyguanosine on Human Cytomegalovirus Replication

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The nucleoside analog 2′-nor-2′-deoxyguanosine (2′NDG) effectively inhibits the replication of several laboratory and clinical isolates of human cytomegalovirus. These isolates included viruses obtained from congenitally infected infants and patients suffering from acquired immune deficiency syndrome. The dose of 2′NDG that inhibited cytomegalovirus plaque formation ranged from 0.1 to 1.6 μg/ml. At 10 μg/ml, 2′NDG completely blocked the production of virus progeny but not the expression of immediate early and early virus gene functions. Cytomegalovirus DNA was not detectable in 2′NDG-treated virus-infected human embryo lung cells when assayed by CsCl density gradient centrifugation. In contrast, the guanosine analog acyclovir at 100 μg/ml did not inhibit the production of virus or the synthesis of cytomegalovirus DNA. In virus-infected cells, 2′NDG and acyclovir at 10 and 100 μg/ml, respectively, inhibited the incorporation of [^{3}H]thymidine and $^{32}P_i$ into cellular DNA by ca. 50%. Uninfected human embryo lung cells grown in these concentrations of acyclovir or 2′NDG exhibited a slightly transient lag phase but, overall, cell growth was not retarded, and there was no decrease in cell viability. The extended lag in cell division was not due to inactivation or breakdown of the antiviral compounds but may be due in part to a temporary decrease in cellular DNA synthesis.

Recently, a nucleoside analog of guanosine, 2′-nor-2′-deoxyguanosine (2′NDG), was reported to effectively inhibit herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) replication in a manner apparently similar to acyclovir (ACV) (1, 4, 8, 12). 2′NDG was thought to be ineffective in preventing human cytomegalovirus (CMV) replication (13). Like ACV, 2′NDG is efficiently phosphorylated by the HSV-1 virus thymidine kinase (TK) but is not a substrate for the cellular enzyme (1, 6). The monophosphate of 2′NDG is rapidly converted to the triphosphate by cellular kinases and then acts as a selective inhibitor of the HSV-1 DNA polymerase (6). Unlike HSV-1 and HSV-2, human CMV lacks a virus-specific TK (5, 20). Instead, human CMV induces elevated levels of the TK of the host cell (5). This fact circumvents the usefulness of antiviral nucleoside analogs, such as ACV, which rely on a virus-specific TK for activation (12). Our studies show that 2′NDG is a potent and effective inhibitor of human CMV replication. The results of these experiments contradict the findings reported by Smith et al. (13) and support the studies of Cheng et al. (2). The experiments described in this manuscript indicate that 2′NDG blocks CMV replication by preventing virus DNA synthesis. These experiments also provide evidence which supports previous studies (2, 6) suggesting a second mechanism of activation for 2′NDG that does not involve a virus-specific TK.

MATERIALS AND METHODS

Cells and viruses. Human embryonic lung (HEL) and MRC-5 cells were cultured in Dulbecco modified Eagle medium as mentioned previously (18). Viable cells, determined by trypan blue exclusion, were counted microscopically with a hemacytometer (11). Methods for the propagation of human CMV strains, as well as for HSV types 1 and 2, have been described elsewhere (16, 18). Virus infectivity was quantitated by plaque assay (19). Plaque reduction assays were performed in 60-mm plastic tissue culture dishes with ca. 100 PFU of virus per dish. Plates were incubated at least 7 days after infection before the plaques were counted.

Analysis of virus and cell DNA. The procedure for analyzing DNA was that described by Crouch and Rapp (3). Briefly, cells infected at a virus-to-cell ratio of 1 PFU per cell were pulse-labeled at various times after infection for 12 h in medium containing 10 μCi of [*methyl*-^{3}H]thymidine ([^{3}H]TdR) (New England Nuclear Corp.) per ml or 5 μCi of $^{32}P_i$ per ml. Cells were harvested at daily intervals, and the amount of [^{3}H]TdR or ^{32}P incorporated into acid-insoluble material was determined (16). Samples from ^{32}P-labeled cultures were treated with RNases A and T_1 and extracted with phenol three times to remove RNA. Equal amounts of each culture were analyzed by CsCl density centrifugation (3, 16, 18) for CMV and cell DNA (16).

