Synergistic Antiviral Activity of Acyclovir and Interferon on Human Cytomegalovirus

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The efficacy of human alpha interferon (IFN-α) combined with 9-(2'-hydroxyethoxymethyl)guanine (acyclovir; ACV), (E)-5-(2-bromovinyl)-2'-deoxyuridine, 9-β-D-arabinofuranosyladenine, or 1-β-D-arabinofuranosylcytosine on the inhibition of human cytomegalovirus (HCMV) replication in human embryonic lung cells was analyzed by plaque reduction assays. IFN-α combined with 9-β-D-arabinofuranosyladenine or 1-β-D-arabinofuranosylcytosine produced an additive antiviral activity with respect to HCMV plaque formation. IFN-α combined with (E)-5-(2-bromovinyl)-2'-deoxyuridine also exhibited additive antiviral activity. However, IFN-α combined with ACV at concentrations higher than 10 μM consistently yielded synergistic activity in HCMV plaque reduction assays. Kinetic analyses of HCMV replication demonstrated that approximately a 1,000-fold reduction can be attained through the synergistic interaction between ACV (200 μM) and IFN-α (42 IU/ml). These data suggest that combined ACV and IFN-α treatment may be useful against HCMV infection.

Human cytomegalovirus (HCMV) has been implicated in a wide range of clinical diseases, including intrauterine infections, perinatal infections, infections of immunosuppressed transplant and cancer patients, birth defects, and, more recently, acquired immunodeficiency disease syndrome and Kaposi sarcoma (2, 14, 20, 24). In general, treatment of HCMV with 1-β-D-arabinofuranosylcytosine (ara-C [25]), 9-β-D-arabinofuranosyladenine (ara-A [19]) or human alpha interferon (IFN-α [21]) has not been successful. In addition, the newly licensed drug 9-(2'-hydroxyethoxymethyl)guanine (acyclovir; ACV) has not been effective in the treatment of HCMV pneumonia (32).

Each of these compounds was initially shown to be an effective inhibitor of herpes simplex virus (HSV) and has been used to treat HSV infections (4, 5, 10, 13, 23). However, due to problems of low therapeutic indices and isolation of HSV resistant to these antiviral agents, studies have been undertaken to examine the potential of polychemotherapy in the treatment of HSV infections. Combination therapy has proven to be effective against bacterial infections and certain cancers (9, 26) and may allow decreased dosage and duration of treatment, thus reducing toxicity and the incidence of resistant virus strains.

Numerous in vitro studies have examined the type of interaction (additive, synergistic, or antagonistic) of various antiherpetic compounds when used in combination. Ara-A in combination with human beta interferon (IFN-β [3]) or ACV (27) has been shown to have an additive inhibitory effect on HSV replication. Synergistic inhibitory interactions have been reported with ara-A in combination with IFN-α (16) or (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU [27]). ACV combined with IFN-α (17) or IFN-β (29) has also been shown to have a synergistic inhibitory effect on HSV replication. However, the combined interaction of ACV with BVDU has been shown to be only additive (27). ACV in combination with BVDU has also been demonstrated to have only an additive inhibitory effect on the replication of varicella-zoster virus (1). In addition, the anti-varicella-zoster virus effect of ACV combined with trifluorothymidine has been shown to be additive. However, ACV in combination with ara-A has been shown to have additive to synergistic inhibitory effects on varicella-zoster virus replication (1).

Similar studies have been performed with HCMV, the majority of which examined ACV in combination with other antiviral agents. The anti-HCMV effect of ACV combined with IFN-α (17) or IFN-β (28) has been demonstrated to be additive and, depending on the HCMV strain, to be additive to synergistic when combined with phosphonoformic acid or trifluorothymidine (28). Although BVDU is an effective inhibitor of HSV and is relatively noncytotoxic (8), few studies have tested the effect of BVDU on

HCMV replication when used in polychemotherapeutic regimens.

