Alpha Interferon and Acyclovir Treatment of Herpes Simplex Virus in Lymphoid Cell Cultures

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The T-lymphoblastoid cell line CEM, persistently infected with herpes simplex virus type 1, has been used to examine the antiviral efficacy of human alpha interferon and acyclovir, both alone and in combination. Acyclovir and interferon each produced dose-dependent decreases in virus titer at concentration ranges of 1 to 100 μM (≈ 0.225 to 22.5 $\mu\text{g/ml}$) and 10 to 10,000 U/ml, respectively. Mean reductions in titer of 1.9 and 4.2 $\log_{10}$ PFU/ml were observed with 100 μM acyclovir and 10,000 U of interferon per ml, respectively, on day 10 of treatment. The combination of 100 μM acyclovir and 10,000 U of interferon per ml produced the most rapid fall in virus titer of all regimens examined and elimination of infectious virus by day 7. Prolonged treatment (>10 days) with acyclovir or alpha interferon was accompanied by a gradual return of virus titer to control levels despite the continuous presence of drug. Virus preparations isolated from such cultures were tested for antiviral agent sensitivity by a plaque reduction method. Acyclovir-exposed isolates were found to be acyclovir resistant, with 50% inhibitory doses of >200 μM , and to be thymidine kinase deficient. Alpha interferon-exposed isolates were not interferon resistant. These results suggest that, in persistently herpes simplex virus-infected CEM cells: (i) combination treatment with 100 μM acyclovir and 10,000 U of alpha interferon per ml is more effective in reducing virus titer than either agent alone; (ii) prolonged exposure to drug may result in development of resistance by either the virus strain or the host cell system; and (iii) development of acyclovir resistance by herpes simplex virus in lymphoid cells is mediated by thymidine kinase.

The pathogenesis of herpes simplex virus (HSV) infections is incompletely understood. Human sensory and autonomic ganglia harbor the viral genome (2, 3, 37), but questions concerning other sites of persistence, the state of the viral DNA, and the controls of latency and reactivation remain. Attempts to examine these questions have led to the development of cell culture models of infection (9, 23, 24, 27, 29, 35). One such system, the persistently HSV-infected T-lymphoblastoid cell line CEM, is a useful model of latency and reactivation of HSV in lymphoid cells (12).

In vitro and in vivo models of HSV infection are also useful in evaluating the efficacy and mechanisms of antiviral agents (31). Among the most promising agents for the control of herpetic disease are the nucleoside derivatives and interferons (16). We have examined the effects of two of these agents, acyclovir [9-(2-hydroxyethoxymethyl-guanine); ACV] and human alpha interferon (Hu IFN- α), both alone and in combination, on productively infected CEM lymphoblastoid cells.

MATERIALS AND METHODS

Cells and media. Characteristics of the T-lymphoblastoid cell line CEM have been described (12). The line was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 250 U of penicillin per ml, 50 μg of gentamicin per ml, and 10% gamma-irradiated fetal calf serum (M.A. Bioproducts, Walkersville, Md.) Cell viability was determined by trypan blue dye exclusion.

Human embryonic lung cells were used for determination of virus titer in the persistently infected CEM cell line. Vero (African green monkey kidney) cells were used in plaque assay determinations of antiviral activity. Both were maintained in Eagle minimal essential medium with four times the usual concentration of vitamins and amino acids (MEM $\times 4$) and supplemented with 2 mM L-glutamine, 250 U of penicillin per ml, 250 μg of streptomycin per ml, and 5% newborn calf serum.

Viruses. A clinical strain of HSV type 1 (HSV-1) was used to establish the persistent infection of CEM cells. The origin of this virus strain has been described previously (12). The titer was 5×10^7 PFU/ml on human embryonic lung cells. After passage in lymphoid cells, isolates were obtained as follows: samples of infected cell suspensions were subjected to sonica-

tion for 2 min 45 s at an output current of 1.0 to 1.2 A in a model DF-101, 200-W, 10-kc sonic oscillator (Raytheon Co., Burlington, Mass.). The preparations were clarified by centrifugation at 1,500 rpm for 10 min at 4°C, quick frozen in a dry ice-ethanol mixture, and stored at -70°C. Isolates of the original input strain of HSV-1 and lymphoid cell-passaged isolates (untreated, ACV treated, and IFN treated) were each titrated in Vero cells. Five individual clones of each virus isolate were plaque purified three times in Vero cells; virus pools were prepared and stored at -70°C.