Chemicals. ACV and 2′NDG were provided by Richard L. Tolman, Merck Sharp & Dohme Research Laboratories, Rahway, N.J. (1). Compounds were dissolved in phosphate-buffered saline (pH 11.0) or dimethyl sulfoxide and stored at −20°C. In all experiments, 2′NDG and ACV were added to the culture medium after the 1-h virus adsorption period.

RESULTS

Initial experiments demonstrated that 2′NDG was a potent inhibitor of HSV-1 and HSV-2 replication (1, 2, 6). Subsequent studies suggested that like ACV, 2′NDG was activated specifically by a herpes-specific TK (1, 6–8, 12) but not by the cellular TK. 2′NDG was also shown to be active against both the Towne and AD169 strains of human CMV (2, 6). In the following experiments, 2′NDG and ACV were tested in plaque reduction assays against several different strains of

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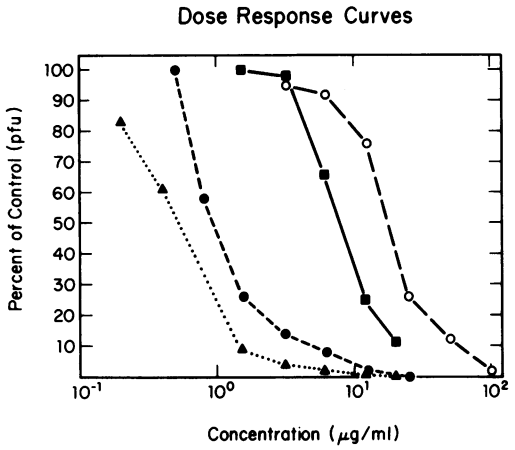

FIG. 1. Dose-response curves of 2'NDG and ACV for human CMV strains AD169 and Eisenhardt. Inhibition of CMV plaque formation on HEL cells was determined by plaque-reduction assay. Cells were infected with ca. 100 PFU of virus and individual cultures maintained in media containing increasing concentrations of either 2'NDG or ACV. The number of CMV plaques found in duplicate drug-treated cultures were counted and compared with control cultures that received no drug treatment, and the number of plaques was expressed as a percent of the control. Symbols: ▲, Eisenhardt strain plus 2'NDG; ●, AD169 strain plus 2'NDG; ■, Eisenhardt strain plus ACV; O, AD169 strain plus ACV.

human CMV. 2'NDG inhibited the replication of CMV AD169, a standard laboratory strain, as well as the Eisenhardt strain, a recent clinical isolate, in a dose-dependent manner (Fig. 1). These studies showed that 2'NDG is 10- to 100-fold more potent than ACV when tested against a number of laboratory and clinical CMV isolates (Table 1). The clinical isolates tested included viruses obtained from congenitally infected infants with cytomegalic inclusion disease (Eisenhardt and CHMC) and patients suffering from acquired immunodeficiency syndrome (AIDS-O.C. and AIDS-D.L.).

In the following experiments, 2'NDG and ACV were used at 10 and 100 μg/ml, respectively. At these concentrations, CMV plaque formation is inhibited by 95% or more. ACV at 10 μg/ml did not effectively inhibit CMV replication (Fig. 1). The addition of 2'NDG (10 μg/ml) to CMV-infected cell cultures (multiplicity of infection = 0.1 PFU per cell) after virus adsorption blocked the spread of CMV-specific cytopathic effects (CPE) to adjacent cells but did not block the appearance of CPE in infected cells (Fig. 2). Cells in the culture which were initially infected by CMV developed typical CMV-induced CPE, indicating that immediate early and, at least, partial early virus gene expression occurred in 2'NDG-treated cells (13, 18). The failure of CPE to spread to adjacent cells even after 1 week of incubation and the inhibition of CMV plaque formation suggested that late virus gene expression may be blocked in 2'NDG-treated cells.