These studies were undertaken to further examine the type of interactions involved in combination chemotherapy of HCMV-infected cells. Reports that ACV in combination with human IFNs produces at least additive inhibitory effects on HCMV replication (17, 28) prompted our investigation of the combined use of ACV and IFN-α in plaque reduction and replication kinetic assays. Since ara-A combined with IFN-α or BVDU has been shown to act in a synergistic manner to inhibit HSV (16, 27) but has not yet been examined against HCMV, in initial studies we used IFN-α combined with ara-A, BVDU, or ara-C to treat HCMV-infected cells.

MATERIALS AND METHODS

Cells and virus. Human embryonic lung (HEL) cells (Flow 2000; Flow Laboratories, Inc., McLean, Va.) were grown in Dulbecco medium containing 10% heat-inactivated fetal calf serum as previously described (6, 7, 30). Maintenance medium contained 5% fetal calf serum with supplements as previously described (6, 7). HCMV strain AD169 was obtained from the American Type Culture Collection, Rockville, Md. The virus was plaque purified, and stocks were prepared and titrated in HEL cells as previously described (6, 30).

Antiviral agents. Ara-C and ara-A were purchased from Sigma Chemical Co., St. Louis, Mo. IFN-α (specific activity, 6.5×10^5 IU/mg of protein) was obtained from Life Sciences, Inc., St. Petersburg, Fla. ACV and BVDU were generously provided by G. Elion, Burroughs Wellcome Co., Research Triangle Park, N.C., and E. De Clercq, Rega Institute for Medical Research, Katholieka Universiteit Leuven, Leuven, Belgium, respectively.

Plaque reduction assays. All plaque reduction assays were performed in 35-mm plates seeded with approximately 2.3×10^5 HEL cells. These cultures were incubated for 24 h before infection with 150 to 200 PFU of HCMV. After a 2-h virus adsorption period, the cultures were overlaid with 2 ml of 0.3% agarose medium supplemented with 5% fetal calf serum and no inhibitors, with IFN-α alone, or with ara-A, ara-C, ACV, or BVDU alone or in combination with IFN-α as previously described (33). The cultures were then incubated at 37°C, and every 72 h, 1.0 ml of fresh 0.3% agarose medium containing the appropriate concentration of the nucleoside analog was added. After incubation at 37°C for 10 days, the infected cultures were fixed with 5% Formalin in phosphate-buffered saline and stained with methylene blue. Discrete foci were counted under a dissecting microscope. The mean plaque number was calculated from quadruple samples and converted to a percentage of untreated controls.

Kinetic analysis of HCMV replication. Approximately 2.3×10^5 HEL cells were seeded in 35-mm plates, incubated for 24 h at 37°C, and infected with HCMV at a multiplicity of infection (MOI) of 3. After a 2-h adsorption period at 37°C, the cultures were washed twice with phosphate-buffered saline; overlaid with 3 ml of maintenance medium containing no inhibitors, IFN-α alone, ACV alone, or ACV in combination with IFN-α; and incubated at 37°C. At 72-h intervals, 1 ml

of medium with or without ACV was added to the remaining cultures. Triplicate samples were frozen after adsorption and at 24-h intervals and then stored at −70°C until the infectious virus content of each culture was determined.

The total virus content of each culture was determined as previously described (6, 30) with several modifications. Briefly, the cultures were frozen and thawed three times and sonicated, and cell debris was eliminated by centrifugation at $1,000 \times g$ for 10 min. The infectious virus was quantitated by plaque assay in HEL cells (30). The mean total virus content of the cultures was calculated and converted to a percentage of untreated controls.