The KOS strain of HSV-1 (kindly provided by P. Schaffer and D. Coen, Sidney Farber Cancer Institute, Boston, Mass.) was used in thymidine kinase (TK) and plaque assay determinations of ACV sensitivity. Its titer in Vero cells was 5×10^8 PFU/ml. The Indiana strain of vesicular stomatitis virus was used in plaque assay determinations of IFN sensitivity and possessed a titer of 2×10^7 PFU/ml in Vero cells.

Persistent infection. The establishment of the persistent infection of CEM cells has been described (12). In brief, 12×10^6 CEM cells were washed in RPMI 1640 medium, pelleted, and incubated with HSV-1 in a volume of 1 ml (multiplicity of infection of 1 PFU per cell) for 1 h on a shaker apparatus at 37°C. An equal number of cells were mock infected with virus-free medium. Cells were washed three times in medium, resuspended to a concentration of 6×10^5 cells per ml, and incubated at 37°C in a 95% air-5% CO₂ atmosphere. At 4- to 7-day intervals, cells were passaged by adding fresh medium to a sample of cell suspension or by pelleting and resuspension to achieve a final concentration of 4×10^5 cells per ml.

ACV and IFN- α treatments. A lyophilized powder of ACV (kindly provided by G. Elion, Burroughs-Wellcome Co., Research Triangle Park, N.C.) was dissolved in 1 N NaOH, diluted in tissue culture medium, and filter sterilized. Hu IFN- α was obtained from K. Cantell and possessed a specific activity of 1.6×10^6 U/mg of protein.

Antiviral treatments were performed as follows. Samples of productively infected and control cultures were adjusted to a concentration of 4×10^5 cells per ml in RPMI 1640 medium with 10% fetal calf serum. ACV was added to achieve final concentrations of 1, 10, and 100 μ M (≈ 0.225 , 2.25, and 22.5 μ g/ml). IFN- α was added to achieve final concentrations of 10, 1,000, and 10,000 U/ml. Supplementary drug was added at the time of each cell passage. Results of studies at each drug concentration represent the mean of two to four experiments, each run at a separate time in duplicate.

Infectivity assay. Virus titers of sonicated cell suspensions were determined by a standard plaque assay. Serial 10-fold dilutions of the sonicates in MEM $\times$ 4 were plated in duplicate 0.1-ml amounts onto human embryonic lung cells in 35-mm six-well plates. After a 1-h adsorption at 37°C, the monolayers were washed twice with phosphate-buffered saline to prevent drug carry-over and then overlaid with 0.5% agarose (Sigma Chemical Co., St. Louis, Mo.) in MEM $\times$ 4 with 5% fetal calf serum. The plates were incubated for 4 days at 37°C, stained with neutral red (GIBCO Laboratories, Grand Island, N.Y.), and reincubated. Plaques were counted 1 day later.

Antiviral agent sensitivities. For determination of ACV sensitivity, 100 PFU of each virus strain in 0.2 ml of MEM $\times$ 4 were inoculated onto Vero cell monolay-

ers in 35-mm six-well plates. After a 1-h adsorption period, the monolayers were overlaid with 0.5% agarose in MEM $\times$ 4 with 5% fetal calf serum containing appropriate concentrations of ACV. Plates were incubated, stained, and analyzed as described above.

For determination of IFN sensitivity, Vero cell monolayers were incubated with appropriate concentrations of IFN- α in tissue culture medium for 18 h. The IFN preparation was then removed, and monolayers were inoculated with 100 PFU of each isolate. After a 1-h adsorption period, the plates were overlaid with 0.5% agarose in MEM $\times$ 4 with 5% fetal calf serum, incubated, and stained with neutral red as described above. Vesicular stomatitis virus plaques were counted on day 2; HSV plaques were counted on day 5.

The 50% inhibitory doses (ID₅₀) were determined by direct reading from the semilogarithmic plot of percentage of surviving plaques versus drug concentration.

Immunofluorescence. With each infectivity assay, cells were prepared for immunofluorescence analysis by washing 0.4-ml samples of infected and control well suspensions three times with Hanks balanced salt solution, allowing them to air dry on glass slides, and fixing them in acetone at -20°C. They were stained for 45 min with fluorescein isothiocyanate-conjugated rabbit anti-HSV serum (Flow Laboratories, Rockville, Md.) at room temperature. The slides were then washed with phosphate-buffered saline (pH 7.4), counterstained with 0.1% Evans blue, washed again, dried, and mounted with phosphate-buffered glycerol. The rabbit preimmunization serum produced no immunofluorescence when tested on a known HSV-positive cell control.