To determine whether 2'NDG prevented CMV DNA synthesis, which is required for late gene expression (13, 18), confluent HEL cells were infected at a multiplicity of infection equal to 0.5 to 1.0 PFU per cell, pulse-labeled with [^{3}H]TdR at 12 to 24 h postinfection (p.i.), 36 to 48 h p.i., and 120 to 136 h p.i., and subsequently harvested at the end of each labeling period. During the same time periods, culture fluids were harvested for virus plaque assays. The incorporation of [^{3}H]TdR into DNA in 2'NDG- and ACV-treated cultures was significantly lower than in nontreated cultures (Fig. 3). To determine what effects these compounds had on virus or cell DNA or both, equal samples of DNA from the above cultures were analyzed on CsCl density gradients. The results of this experiment (Fig. 4) indicate that CMV DNA (ρ = 1.716 g/ml) is not detectable in CsCl density gradients from cultures treated with 2'NDG (Fig. 4A). In contrast, the incorporation of [^{3}H]TdR into virus DNA in ACV-treated cultures was depressed but not totally inhibited (Fig. 4B). When ACV was used at a concentration of 10 μg/ml, the incorporation of [^{3}H]TdR into CMV DNA was barely affected and CsCl gradient profiles were similar to those obtained when no drug was present (Fig. 4C). Analysis of cellular DNA peaks indicated that in drug-treated CMV-infected cultures, the incorporation of [^{3}H]TdR into cellular DNA was depressed at 24, 48, and 136 h p.i. by ca. 50%. The [^{3}H]TdR incorporation data from 136 h p.i. is shown in Table 2. In these experiments, 2'NDG completely inhibited the production of progeny virus as determined by plaque assay, whereas ACV reduced virus replication by ca. 10^3-fold (Fig. 5).

The previous experiments indicated that 2'NDG may affect the incorporation of [^{3}H]TdR into cellular DNA in infected cells. This result suggested that 2'NDG and ACV, at the concentrations used in these experiments, might inhibit cellular DNA synthesis or replication or both in normal uninfected HEL cells. In view of this, experiments were performed to determine the effects of 2'NDG on confluent and growing cells.

Confluent HEL cells were placed in media containing either 2'NDG (10 μg/ml), ACV (100 μg/ml), or phosphate-buffered saline and pulsed with [^{3}H]TdR for 24 h. Under these conditions, HEL cells should undergo only a single round of DNA replication. In these experiments, 2'NDG- or ACV-treated cultures incorporated ca. 20 ± 3% less [^{3}H]TdR than control cultures (Table 2). Cell viability was greater than 95% in all the cultures examined. Even after 5 days of observation, there was also no discernable decrease in total cell number in any of the cultures tested.

The effect(s) of 2'NDG and ACV on exponentially growing HEL cells was also tested. Cells seeded at ca. 1.5×10^5

TABLE 1. Activity of 2'NDG and ACV against human CMV strains

Virus	Source	ED_{50} (μg/ml)[a]	
		2'NDG	ACV
CMV Towne[b]	Urine	0.5	15
CMV Davis[b]	Urine	0.8	ND[d]
CMV AD169[b]	Adenoid	0.8	20
CMV Eisenhardt-CID[c]	Urine	0.5	8.0
CMV AIDS-D.L.[c]	Lung	0.24	26
CMV AIDS-O.C.[c]	Lung	1.40	33
CMV CHMC-CID[c]	Blood	1.50	17.5
HSV-1 Schooler[b]		0.1–0.2	0.4
HSV-2 Curtis[b]		0.4	0.8

[a] ED_{50}, 50% effective dose. The amount of drug required to inhibit CMV plaque formation by 50% when compared with control cultures not treated with drug. Assays were performed by infecting HEL or MRC-5 cells with ca. 100 PFU of virus per 60-mm culture dish. Cells were incubated for 7 to 14 days, fixed, and stained, and the plaques were counted. Values are the average of at least two independent experiments.

[b] Assay performed on MRC-5 cells.

