Synergistic Antiherpes Virus Activity of Acyclovir and Interferon in Human Corneal Stromal Cells

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Synergistic anti-herpes simplex virus type 1 (HSV-1) activity between acyclovir (ACV) and recombinant human interferon alpha A2 (IFN) was detected in cell cultures derived from human corneas. This activity was demonstrated when cells were infected at high multiplicities of infection (5 pfu/cell) in both cytopathic effect reduction and yield reduction assays as well as in plaque reduction assays at low multiplicities. The synergistic effects occurred over a 30-fold range of concentrations of IFN (0.15–200 IU/ml) and ACV (0.15–5 μM) at various ratios of the drugs. The augmentation of antiviral activity was greatest when cells were treated with IFN prior to infection and ACV following infection. The addition of IFN after infection, with simultaneous ACV treatment, also resulted in enhanced anti-HSV-1 activity. The combination of IFN with ACV did not increase the cytotoxicity of ACV. The synergistic antiviral activity in corneal cells may account for the previously reported enhanced efficacy of combined treatment with IFNs and nucleoside analogs for therapy of herpetic keratitis. Invest Ophthalmol Vis Sci 30:365–370, 1989

In clinical trials dendritic corneal ulcers due to herpes simplex virus infection responded favorably to treatment with nucleoside analogs such as trifluorothymidine or acyclovir in 90 to 96% of the cases studied.1,2 Trials using human interferons (IFNs) to topically treat dendritic herpetic keratitis have shown that IFN alone is of limited value and must be given at high dosages to achieve a significant effect.3–7 However, in several human trials topical application of nucleoside analogs in combination with IFN resulted in significantly decreased time to wound closure, decreased time to total healing, and a reduction in virus shedding when compared to nucleoside alone.8–13

These reports suggest that there may be a synergistic antiviral effect of nucleoside analogs and interferon. Reports using several cell culture model systems indicate that this enhanced therapeutic effect may be a reflection of an enhanced antiviral activity demonstrable at the cellular level.14–16 Whether this combined effect represents synergism, i.e., that treatment with both agents has a significantly greater antiviral effect than the sum of their individual effects, or is simply an additive response, has not always been apparent.

The studies reported here were designed to determine whether treatment of fibroblastic cells obtained from the stroma of human donor corneas with IFN and the nucleoside analog acyclovir (ACV) can result in a truly synergistic anti-herpes simplex virus type 1 (HSV-1) activity. We have found that significantly enhanced anti-herpes virus activity occurred when these corneal cells were treated with a broad range of dosages of IFN and acyclovir and that this enhanced activity was synergistic, as mathematically defined.

Materials and Methods

Cells

African green monkey kidney cells (Vero) were grown in Dulbecco's minimum essential medium (DMEM) containing 10% newborn calf serum (Gibco, Grand Island, NY), penicillin and streptomycin. Human corneal stromal fibroblast cell cultures were established from donor corneas following digestion with collagenase, grown in DMEM with 10% fetal bovine serum (FBS), penicillin, streptomycin and gentamycin and used at passage level 7 or less.17 All cell lines and corneal isolates were tested and found to be mycoplasma-free.

Virus

A plaque-purified stock of the McKrae strain of HSV-1 was used throughout the study. The stock virus was propagated in Chang conjunctival cells, a
continuous human cell line (CCL20.2, American Type Culture Collection, Rockville, MD), and assayed by plaque formation in Vero cells.

**Interferon**

The IFN used for these studies was recombinant human IFN-alpha A2 which was generously supplied by Hoffmann LaRoche (Nutley, NJ). IFN was assayed using the hemagglutinin yield-reduction assay or the naphthol blue-black dye uptake assay in A549 human lung carcinoma cells with encephalomyocarditis virus as the challenge virus. Titer of IFN were adjusted to the appropriate NIH/WHO IFN standard, Gxa01-901-353.

**Nucleoside Analog**

9-(2-hydroxyethoxymethyl) guanine (acyclovir, ACV) was obtained from Burroughs Wellcome Company (Research Triangle Park, NC). Solutions of ACV were prepared in DMEM without serum. The concentration and purity was determined by high performance liquid chromatography and ultraviolet spectral analysis in a manner similar to that previously described.

**Assays for Antiviral Activity of Combinations**

Inhibition of virus replication was evaluated by three forms of assay, yield reduction, cytopathic effect reduction, and plaque reduction.