TK assay. Vero cells were grown to confluence in tissue culture tubes (16 by 125 mm) and were either mock infected or infected with HSV-1 at a multiplicity of infection of 5 PFU per cell. After 16 h at 37°C, floating cells were pelleted and the medium was discarded. The monolayers were washed, scraped into 0.5 ml of 10 mM sodium phosphate buffer, pH 6.0, and sonicated at 10 kc for 1 min, as described by Coen and Schaffer (5). Extracts were centrifuged in a Beckman Microfuge (12,000 $\times$ g) for 1 min, and the supernatant fluids were used for TK determinations. The reaction mixture had a total volume of 30 μ l and contained: enzyme sample (1 to 16 μ l), bovine serum albumin (0.1 mg/ml), 10 mM ATP, 10 mM magnesium acetate, 100 μ M sodium phosphate buffer (pH 6.0), 0.3 μ Ci of [methyl-³H]thymidine (50 Ci/nmol; New England Nuclear Corp., Boston, Mass.), and 40 μ M TTP (to suppress host TK activity). The assay mixture was incubated at 30°C, and at various times (15 to 60 min), the reaction was terminated by removing 5 μ l of the assay mixture, transferring it into 20 μ l of 1 mM thymidine, and boiling for 2 min. After a low-speed centrifugation, the entire sample was spotted onto Whatman DE 81 paper disks and air dried. The disks were washed three times in 4 mM ammonium formate (pH 4.0)-10 μ M thymidine for 5 min at room temperature, dried, and measured for radioactivity in a liquid scintillation counter (model 2540; Packard Instrument Co., Inc., Downers Grove, Ill.). TK activity was normalized to the amount of protein present in each sample as determined by the method of Lowry et al. (20).

RESULTS

ACV treatments. Treatment of the HSV-productive CEM line with ACV at concentrations of 1, 10, and 100 μM produced a dose-dependent decrease in virus titer over the first 10 days (Fig. 1A). A concentration of 1 μM was ineffective, whereas 10 μM ACV produced mean reductions in virus titer of 1.1 and 1.0 $\log_{10}$ PFU/ml on days 4 and 10, respectively. Corresponding reductions for 100 μM ACV were 1.6 and 1.9 $\log_{10}$ PFU/ml, respectively. Cell viability was unaffected by any of the ACV concentrations tested (data not shown).

Treatment with 100 μM ACV was carried beyond 10 days to determine the effect of chronic drug administration. In these studies, two different patterns were observed and confirmed by repeat investigations (Fig. 2A). In the first, mean virus titer, after an early decrease, gradually returned to control levels, where it remained after day 39. In the second, mean virus titer progressively diminished, disappearing entirely by day 18. Viral antigen diminished more slowly, disappearing by day 39. In the first studies, virus titer at the time of drug treatment averaged 5.7 $\log_{10}$ PFU/ml. In the second studies, initial virus titer was lower by an average of 1.7 $\log_{10}$

PFU/ml. This difference in virus titer at the time of drug treatment may have influenced the drug efficacy of prolonged administration.

IFN treatments. IFN- α treatment of the HSV-producer line at concentrations of 10, 1,000, and 10,000 U/ml also produced a dose-dependent decrease in virus titer over the first 10 days (Fig. 1B). A 10-U/ml concentration of IFN- α was ineffective in reducing virus titer. A 1,000-U/ml concentration achieved mean reductions of 0.6 and 2.3 $\log_{10}$ PFU/ml on days 7 and 10, respectively, and 10,000 U/ml produced mean decreases in titer of 1.3 and 4.2 $\log_{10}$ PFU/ml for the corresponding days. Cell viability remained unaffected by any of the IFN concentrations examined (data not shown).

FIG. 1. Mean change in HSV titer (treated - control) during 10-day treatment of the persistently HSV-infected CEM lymphoblastoid cell line with (A) ACV, (B) IFN- α , and (C) ACV plus IFN- α . Supplementary ACV or IFN- α (or both) was added at the time of each cell passage.

FIG. 2. Mean change in HSV titer (treated - control) during prolonged treatment (>10 days) of the persistently HSV-infected CEM lymphoblastoid cell line with (A) ACV and (B) IFN- α . Supplementary ACV or IFN- α was added at the time of each cell passage.