[c] Assay performed on HEL cells. AIDS, Acquired immune deficiency syndrome. CID, Cytomegalic inclusion disease.

[d] ND, Not done.

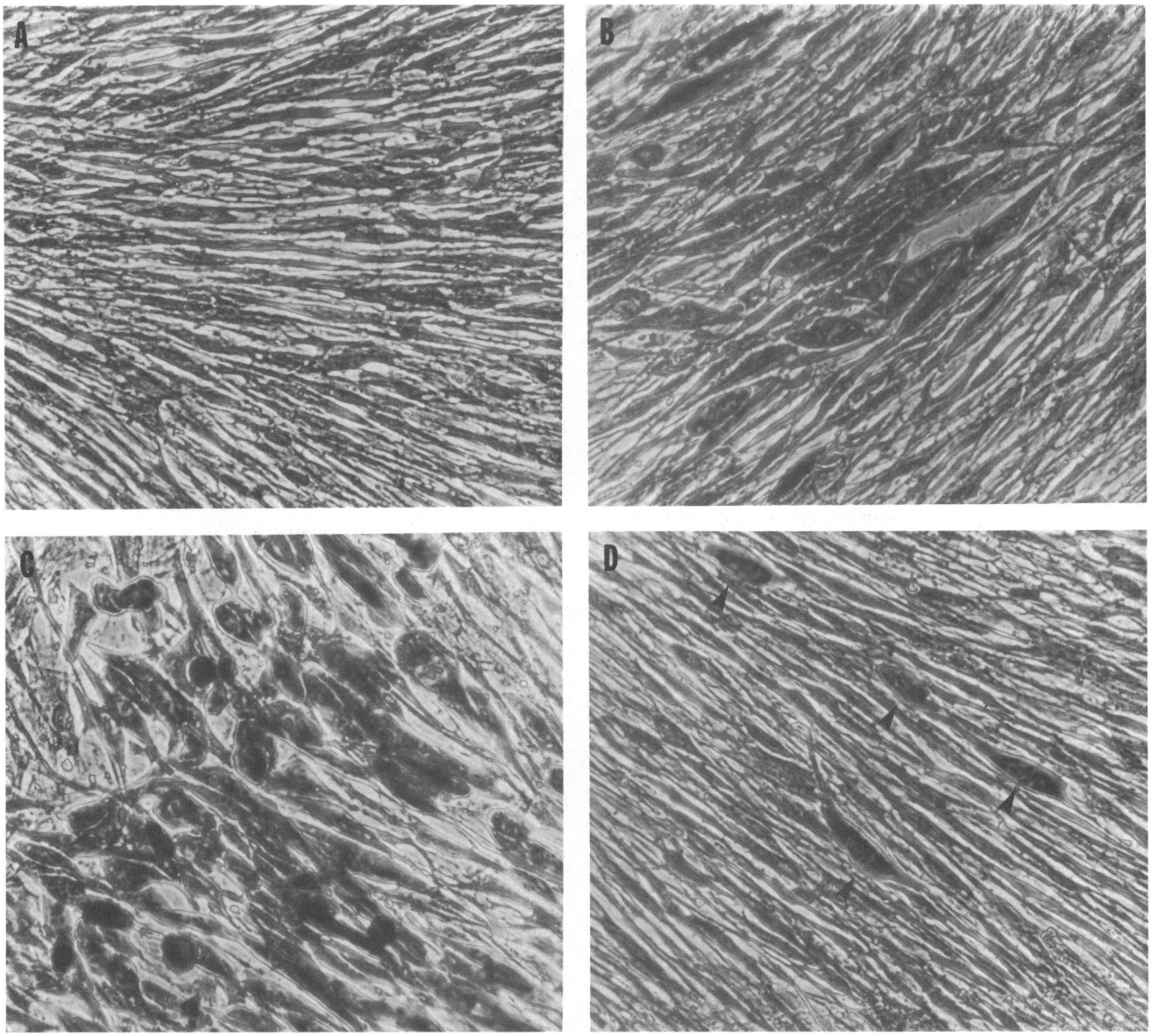

FIG. 2. Effects of 2'NDG and ACV on CMV-specific CPE. (A) Uninfected HEL cells. (B) CMV-infected HEL cells treated with 100 μg of ACV per ml, 7 days p.i. (C) CMV-infected HEL cells, 7 days p.i. (D) CMV-infected HEL cells treated with 10 μg of 2'NDG per ml, 7 days p.i. Cells infected with CMV or mock infected were placed in media containing either 2'NDG or ACV. Cultures were incubated for 7 days at 37°C. The cells were then fixed and stained with methylene blue. Arrows indicate individual cells or foci of cells showing CMV-specific CPE. Uninfected cells grown in the presence of either drug look similar to (A) and showed no CPE.

cells per flask and grown in the presence of 2'NDG or ACV remained viable throughout the experiment; however, cell growth in 2'NDG- and ACV-treated cultures lagged behind control cultures for ca. 3 to 4 days (Fig. 6). The lag in cell division appeared transient, and drug-treated cultures reached approximately the same cell density as untreated cultures after 4 or 5 days of incubation in the continuous presence of the drug. Total cell and viable cell number in 2'NDG- and ACV-treated cultures were greater than 90% of control cultures after 5 days of observation. If cells were fed each day with media containing fresh drug (ACV or 2'NDG), the same transient lag in cell division was observed.