Analysis of combined antiviral activity of IFN-α and nucleoside analogs. The combined HCMV inhibitory effect of IFN-α and nucleoside analogs was assessed by the criteria used by Spector et al. (28). $\bar{A}$ and $\bar{B}$ are the mean virus yields in the presence of antiviral agents A and B, respectively. Similarly, $\bar{A}\bar{B}$ and $\bar{V}\bar{C}$ are the mean virus yields produced in the presence of both A and B and in the absence of agents (virus control), respectively. When agents A and B are used in combination, their activities are defined as additive if $(\bar{A}/\bar{V}\bar{C})(\bar{B}/\bar{V}\bar{C}) = (\bar{A}\bar{B}/\bar{V}\bar{C})$. Assuming additive activity, this equation simplifies to $(\bar{A})(\bar{B})/(\bar{A}\bar{B})(\bar{V}\bar{C}) = 1$. Taking the natural log of this equation, $\ln(\bar{A}) + \ln(\bar{B}) - \ln(\bar{A}\bar{B}) - \ln(\bar{V}\bar{C}) = \ln(1) = 0$ for additive antiviral combinations. The numerical result of this final equation is defined as the combination index (CI). When $\ln(\bar{A}\bar{B})$ decreases, the CI increases and approaches synergism. However, if $\ln(\bar{A}\bar{B})$ increases, the CI decreases and approaches antagonism. The activity of two antiviral agents is defined as additive if the CI is within two standard errors (SE) of zero (0 + 2 SE ≥ CI ≥ 0 − 2 SE), as synergistic if the CI is more than two SE greater than zero (CI > 0 + 2 SE), or as antagonistic if the CI is more than two SE less than zero (CI < 0 − 2 SE).

RESULTS

Inhibitory effects of IFN-α combined with nucleoside analogs on HCMV plaque formation. Plaque reduction assays were performed for preliminary assessment of the antiviral activity of IFN-α in combination with ara-A, ara-C, BVDU, or ACV on HCMV replication in HEL cells. Those concentrations of the nucleoside analogs that exhibited minimal toxicity to confluent cultures of HEL cells were combined with low levels of IFN-α.

Initial studies demonstrated that HCMV plaque formation was inhibited to the same degree in HEL cells pretreated with IFN-α for 24 h before HCMV infection and the nucleoside analog addition as in cultures that received IFN-α with the nucleoside analog immediately after virus adsorption (data not shown). Therefore, in all experiments, IFN-α was added with the nucleoside analog after the virus adsorption period. The data presented in the tables were calculated from at least three different experimental determinations. The average percentage of HCMV plaque formation, relative to controls,

varied by a standard deviation of 10 to 15%.

It was apparent that little, if any, additional antiviral activity could be attained by a 2- or 10-fold increase in the concentration of ara-C or ara-A, respectively (Table 1). Due to cellular toxicity, it was not possible to examine further increases in the concentration of these nucleosides. However, noncytotoxic concentrations of ara-A or ara-C when combined with IFN-α had an additive effect on the inhibition of HCMV plaque formation. The number of SE from zero for the combination indices was within two. Maximal HCMV plaque reduction was attained by treatment with 10 μM ara-A combined with 32 IU of IFN-α per ml or 0.02 μM ara-C combined with 16 IU of IFN-α per ml, which reduced HCMV plaque formation to approximately 20% of that observed in untreated control cells.

Similar results were obtained in plaque reduction assays in which BVDU was combined with IFN-α (Table 2). IFN-α in combination with BVDU acted in an additive manner to inhibit HCMV plaque formation. At all concentration combinations of BVDU and IFN-α, the combination indices were within two SE from zero. Combinations of 20 or 75 μM BVDU with 32 IU of IFN-α per ml inhibited HCMV plaque formation to approximately the same degree (85% relative to untreated controls).

ACV at 10 μM in combination with IFN-α also demonstrated additive antiviral activity (Table 2). However, synergistic effects with IFN-α were obtained consistently by increasing the ACV concentrations, independent of the IFN-α concentration. Maximal HCMV plaque reduction was obtained at 75 μM ACV, with cultures exhibiting fewer than 10% of plaques formed in control cultures. Since ACV at concentrations above 10 μM acted in a synergistic manner in combination with IFN-α and was relatively noncytotoxic to HEL cells, these two agents were used to examine the inhibition of the HCMV replication cycle.