Results of treating the HSV-productive CEM line with IFN- α at 10,000 U/ml beyond 10 days are shown in Fig. 2B. Maximum reduction in mean virus titer was seen on day 10 but, despite continued addition of IFN with each cell passage, titer gradually returned to control levels by day 39. The proportion of cells producing viral antigen paralleled these observed changes in virus titer. Similar results were obtained when virus titer at the time of IFN treatment averaged either 5.7 or 4.0 log₁₀ PFU/ml.

Combination ACV and IFN- α . Combination treatment of the HSV-producer line with 1 μ M ACV and 10 U of IFN- α per ml produced no demonstrable decreases in either virus titer (Fig. 1C) or viral antigen production.

Treatment of the cell line with 10 μ M ACV and 1,000 U of IFN- α per ml (Fig. 1C) resulted in a pattern of virus titer reduction similar to that observed with 1,000 U of IFN- α per ml alone (Fig. 1B); on day 10, this reduction was 2.3 log₁₀ PFU/ml.

Treatment with 100 μ M ACV and 10,000 U of IFN- α per ml in combination produced the most rapid fall in virus titer of all regimens examined (Fig. 1C). Titers were reduced an average of 2.2, 2.0, and 3.3 log₁₀ PFU/ml on days 1, 2, and 3, respectively, and infectious virus was eliminated by day 7. The reduction in log₁₀ HSV titer produced by 100 μ M ACV and 10,000 U of IFN- α per ml on each of these days was greater than the sum of the log₁₀ reductions of each agent individually; their combined effect can thus be considered synergistic (19, 34, 36). This combination was not found to reduce cell viability (data not shown).

Antiviral agent sensitivity. To determine the sensitivity of virus isolates exposed to prolonged drug treatment, virus was harvested from lymphoid cell cultures that had been exposed to 100 μ M ACV or 10,000 U of IFN- α per ml for 73 days. This was 34 days after virus titer had returned to control levels (Fig. 2). These isolates, along with virus simultaneously isolated from lymphoid cell cultures not exposed to any antiviral agent and the original input strain of HSV-1 were titrated in Vero cells. Five cloned progeny from each of these four virus isolates were subsequently plaque purified three times in Vero cells and then analyzed for their ACV or IFN sensitivity or for both. This was done in an effort to detect different sensitivities among virion subpopulations.

(i) **ACV.** The ID₅₀s for the input HSV-1, lymphoid cell-passaged virus, and ACV-exposed virus isolates are listed in Table 1. The ACV passaged isolates were all markedly resistant to ACV, with ID₅₀s of >200 μ M. The input and lymphoid cell-passaged isolates were much more sensitive but still had relatively high ID₅₀s

TABLE 1. ACV and IFN- α sensitivities of input, lymphoid cell-passaged, ACV-passaged, and IFN-passaged plaque-purified HSV isolates

HSV isolate	Clone no.	ID ₅₀	
		ACV (μ M)	IFN- α (U/ml)
Input	1	7.2	60
	2	15.7	20
	3	16.2	33
	4	5.4	31
	5	6.8	12
Avg		10.3	31
Lymphoid cell passaged	1	14.5	90
	2	8.2	22
	3	32.0	190
	4	11.0	41
	5	21.0	40
Avg		17.3	77
ACV passaged	1	>200	
	2	>200	
	3	>200	
	4	>200	
	5	>200	
Avg		>200	
IFN- α passaged	1		32
	2		270
	3		40
	4		12
	5		90
Avg			89

compared with typical HSV-1 isolates (8).

(ii) **IFN- α .** In contrast to ACV, prolonged exposure to IFN did not lead to the development of interferon resistance within the virion population. ID₅₀s for input, lymphoid cell-passaged, and IFN-passaged plaque-purified isolates were all similar (Table 1).

TK assay. In an effort to determine the mechanism of resistance to ACV among the ACV-exposed plaque isolates, TK determinations were performed. Table 2 lists the TK activity of these viruses in comparison with the plaque-purified isolates obtained from the input strain. It is evident that input isolates were equivalent to the standard KOS strain in TK activity. ACV-passaged strains were TK deficient.

DISCUSSION

Diseases caused by HSV are a continued cause of morbidity and, to a lesser extent, mortality worldwide. In normal individuals, the syndromes of genital herpes, neonatal disease, keratitis, labialis, and encephalitis are of major physical, psychosocial, and epidemiological importance. In immunocompromised persons,

TABLE 2. TK activities of input and ACV-passaged plaque-purified HSV isolates

HSV isolate	Clone no.	% TK activity ^a
Input	1	85
	2	100
	3	102
	4	78
	5	105
KOS		100
ACV passaged	1	0.3
	2	0.4
	3	0.9
	4	0.5
	5	0.4

^a Viral TK activity was normalized to that induced by the KOS strain of HSV-1. Mock infection activity was <1% of KOS-induced activity.