To exclude the possibility that either ACV or 2'NDG interfered with thymidine metabolism, cells were labeled with $^{32}P_i$. Analysis of cell and virus DNA synthesis was performed as previously described with ^{32}P-labeled samples. The results obtained with $^{32}P_i$ were identical to those obtained with [^{3}H]TdR. Therefore, the effects observed with ACV and 2'NDG on CMV and cell DNA do not appear to be the result of reduced thymidine metabolism or uptake by drug-treated cells.

DISCUSSION

The results of our studies indicate that the nucleoside analog 2'NDG inhibits the replication of several laboratory and clinical isolates of human CMV by blocking virus DNA synthesis. In contrast with our data and those of Cheng et al. (2), a previous report (13) indicated that 2'NDG did not prevent CMV replication. These investigators monitored virus replication by CMV-specific immunofluorescence and

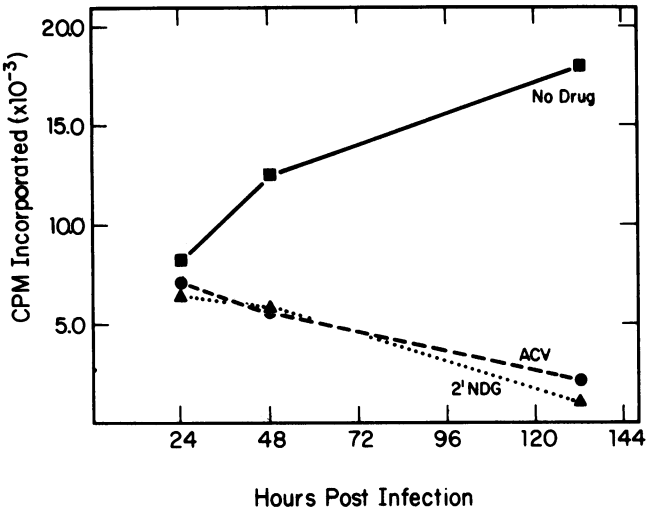

FIG. 3. DNA synthesis in 2'NDG- and ACV-treated CMV-infected HEL cells. CMV (Eisenhardt)-infected HEL cells were treated with 2'NDG, ACV, or phosphate-buffered saline and pulsed for 12 h at various times after infection with [^{3}H]TdR. The cell monolayers were rinsed three times with phosphate-buffered saline and lysed with pronase (2 mg/ml), Sarkosyl (1%), and 0.02 M EDTA. Portions of each sample were spotted on Whatman no. 1 filter paper disks, washed with 10% trichloroacetic acid, and counted in a liquid scintillation counter. Symbols: ■, control cultures treated with phosphate-buffered saline; ▲, 2'NDG (10 μg/ml); ●, ACV (100 μg/ml).