Inhibitory effect of combined IFN-α and ACV treatment on HCMV replication. The plaque reduction assay is a common method used for the evaluation of antiviral compounds. However, in this assay only very few cells are infected, and it is difficult to quantitate the overall effect of the inhibitor. Therefore, virus growth studies were carried out to more accurately quantitate the inhibitory effect of combined ACV and IFN-α treatment on HCMV. Based on the results of the plaque reduction assays, these studies were first performed at the lowest concentrations of ACV and IFN-α that exhibited synergistic antiviral activity (20 μM and 16 IU/ml, respectively).

Representative analyses of three growth ki-

TABLE 1. Combined antiviral activity of ara-A or ara-C with IFN-α on HCMV plaque formation

Nucleoside	Concn (μM)	IFN-α (IU/ml)	% of control[a]	CI[b]	2 × SE[c]	Combined effect[d]
Ara-A	1.0		75.8			
	10.0		64.7			
		16	53.7			
		32	39.6			
	1.0	16	52.0	−0.245	0.328	Additive
	1.0	32	33.9	−0.174	0.304	Additive
	10.0	16	42.0	−0.189	0.513	Additive
	10.0	32	22.4	−0.018	0.364	Additive
Ara-C	0.01		71.7			
	0.02		48.9			
		8	62.5			
		16	46.4			
	0.01	8	44.9	−0.003	0.321	Additive
	0.01	16	33.1	−0.454	0.876	Additive
	0.02	8	36.5	−0.177	0.344	Additive
	0.02	16	17.4	−0.102	0.886	Additive

[a] Percentage of HCMV plaques formed in the presence of the nucleoside and IFN-α relative to the number of plaques formed in the absence of inhibitors.
[b] Computed as described in text.
[c] Twice the SE (see text).
[d] Combined inhibitory effect of the nucleoside and IFN-α on HCMV plaque formation (see text).

TABLE 2. Combined antiviral activity of BVDU or ACV with IFN-α on HCMV plaque formation

Nucleoside	Concn (μM)	IFN-α (IU/ml)	% of control[a]	CI[b]	2 × SE[c]	Combined effect[d]
BVDU	10		75.7			
	20		70.7			
	75		47.0			
		16	45.6			
		32	36.6			
	10	16	30.2	0.135	0.355	Additive
	10	32	21.9	0.056	0.870	Additive
	20	16	29.9	0.074	0.467	Additive
	20	32	15.0	0.397	0.965	Additive
	75	16	21.1	0.005	1.029	Additive
	75	32	14.8	0.180	1.157	Additive
ACV	10		76.2			
	20		65.0			
	75		16.0			
		16	47.0			
		32	42.0			
	10	16	29.9	0.175	0.262	Additive
	10	32	28.6	0.147	0.454	Additive
	20	16	17.5	0.557	0.235	Synergistic
	20	32	17.3	0.454	0.442	Synergistic
	75	16	9.5	1.504	0.246	Synergistic
	75	32	1.3	2.050	1.157	Synergistic

[a] Percentage of HCMV plaques formed in the presence of the nucleoside and IFN-α relative to the number of plaques formed in the absence of inhibitors.
[b] Computed as described in text.
[c] Twice the SE (see text).
[d] Combined inhibitory effect of the nucleoside and IFN-α on HCMV plaque formation (see text).

netic studies in which 20 μM ACV and 16 IU of IFN-α per ml were used are shown in Fig. 1. By day 3 postinfection, exponential HCMV replication was observed in the presence of ACV, IFN-α, or combined ACV and IFN-α. At days 3 to 5 postinfection, the total virus yield in cultures treated with combined ACV and IFN-α remained within approximately 20% of the control, and the inhibitor interaction was additive (Table 3).

Plaque reduction assays indicated that the additive interaction of ACV and IFN-α could be changed to a synergistic interaction by increasing the concentration of ACV. Therefore, the effect of increased concentrations of ACV and IFN-α (100 μM and 42 IU/ml, respectively) on HCMV replication was determined. The virus yield in cultures treated with both agents at these increased concentrations resulted in approximately a 20-fold increase in the inhibition of HCMV replication relative to that observed with 20 μM ACV and 16 IU of IFN-α per ml

during days 3 to 5 postinfection (Fig. 2). Thus, the replication of HCMV in HEL cells treated with 100 μM ACV and 42 IU of IFN-α per ml was approximately 1% of the untreated control cells. Depending on the time postinfection, the degree of inhibition of HCMV replication was the result of an additive to synergistic interaction between ACV and IFN-α (Table 3).