HSV not infrequently causes chronic mucocutaneous ulceration and may cause fatal, disseminated disease (15). The pressure to develop prophylactic and therapeutic regimens effective in controlling herpetic disease has thus been intense. Prevention of HSV reactivation has been demonstrated in normal hosts with IFN- α and in immunocompromised individuals with ACV (26, 30). Vidarabine has shown therapeutic benefit in several controlled trials (16), and ACV, a more potent and selective inhibitor of HSV replication, is beginning to show efficacy in controlled trials (4, 21).

The persistent HSV infection of CEM cells is an interesting system in which to examine certain perplexing questions concerning HSV pathogenesis. These cells can be either productively or latently infected, and virus can be activated from latently infected cells with phytohemagglutinin (12). As leukocytes remain probable vehicles of viremic spread (7, 22) and potential sites of persistence within the human host, it was of interest to examine the effects of antiviral agents in our system.

ACV added to an already productive culture in increasing concentrations was shown to produce a dose-dependent decrease in virus titer over the first 10 days of treatment. When the HSV-productive cultures were subjected to prolonged exposure to 100 μ M ACV, two contrasting patterns were seen: virus titer either gradually returned to control levels or was eliminated. The higher average virus titer present at the time of treatment in the former studies (5.7 versus 4.0 log₁₀ PFU/ml) may have made it more difficult to completely suppress virus titer (13) as well as facilitated the emergence of resistance upon exposure to the drug.

Five individually cloned isolates from the long-term ACV-treated cultures that returned to control titer were examined for their level of sensitivity to ACV and were found to be uniformly resistant, with ID₅₀s of >200 μ M. Further, these isolates were all shown to be TK deficient. These results are consistent with previously published work indicating that resistance by HSV to ACV readily occurs upon in vitro exposure to the drug and that the resistance is generally TK mediated (5, 32, 33). The original input strain of HSV-1 used to establish the persistent infection had an unusually high ID₅₀ for a clinical strain (8). However, relative resistance to this drug in clinical isolates, not previously exposed to ACV, has also been described (33). Whether this diminished level of sensitivity represents decreased affinity of the viral DNA polymerase for ACV-triphosphate is not yet clear.

IFN- α treatment of HSV-productive cultures also produced a dose-dependent decrease in virus titer over the first 10 days of treatment but with a different time course than that observed for ACV; maximal decreases occurred on day 10. Prolonged treatment with 10,000 U of IFN per ml resulted in a gradual return to control virus titer by day 39. This effect was observed irrespective of the virus titer present at the time of antiviral treatment. In contrast to ACV-treated cultures in which drug resistance developed, cloned isolates from long-term IFN-treated cultures were unchanged in their IFN sensitivity. Similar observations have been made for HSV-1 grown in the presence of high concentrations of IFN in Vero cells (18). It thus appears that the cell system developed resistance to the antiviral effects of IFN.

The combination of 100 μ M ACV and 10,000 U of IFN- α per ml produced the most rapid decrease in virus titer and the earliest disappearance of infectious virus. The efficacy of this combination in our system agrees with recent studies demonstrating a synergistic antiviral effect of ACV and either IFN- α or IFN- β against HSV in fibroblasts and Vero cells, respectively (19, 34).

Preliminary DNA-DNA hybridization studies indicate that combination treatment of HSV-productive cultures with 100 μ M ACV and 10,000 U of IFN- α per ml reduced viral genome copy numbers after 55 days of drug exposure (C. Mulder and H. Youssoufian, personal communication). Although the number of viral genomes is reduced by this combination, one would not expect to cure the cell line, as each of these agents individually has been incapable of eliminating viral DNA completely in cell cultures, animals, and humans chronically infected with virus (1, 6, 10, 11, 14, 17, 25, 30).

In summary, we have used the persistently HSV-infected CEM cell line to examine the effects of ACV and IFN- α , both singly and in combination. Our results suggest that, in our system, combination high-dose treatment is more effective in reducing virus titer than either agent alone, that prolonged exposure to drug may result in development of resistance by either the virus strain or the host cell system, and that development of ACV resistance by HSV in lymphoid cells is TK mediated. The CEM-HSV system should prove useful in the evaluation of newer antiviral therapies as they become available.

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