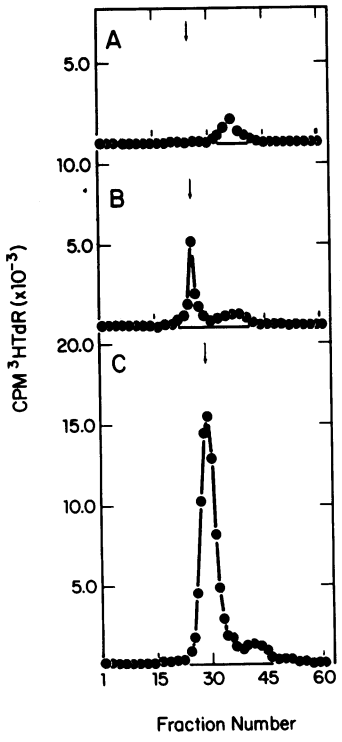

FIG. 4. Analysis of CMV DNA in drug-treated HEL cells. CMV-infected HEL cells were pulse-labeled for 24 h with [^{3}H]TdR at 48 h p.i. Cellular and virus DNAs were analyzed by isopycnic centrifugation in CsCl. (A) CMV-infected HEL cells treated with 10 μg of 2'NDG per ml. (B) CMV-infected HEL cells treated with 100 μg of ACV per ml. (C) CMV-infected HEL cells with no drug treatment. The arrow indicates the position of the CMV DNA marker (1.76 g/ml).

TABLE 2. Incorporation of [^{3}H]TdR into cellular DNA in infected and uninfected HEL cells

Cells	Treatment (μg/ml)	Acid-insoluble counts[a]	% of control[b]
CMV infected[c]	2'NDG (10)	6.31×10^3	48.9
	ACV (100)	6.68×10^3	51.1
	No drug	1.30×10^4	100.0
Uninfected[c]	2'NDG (10)	1.26×10^4	79.4
	ACV (100)	1.28×10^4	80.6
	No drug	1.58×10^4	100.0

[a] Counts were determined by trichloroacetic acid precipitation.
[b] Counts were determined by total number of counts incorporated into cellular DNA peaks from CsCl gradients. Data presented represent cellular DNA determinations from a 24-h pulse at 120 to 136 h p.i.
[c] Results represent the average of at least two independent experiments. Assays for each individual point were performed in triplicate.

radioimmunoassay, two methods which detect the expression of virus polypeptides. It is quite possible that these investigators were detecting CMV immediate early and early antigens which do not require virus DNA synthesis for their expression in 2'NDG-treated cell (15, 17). Our results show that 2'NDG inhibits the incorporation of $^{32}P_i$ and [^{3}H]TdR into CMV DNA and concomitantly blocks the production of virus progeny. The inhibition of CMV replication may be the result of selective inhibition of CMV DNA synthesis. Since CMV does not encode a virus-specific TK, one must postulate an alternative mechanism of action for 2'NDG in CMV-infected cells. Conceivably, 2'NDG could inhibit the CMV-specific DNA polymerase directly or be activated by another virus-specific or virus-modified host enzyme(s) which is as yet unidentified.

The uptake of ^{32}P and [^{3}H]TdR by untreated and drug-treated cells in these experiments was assumed to be a reflection of DNA synthesis. It can be argued, though, that the intracellular nucleotide pools are affected differently in ACV-treated, 2'NDG-treated and untreated cultures. La-

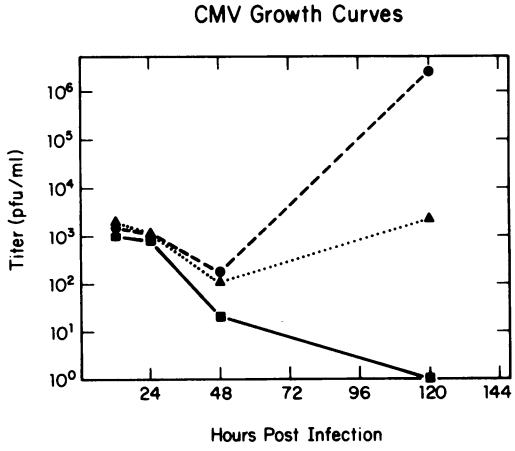

FIG. 5. CMV growth curves. Culture media containing extracellular virus was assayed at various times after infection from 2'NDG-treated HEL cells (10 μg/ml) (■), ACV-treated HEL cells (100 μg/ml) (▲), or control phosphate-buffered saline-treated HEL cells (●).