A comparison of the HCMV replication kinetics (Fig. 1 and 2) shows that increasing the concentration of IFN-α alone from 16 to 42 IU/ml had little or no effect on the degree of inhibition observed and that a maximal effect appeared to be attained at concentrations of between 16 and 42 IU/ml. Increasing the ACV concentration from 20 to 100 μM greatly decreased the HCMV yield in infected cells treated with ACV alone. However, the anti-HCMV effect of 42 IU of IFN-α per ml combined with 100 μM ACV fluctuated between additive and synergistic on each day during the exponential phase of the replication cycle. Subsequently,
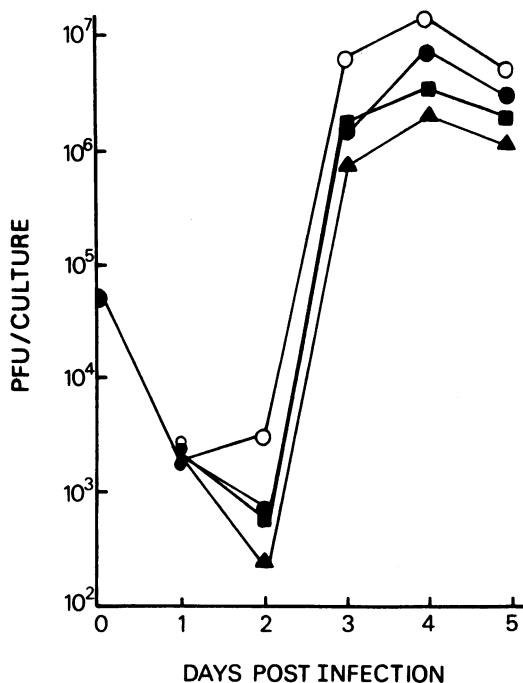
FIG. 1. HCMV replication in HEL cells treated with 20 μM ACV and 16 IU of IFN-α per ml. Cell cultures were infected with HCMV (MOI = 3) and incubated with medium containing no inhibitors (O), 16 IU of IFN-α per ml (●), 20 μM ACV (■), or 16 IU of IFN-α per ml combined with 20 μM ACV (▲). At time zero postinfection and at 24-h intervals, the total virus content in triplicate samples was determined, and the average value was plotted.

growth studies were performed at an increased concentration of ACV (200 μM) combined with the same concentration of IFN-α (42 IU/ml) to determine whether an increased inhibition of

HCMV could be attained while maintaining a synergistic interaction between ACV and IFN-α.

As shown in Fig. 3, 200 μM ACV in combination with 42 IU of IFN-α per ml resulted in extensive inhibition of HCMV replication during days 3 to 5 postinfection. Relative to untreated control culture, HCMV titers in cells treated with ACV and IFN-α were approximately 3 logs lower. In each of three experiments, this greater inhibition was the result of synergistic interaction between ACV and IFN-α throughout the replication cycle (Table 3) and was not due to drug cytotoxicity. Both trypan blue dye exclusion and plating efficiency studies demonstrated that ACV (200 μM) and IFN-α (42 IU/ml) in combination were relatively noncytotoxic to HEL cells incubated over a 6-day period under these experimental conditions.

DISCUSSION

Antiviral chemotherapy is used in an attempt to inhibit virus replication without impeding host cell functions. One approach to this problem has been the simultaneous application of two antiviral agents. When agents differ in their modes of action, a synergistic antiviral effect may occur, allowing the use of a lower dose of each drug to minimize toxicity. Polychemotherapy for the treatment of HCMV infections is a strategy worthy of investigation since to date there is no effective single-agent therapy. In addition, HCMV has been shown to be several times more resistant than HSV to antiherpes agents such as ACV and trifluorothymidine (15, 31). The experiments reported here were designed to test whether IFN-α in combination with nucleosides demonstrated synergistic antiviral effects on the replication of HCMV.