**Yield reduction assay:** Cells were planted at confluence (3.4 × 10^6 cells/cm^2) in 24-well dishes. The following day IFN dilutions, prepared in maintenance medium (DMEM with 2% FBS), were added to cultures. After 24 hr of IFN treatment, medium containing IFN was removed and cells were infected with HSV-1 at a multiplicity of infection (MOI) of five plaque-forming units (pfu)/cell. One hour after infection the unadsorbed virus was removed and cells were overlayed with medium containing various concentrations of ACV. Cultures were incubated for 24 hr and then frozen at −80°C until virus yield was assayed by plaque formation in Vero cells.

**Cytopathic effect (CPE) reduction assay:** Cells were planted in 96-well dishes and treated with IFN as described above. After 24 hr the IFN was removed and cells were infected with HSV-1 at 0.05 to 5 pfu/cell. Following the 1 hr adsorption period unadsorbed virus was removed and cells were overlayed with maintenance medium with or without ACV. For some studies IFN was also included in the overlay medium. After 48 hr to 4 days, depending upon the MOI used, CPE was evaluated by two methods, direct microscopic observation or dye uptake. For microscopic evaluations of CPE before staining, cells were examined and CPE was rated on a 4+ scale with 0 = no CPE and 4+ = 100% cell destruction. For the dye uptake method cells were stained with 0.15% neutral red in Hank's balanced salt solution (HBSS) for 30 min to 1 hr. Cells were then washed three times with phosphate buffered saline and dye was eluted with formaldehyde:ethanol:acetic acid (2:20:1). Dye concentration was measured spectrophotometrically directly in the wells of the 96-well plates using a BioTek EIA reader (Burlington, VT) at 540 nm. Caution was exercised when using the dye uptake method because the cells must be incubated until infected cells detached from the growth surface. Prior to that time infected cells became enlarged and took up more dye than uninfected cells, which resulted in artificially high optical density readings.

**Plaque reduction assay:** Cells were planted at confluence in 12-well plates in growth medium. The following day cells were treated with IFN as described above. After 24 hr IFN was removed, cells were washed once with HBSS, and 50 to 100 pfu of HSV-1 were added to each well. After 1 hr unadsorbed virus was removed and cells were overlayed with maintenance medium containing various concentrations of ACV and methylcellulose. After incubation for 3 days, cells were fixed, stained with crystal violet, and plaques were counted. Sigmoid dose response curves were plotted and ED<sub>50</sub> values determined as previously described.

**Measurement of Synergy**

Data were evaluated to determine combination index as described by Chou and Talalay using a computer program designed by Chou and Chou. This method requires that (1) the drugs be used in combination at a constant ratio; and (2) dose-response curves with positive slopes must be obtained for each drug alone and for combinations. Dose-response curves were plotted using the median effect equation. The median doses determined from the median effect equation were then used to calculate the combination index (CI) by the following equation:

\[
CI = \frac{[\text{IFN}]_{C} + [\text{ACV}]_{C} + \alpha[\text{IFN}][\text{ACV}]}{[\text{IFN}][\text{ACV}]} \]

where [IFN] and [ACV] are the doses of IFN and ACV which cause a specific level of protection when used alone; [IFN]<sub>C</sub> and [ACV]<sub>C</sub> are the doses of the two drugs which cause a similar protection when used in combination. The value of α is equal to 0 for mutually exclusive drugs or 1 for mutually nonexclusive drugs. Thus, when the value of CI is equal to one the two drugs are additive in their activity; however,
when CI is less than one the drugs are synergistic in their action.

Assays for Cytotoxicity

Cytotoxicity was evaluated by measuring growth and viability of cells following culture for 1 week in the presence of IFN, ACV, or various combinations of the two agents as previously described. 25

Results

Synergy as Detected by Inhibition of CPE

The CPE inhibition assay facilitates the screening of large numbers of combinations of IFN with ACV. Cell destruction resulting from virus infection was examined microscopically and was spectrophotometrically determined by reduction in dye uptake. Synergistic antiviral activity as measured by an inhibition of this destruction was demonstrated over a wide range of concentrations of ACV and IFN. Table 1 shows the percentage of the cell monolayer protected from viral CPE as measured by dye uptake at various concentrations of both drugs. The CIs determined for each treatment condition were all less than one, indicating that synergism occurred throughout the range of doses used. For example, treatment of cells with relatively high levels of both drugs (ie, IFN ≥ 50 IU/ml prior to infection and ACV at ≥1.25 μM post-infection) resulted in complete to nearly complete protection from viral CPE and CI values of less than 0.1. At lower concentrations of drugs the level of protection decreased and the CI values increased but remained less than one throughout the range of concentrations used.