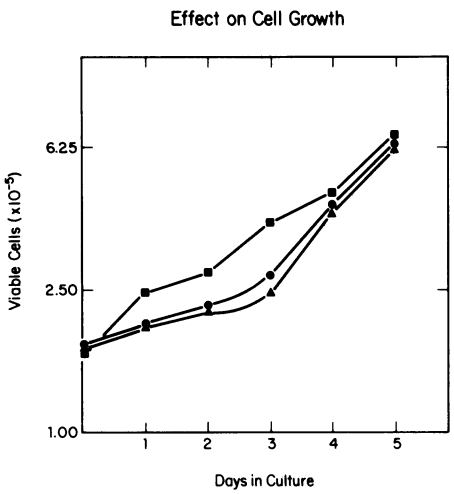

FIG. 6. The effects of 2'NDG and ACV on the growth of HEL cells. Cells were seeded at 1.5×10^5 per 60-mm tissue culture dish in the presence of 10 μg of 2'NDG (●) per ml, 100 μg of ACV (▲) per ml, or phosphate-buffered saline (■). Each day thereafter cells were trypsinized and counted with a hemacytometer.

beled precursors such as $^{32}P_i$ and [^{3}H]TdR must travel through several complex biochemical pathways before being incorporated into DNA. Even under the best of conditions, the labeled precursors represent only a very small fraction of the total material incorporated. Both ACV and 2'NDG are very poor substrates for the cellular TK. Therefore, in this instance, it is not clear if interference with intracellular nucleotide pools is occurring. The experimental results showing that $^{32}P_i$ and [^{3}H]TdR incorporation into DNA are similar between the respective samples in 2'NDG-treated, ACV-treated, and untreated cells suggest that incorporation may reflect synthesis in these studies. That is to say that incorporation did not appear to be affected by the labeled isotope. This contention is supported by the fact that as virus titer is reduced in ACV- and 2'NDG-treated cells, the amount of label incorporated into CMV DNA is also reduced.

The toxicity of 2'NDG in vitro appears similar to ACV at concentrations that give comparable antiviral activity. Cell division in growing HEL cells was slowed at relatively high doses of 2'NDG and ACV but did not result in cell death. This temporary lag did not appear to be due to drug breakdown. Ultimately, drug-treated cells reached the same cell density as untreated cells. The slower rate of cell division in logarithmic phase cells may be due in part to the slight decrease in cellular DNA synthesis as seen in pulse-labeling experiments of confluent HEL cells. In uninfected HEL cells, the incorporation of [^{3}H]TdR was depressed by ca. 20% in 2'NDG- and ACV-treated confluent cell cultures. Despite this observation, cell viability does not appear to be affected by either 2'NDG or ACV at the concentrations used in these experiments. By contrast, the incorporation of [^{3}H]TdR into cellular DNA in virus-infected cells was only 50% of that seen in untreated CMV-infected cultures. There are several possible explanations for this latter result. Nucleoside analogs such as these may (i) exhibit increased toxicity in virus-infected cells, (ii) selectively inhibit cellular enzymes modified by the virus, or (iii) be preferentially taken up by infected cells (7). Alternatively, these compounds may inhibit a CMV gene product that is responsible for the induction of cellular DNA synthesis and thus inhibit the incorporation of [^{3}H]TdR into DNA in infected cells to a greater extent than in uninfected cells.

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ADDENDUM

One week before the acceptance of this manuscript, a paper by Mar et al. appeared (Antimicrob. Agents Chemother. **24**:518–521, 1983) which presented results similar to those obtained in our experiments.

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