Our results demonstrate that IFN-α combined

TABLE 3. Combined antiviral activity of ACV and IFN-α

Days postinfection	ACV (μM)	IFN-α (IU/ml)	% of control[a]	CI[b]	2 × SE[c]	Combined effect[d]
3	20	16	20.3	0.015	0.255	Additive
4			19.3	0.044	0.639	Additive
5			24.5	0.307	0.440	Additive
3	100	42	0.7	0.941	0.068	Synergistic
4			1.0	0.006	0.081	Additive
5			0.6	0.810	0.152	Synergistic
3	200	42	0.03	1.990	1.027	Synergistic
4			0.21	1.385	0.591	Synergistic
5			0.12	0.903	0.757	Synergistic

[a] Percentage of HCMV yield in the presence of the nucleoside and IFN-α relative to the yield titer in the absence of inhibitors.
[b] Computed as described in text.
[c] Twice the SE (see text).
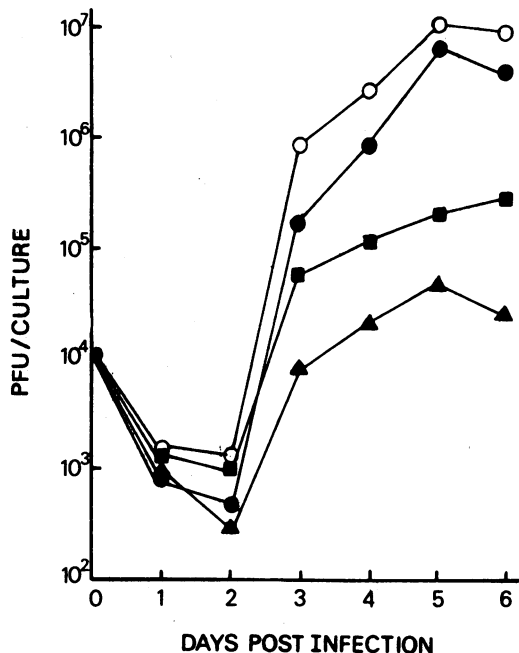[d] Combined inhibitory effect of the nucleoside and IFN-α on HCMV replication (see text).

FIG. 2. HCMV replication in HEL cells treated with 100 μM ACV and 42 IU of IFN-α per ml. Cell cultures were infected with HCMV (MOI = 3) and incubated with medium containing no inhibitors (O), 42 IU of IFN-α per ml (●), 100 μM ACV (■), or 42 IU of IFN-α per ml combined with 100 μM ACV (▲). At time zero postinfection and at 24-h intervals, the total virus content of triplicate samples was determined, and the average value was plotted.

with ara-A or ara-C at all concentrations tested had additive antiviral effects, resulting in a maximum of 80% HCMV plaque reduction relative to controls. Higher concentrations of these agents in combination were cytotoxic and could not be examined. These results indicate that IFN-α combined with ara-A or ara-C would not be useful as a synergistic combination treatment of HCMV infection.

Examination of BVDU inhibition of HCMV demonstrated that, when used as a single agent, 10 μM was required for 50% plaque reduction (data not shown). Therefore, the sensitivity of HCMV to BVDU was approximately 3- or 300-fold less than the sensitivity reported for HSV-2 or HSV-1, respectively, but 30-fold greater than that reported for thymidine kinase-deficient HSV type 1 (8). In general, BVDU antiviral interactions with IFN-α were additive, resulting in 70 to 85% inhibition of HCMV plaque formation. These results do not preclude a possible synergistic interaction between BVDU and IFN-α at higher noncytotoxic concentrations. We did not pursue this possibility because the efficacy of combined ACV and IFN-α appeared to be greater in the plaque reduction analyses.

