Inhibition of Human Herpesviruses by Combinations of Acyclovir and Human Leukocyte Interferon

MYRON J. LEVIN* AND PATRICIA L. LEARY

Laboratory of Clinical Microbiology, Sidney Farber Cancer Institute, Boston, Massachusetts 02115

Human leukocyte interferon in low concentrations (1 to 5 U/ml) enhanced the antiviral effect of acyclovir against herpes simplex virus, varicella-zoster virus, and cytomegalovirus grown in human fibroblasts. This occurred without additive inhibition of the division of human fibroblasts or proliferation of peripheral blood mononuclear cells. The combined antiviral effect was additive against clinical isolates of cytomegalovirus and was synergistic against clinical isolates of the other two viruses. The magnitude of the effect with cytomegalovirus was the same when laboratory and wild-type virus were compared. The persistence of varicella-zoster virus in the presence of acyclovir in infected human cells was also reduced by the addition of interferon.

The in vivo response in uncontrolled trials of acyclovir (ACV) for treating severe herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections correlates with the observation that these viruses are inhibited by ACV in vitro at concentrations readily obtained in serum by parenteral administration (3, 4). Oral therapy of HSV and VZV has just begun, and serum levels obtained by this route are lower and less predictable because of poor absorption (Burroughs Wellcome Co., unpublished data). Even with parenteral ACV therapy, some cases of severe HSV infection in immunocompromised patients recrudesce after treatment, presumably because virus has persisted in situ in spite of ACV (M. J. Levin and B. J. Hershey, unpublished data). Furthermore, the high drug concentrations required in vitro to inhibit another herpesvirus, cytomegalovirus (CMV), may indicate that ACV will not be useful in treating clinical CMV infections (3). Increasing the dose or the duration of ACV treatment, or both, may solve one or all of these problems. An alternate approach would be to use a combination of ACV and a second antiviral agent, providing that the other agent inhibits herpesviruses by a mechanism different from that of ACV and is not toxic. This report describes the inhibitory effect in vitro of combinations of human leukocyte interferon (HLI) with ACV on the growth of HSV, VZV, and CMV.

MATERIALS AND METHODS

Cells. A strain of human foreskin fibroblasts (350Q) derived by M. Myers was used at passage 17 to 22. Cell cultures were maintained in Dulbecco modified Eagle medium supplemented with fetal calf serum and antibiotics.

Virus. (i) HSV type I. Strain LA was isolated in human fibroblasts from a patient with disseminated disease. A cell-free pool was prepared by a single passage in 350Q cells, clarified after freeze-thaw, and frozen at −70°C at 4.6×10^7 plaque-forming units (PFU) per ml.

(ii) VZV. Strain FM was isolated in human fibroblasts from a patient with herpes zoster. Cell-associated virus (2.6×10^4 PFU/ml) was prepared by trypsinization and stored at −70°C in medium containing 8% dimethyl sulfoxide and 30% serum. Cell-free VZV was prepared from 350Q cultures which were scraped, sonicated (model W185, Heat Systems-Ultrasonics, Inc.; 60 W, 10 s), and clarified ($600 \times g$ for 10 min). The supernatant was stored in sorbitol (7%, final concentration) at −70°C at 1.24×10^3 PFU/ml.

(iii) CMV. A cell-free pool of laboratory strain AD 169 was prepared in human embryonic lung fibroblasts (58×10^3 PFU/ml) as described for VZV grown in 350Q cells. Cell-associated virus was prepared and stored (10^6 cells/ml; 3.3×10^4 PFU/ml) as described for VZV. Wild type-virus was isolated in human fibroblasts from the urine of patients with mononucleosis and passed three times before pools were prepared as described above.

Antiviral agents. ACV [9-(2-hydroxyethoxymethyl)guanine; BW 248U, acycloguanosine] was supplied by Burroughs Wellcome Co. A 10 mM stock solution of ACV was prepared in distilled water and stored at −20°C. HLI (Kaufman 73F 27/A) was obtained from June Dunnick, Antivirals Substance Program of the National Institute of Allergy and Infectious Diseases, Bethesda, Md., and titrated with a microtiter assay (6, 8), using human embryonic lung fibroblasts and purified vesicular stomatitis virus (Indiana strain). Titer was expressed in relation to the titer of our HLI standard (National Institutes of Health reference standard G023-901-527).

Determination of antiviral activity. Monolayers of 350Q cells were prepared in six-well (35 mm) plastic tissue culture plates (Linbro Division, Flow Laboratories, Inc.) by seeding each well with 3×10^5 cells in

3 ml. Four days later, confluent monolayers were drained and inoculated with approximately 100 PFU of HSV, VZV, or CMV in 0.1 ml of medium. After 2 h at 37°C, the inoculum was removed and the wells were refed with 3 ml of medium containing 2% serum plus 1% methyl cellulose for HSV, or medium and serum alone for VZV and CMV, plus concentrations of antiviral agents. After 4 days (VZV and HSV) or 7 days (CMV) at 37°C in 10% CO_2–90% air, the monolayers were fixed with 10% formaldehyde and stained with 1% crystal violet, and the plaques were counted. The amount of ACV required to reduce plaques by 50% (ID_{50}) from the number present in the control wells was calculated from a curve relating plaque number to the concentration of ACV in the medium.

Persistence of input virus. Confluent monolayers of 350Q cells were infected with VZV as described above. ACV, with or without HLI, was added to the medium. At intervals ranging from 1 to 9 days, the inhibitors were removed and the infected monolayers were washed twice with 3 ml of inhibitor-free medium and refed. Plaque number was determined 3 to 5 days later.

Cell growth experiments. Five milliliters of medium with 10% fetal bovine serum and 7.3×10^4 350Q cells was added to 60-mm tissue culture dishes (Falcon Plastics). Twenty-four hours later some of these dishes received ACV, HLI, or both in 0.05 ml. After an additional 24 or 72 h, triplicate plates at each inhibitor concentration were trypsinized. The viable cells, determined by trypan blue exclusion, were counted in duplicate with a Thoma hemacytometer.

Proliferation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation and suspended in RPMI 1640 with 10% pooled AB serum as previously described (7). One-milliliter

samples containing 10^6 cells were stimulated with phytohemagglutinin for 3 days or with herpes simplex antigen for 6 days (7). Six hours before harvest, duplicate cultures received 2.0 µCi of [methyl-^{3}H]thymidine. Triplicate 0.1-ml samples from each culture were harvested with a multiple automated sample harvester, and incorporation of radioactivity was determined.

RESULTS

Plaque reduction titrations were performed on each of three herpesviruses grown in the presence of HLI, ACV, or combinations of both (Fig. 1). Inhibition of plaque formation by HSV was very sensitive to both HLI and ACV. The 50% inhibitory dose (ID_{50}) of HLI was 1 U/ml, and that of ACV was 0.4 µM. The ID_{50} with ACV was similar to that previously published (3), whereas the ID_{50} with HLI was similar to that reported with fibroblast interferon (10). Because of the sensitivity of HSV to these agents, it was difficult to demonstrate their combined effect. However, the dose-response curve to ACV at concentrations in the range of 0.25 to 0.75 µM was clearly shifted to the left by 1 U of HLI per ml. Plaque number was reduced 75% by 0.75 µM ACV alone, whereas reduction of this magnitude was achieved with 0.10 µM ACV when it was combined with 1 U of HLI per ml. These results were obtained only when the fibroblasts were treated with HLI before HSV infection, and the results were the same when either 1 or 3 U of HLI per ml was added to the cultures with ACV (data not shown).

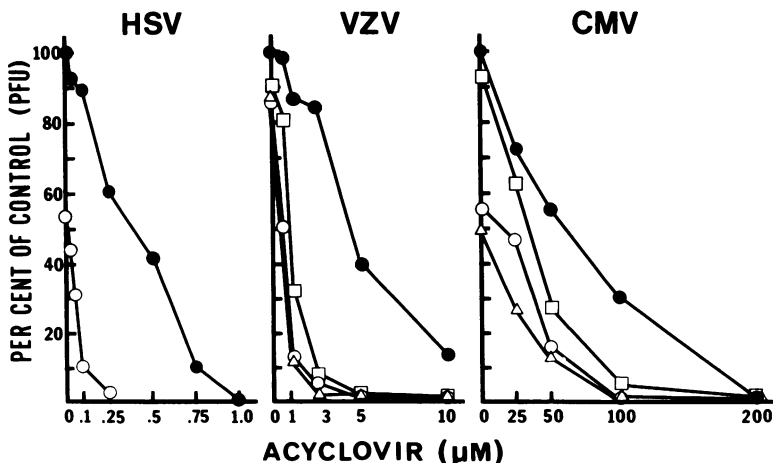The ID_{50} of ACV against cell-associated VZV