ACV in combination with IFN-α consistently exhibited synergistic antiviral effects in plaque reduction assays at ACV concentrations above 10 μM. These results contrast with previous reports of additive inhibitory effects with ACV and human IFN on HCMV plaque formation (17, 28). This discrepancy may be due to either the use of IFN-α as opposed to IFN-β (28) or the addition of 1 ml of medium containing ACV at 72-h intervals over a 10-day incubation period versus a 4- to 7-day incubation period with no addition of ACV to the medium (17, 28). The necessity to supplement cultures with additional ACV to demonstrate inhibition of HCMV plaque formation by ACV corroborates the finding that ACV exerts only a transient 48- to 72-h inhibition on the synthesis of two HCMV-specific late polypeptides (18). Another source of discrepancy for the demonstration of an additive or synergistic antiviral effect is the MOI. In plaque reduction assays synergism was observed at concentrations of 20 μM ACV and 16 IU of IFN-α per ml. In contrast, in growth kinetic studies in which a much higher MOI was used, these
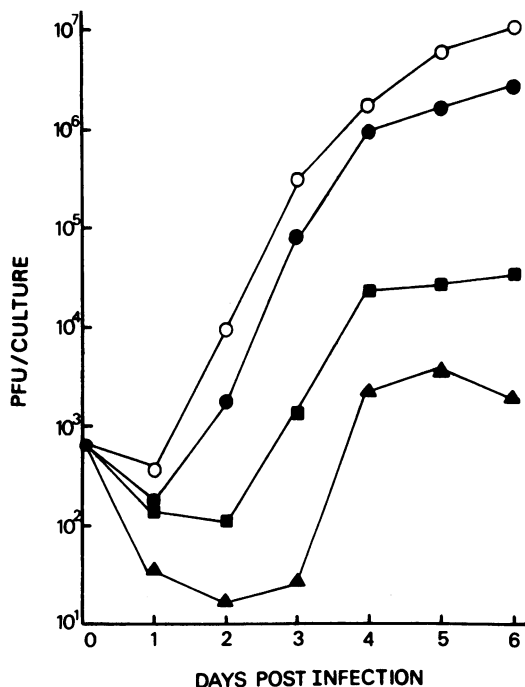

FIG. 3. HCMV replication in HEL cells treated with 200 μM ACV and 42 IU of IFN-α per ml. Cell cultures were infected with HCMV (MOI = 3) and incubated with medium containing no inhibitors (O), 42 IU of IFN-α per ml (●), 200 μM ACV (■), or 42 IU of IFN-α per ml combined with 200 μM ACV (▲). At time zero postinfection and at 24-h intervals, the total virus content of triplicate samples was determined, and the average value was plotted.

concentrations were not sufficient for synergistic inhibition of HCMV replication.

The exact mechanism by which ACV inhibits HCMV replication is unknown. Elion et al. (12) have demonstrated that the high potency and selectivity of ACV for HSV are due to the ability of these viruses to code for a viral thymidine kinase which has the capability to phosphorylate ACV to the nucleoside monophosphate, whereas cellular thymidine kinase does not have this capability. For HCMV, which does not specify its own thymidine kinase (22, 34), the amount of ACV phosphorylated in infected cells is low (11). However, the HCMV polymerase is 10 times more sensitive than the cellular DNA polymerase to inhibition by the nucleoside 5'-triphosphate (11) which may contribute to inhibition of HCMV by ACV at noncytotoxic concentrations. In addition, ACV may inhibit HCMV by exerting a transient inhibition of the synthesis of two virus-specific late polypeptides (18).

The synergistic effect of ACV and IFN-α on HCMV may represent the combination of effects on two or more different pathways of viral inhibition. IFN appears to inhibit virus replication at the level of translation but does not seem to have a direct effect on viral DNA polymerase. Therefore, the synergistic antiviral effect of ACV combined with IFN-α may include the inhibition of HCMV at the level of both viral DNA replication and transcription.

These studies indicate that both the combined drug concentrations and the method of assay are important factors in determining whether agents have potential synergistic polychemotherapeutic value. Results obtained from investigations of combination treatment of in vitro HCMV infection suggest that at high but therapeutically attainable and tolerated levels in humans (21, 32), ACV combined with IFN-α warrants further examination as a treatment for clinical HCMV infection.

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