FIG. 1. *Inhibition of cell-free herpesviruses by ACV, HLI, and combinations of both. HSV: ●, ACV only present; ○, HLI (1 U/ml) with ACV. ACV was added after virus adsorption and HLI was added 18 h before virus adsorption. Plaque number in the control (no inhibitor) was 103. Each point represents the mean of duplicate wells. VZV: ●, ACV only present; □, HLI (1 U/ml) with ACV; ○, HLI (2.5 U/ml) with ACV; △, HLI (5 U/ml) with ACV. Plaque number in the control (no inhibitor) was 120. CMV: ●, ACV only present; □, HLI (0.6 U/ml) with ACV; ○, HLI (1.25 U/ml) with ACV; △, HLI (2.50 U/ml) with ACV. Plaque number in the control (no inhibitor) was 135.*

was 4 μM, and the ID_{50} of HLI was 17.5 U/ml. These values, determined from the data in Table 1, are similar to those previously reported (1, 3). The combined effect of both inhibitors was apparent from the 87% reduction in plaque number produced by the combination of ACV at 1 μM and HLI at 5 U/ml, compared with the small effect with either alone. The remainder of the dose-response curve of VZV to ACV was also shifted to the left when 5 U of HLI per ml was present simultaneously. The magnitude of the shift was similar when 1, 2, or 5 U of HLI per ml was present. Total plaque suppression was achieved only with the combination. To achieve this effect, it was not necessary to pretreat the fibroblasts with HLI. The same result was obtained with either cell-free or cell-associated VZV (data not shown). An alternate description of the combined inhibitory effect is to indicate that when one-quarter of the ID_{50} of each antiviral agent (i.e., 1 μM ACV and 4.4 U of HLI per ml) was combined, the mean plaque number was reduced to 15, which is less than the plaque number obtained with the ID_{50} of either alone (Table 1; Fig. 1). It is probable that the inhibitory effect of the combination was underestimated by the plaque reduction assay, because in the presence of combinations plaque size decreased markedly before there was a decrease in the number of colonies. Although this phenomenon was not reflected in the plaque reduction count, it presumably reflected partial inhibition of virus growth in the presence of both inhibitors.

Figure 1 depicts the effect of both antiviral agents on CMV replication. The laboratory strain (ID_{50} = 65 μM) showed a shift in the ACV dose-response curve when 0.60, 1.25, or 2.5 U of HLI per ml was added. As a result, a 75% reduction in plaque number in the presence of HLI was achieved at one-fourth the ACV dose required when HLI was not present. To achieve

this effect, it was not necessary to pretreat the fibroblasts with HLI. A 99% plaque reduction in the presence of 100 μM ACV was achieved only when both agents were present. The same results were obtained with cell-associated CMV. Most important, the magnitude of the effect of adding HLI was the same for wild-type and laboratory strains of CMV (data not shown).

The persistence of infectious virus in the presence of ACV was also influenced by the addition of HLI. Whereas 75 to 80% of input VZV remained viable in cultures containing 10 μM ACV for 9 days (Fig. 2), the quantity of persisting input virus was decreased four- to fivefold by the addition of 1 or 5 U of HLI per ml. HLI alone had no effect on persistence of input virus.

ACV at concentrations of 10 μM or greater inhibited the proliferative response of peripheral blood mononuclear cells to herpes simplex antigen (Table 2; 7). However, this effect was not potentiated by the addition of 2 or 5 U of HLI per ml.

The inhibitory effect of these antiviral agents was also determined on the fibroblasts used for the plaque reduction assays. Increase in cell number was measured at 24 and 72 h after plating cells in the presence of one or both
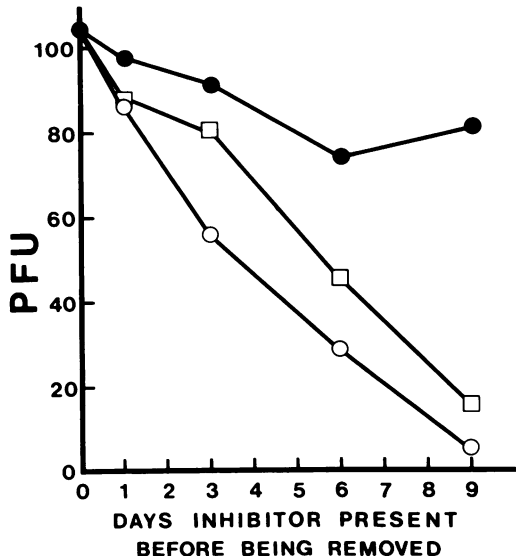
TABLE 1. *VZV plaque number in the presence of HLI and ACV*

ACV concn (μM)	Plaque no. with given interferon concn (U/ml)[a]					
	0	5	25	50	75	100
0	134/105	105/107	47/32	17/10	2/2	2/2
1	132/119	21/9	6/4	0	0	0
3	79/53	2/1	0	0	0	0
5	42/38	4/3	0	0	0	0
10	10/8	0	0	0	0	0

[a] Cells were pretreated with HLI 24 h before VZV was added. ACV was added with the VZV. Both inhibitors were present during the remainder of the experiment. Results are expressed as plaque number in duplicate wells containing inhibitors at the concentrations indicated.

FIG. 2. *Persistence of VZV in cultures containing ACV and HLI. Points represent PFU in wells infected with approximately 100 PFU of VZV and containing ACV or ACV plus HLI. The duration of time inhibitor is present is indicated on the horizontal axis. The wells were washed after inhibitor was removed, and the development of plaques was recorded 3 to 5 days later. Symbols: ●, ACV (10 μM) only present; □, HLI (1 U/ml) present with ACV; ○, HLI (5 U/ml) present with ACV.*

TABLE 2. *Effect of ACV, HLI, and combinations on the proliferative response of peripheral blood mononuclear cells to herpes simplex antigen*

HLI concn (U/ml)	[³H]thymidine incorporation[a] with given ACV concn (μM)				
	None	5	10	20	100
0	21,570 ± 2,734	19,260 ± 1,185	17,747[b] ± 1,055	16,258[b] ± 738	9,969[c] ± 1,116
2	18,892 ± 1,339	18,156 ± 2,002	16,493 ± 1,520	15,392 ± 940	9,961 ± 414
5	18,093 ± 947	19,127 ± 1,365	18,225 ± 618	16,845 ± 1,562	10,418 ± 767

[a] Mean counts per minute per filter with standard deviation; represents triplicate determinations on duplicate tubes at each inhibitor concentration. Incorporation in the presence of mock (uninfected tissue culture) antigen was less than 194 for three patients.
[b] Differs from control at $P < 0.005$.
[c] Differs from control at $P < 0.001$.

inhibitors (Table 3). As previously reported, ACV alone had an inhibitory effect on the cells exposed to ACV at 100 μM (7). HLI alone had no effect on fibroblast proliferation at the concentrations used, nor did it significantly increase the inhibitory effect of ACV on fibroblasts.

DISCUSSION

HLI in low concentrations enhances the in vitro antiviral effect of ACV against HSV, VZV, and CMV. This occurs with either a cell-free or a cell-associated inoculum, and the magnitude of the effect is the same for laboratory and wild-type virus. For HSV and VZV, the combined effect fulfills some arbitrary definitions of synergistic interaction, whereas the combined effect on CMV is additive (2). The addition of interferon does not increase the anticellular effect of ACV, even at high ACV concentrations which inhibit fibroblast division and mitogen- or antigen-induced proliferation of peripheral blood mononuclear cells. Thus, it is appropriate to consider clinical applications for combinations of ACV and interferon. The sensitivity of HSV to ACV would theoretically preclude any role for interferon when ACV is administered parenterally. However, recrudescence of HSV can occur in immunosuppressed patients after parenteral ACV therapy is stopped. If persistence of HSV during therapy of these patients can be influenced by adding a second effective antiviral agent, then dual therapy might be used. Our experiments indicate that the persistence of VZV in the presence of ACV in infected human cells is reduced by the addition of small amounts of HLI. Furthermore, therapy of HSV with orally administered ACV has been unreliable because of the low, irregular levels achieved by this route. Nevertheless, outpatient therapy might be accomplished by combining oral ACV with intramuscular interferon, since intramuscular administration of interferon produces easily detectable levels for extended periods (5). These comments also apply to the treatment of VZV infections,

TABLE 3. *Effect of ACV, HLI, and combinations on the growth of human fibroblasts at 72 h*

ACV concn (μM)	Cell no.[a] with given HLI concn (U/ml)		
	None	1	2.5
0	450 ± 40	402 ± 50[b]	432 ± 98[b]
10	430 ± 50	448 ± 44	388 ± 56[c]
100	358 ± 50[d]	324 ± 28	318 ± 40[e]

[a] Total cell count × 10³ per 60-mm tissue culture dish. The number indicated is the mean of three identical dishes each counted twice; the standard deviation is shown.
[b] Cell count in the presence of HLI differs from control ($P > 0.1$) at 1 and 2.5 U/ml.
[c] Cell count in the presence of HLI differs from count with 10 μM ACV alone at $P > 0.1$.
[d] Differs from control (no ACV) at $P < 0.05$.
[e] Cell count in the presence of HLI differs from count with 100 μM ACV alone at $P > 0.1$.

even though VZV is less sensitive to ACV. The therapy of CMV infection presents a different problem in that this virus will probably not be inhibited by ACV alone. HLI alone has already been shown to be inadequate for treating CMV infections in bone marrow transplant recipients (9). However, since combining these agents enhances the antiviral effect in vitro, and since relatively large interferon and ACV concentrations are tolerated by humans (5, 9), clinical trials of the combination for treating CMV infection are warranted.

ACKNOWLEDGMENTS

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