Enhanced antiviral activity was consistantly found when cells were pretreated with IFN and then treated with ACV after infection. Two alternative schedules of IFN and ACV treatment were compared for induction of synergism: (1) IFN added to cultures 24 hr prior to virus infection followed by replacement with fresh IFN after virus infection and (2) IFN added only after infection. ACV treatment followed virus infection in all treatment schedules. Application of IFN 24 hr before infection followed by combination of IFN with ACV after virus infection did not increase the extent of antiviral activity detected when compared with that found when IFN was present only prior to infection (data not shown). The synergism in each treatment group, ie, IFN pretreatment and IFN pre- and post-treatment, was observed at the same ratios of IFN and ACV concentrations. The drug concentrations tested in these studies were as follows: IFN at initial concentrations of 200 IU/ml and six serial 2-fold dilutions and ACV initially at 5, 2.5, 1.25, 0.6, 0.3, or 0.15 μM and six serial 2-fold dilutions of each initial starting concentration. Combinations indices throughout the ranges of protection were less than one, ranging from 0.01 to 0.95, but generally less than 0.3. Delay of IFN treatment until after infection diminished the combined antiviral action, with enhanced activity microscopically evident only at higher doses of IFN (100 to 200 IU/ml) combined with high doses of ACV (1.25 to 5 μM). When IFN alone was added to cells after infection no antiviral activity was detected even at concentrations as high as 2000 IU/ml. Treatment of cells with ACV alone 24 hr prior to infection did not result in significant protection from virus infection, nor did such pretreatment combined with ACV treatment after infection enhance the antiviral activity of ACV (data not shown).

Synergy Detected by Yield Reduction Assay

Assays which measure the inhibition of CPE are a useful method to screen a large number of drug combinations and treatment schedules. However, a reduction in CPE may not necessarily accurately reflect the extent of decrease in production of infectious virus. Yield reduction assays were performed to measure the inhibition of replication during a single cycle of virus growth. Cells were treated with serial dilutions of IFN beginning at 100 IU/ml and then, after infection, with ACV at 5 μM or less such that the two drugs were used at constant concentration ratios. Treatment prior to infection with 100 IU/ml and serial dilutions of IFN and after infection with 2.5 μM and serial dilutions of ACV resulted in apparent synergism when compared to IFN or ACV alone (Fig. 1). In the same experiment a 2-fold increase in the IFN

Table 1. Synergistic anti-HSV effect in corneal stromal cells treated with IFN before and ACV after infection

<table>
<thead>
<tr>
<th>IFN (IU/ml)</th>
<th>5.0</th>
<th>2.5</th>
<th>1.2</th>
<th>0.6</th>
<th>0.3</th>
<th>0.15</th>
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<td>2000</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>(0.008)</td>
<td>(0.004)</td>
<td>86</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>(0.007)</td>
<td>(0.003)</td>
<td>52</td>
<td>6.5</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>(0.10)</td>
<td>(0.025)</td>
<td>(0.15)</td>
<td>3.4</td>
<td>3.0</td>
<td>2.1</td>
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<tr>
<td>12</td>
<td>(0.87)</td>
<td>(0.36)</td>
<td>(0.42)</td>
<td>6</td>
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<td></td>
<td></td>
</tr>
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* Combination indices are given in parentheses.

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Fig. 1. Yield reduction assay of inhibition of virus replication by IFN, ACV and combinations. Human corneal stromal fibroblasts were treated with IFN at various concentrations (●, ○). After 24 hr IFN was removed and cells were infected with 5 pfu/cell of HSV-1. After 1 hr unadsorbed virus was removed and cells were overlayed with medium containing ACV (△, □). Cultures were frozen at −80°C after 24 hr and virus yields determined by plaque formation on Vero cells.

concentration in the dual treatment led to an additional 2-fold decrease in virus yield (data not shown).

In some assays to measure yield reduction or CPE reduction, pretreatment of cells with IFN alone failed to establish any detectable anti-HSV-1 activity even at concentrations of 2000 IU/ml of IFN. This absence of protection by IFN was not due to inactivation of IFN since the same preparations which showed no detectable antiviral activity retained antiviral activity when assayed in our routine IFN assay against encephalomyocarditis virus. Under these conditions, where IFN alone has no detectable antiviral effect, a median effect plot could not be generated and one of the requirements for the combination index method, i.e., that a dose-response curve with positive slope must be obtained for each drug alone, was not met. Consequently, the combination index method was not valid for this circumstance. However, in this situation IFN was consistently able to potentiate the antiviral effect of ACV ([ACV]C/[ACV] < 1).

Synergy as Detected by Plaque Reduction

A third method, the plaque reduction assay, was used to measure synergistic anti-HSV activity. This method conceptually would best reflect the situation in the eye during dendritic keratitis. In this assay, as in the cornea, the majority of cells are not infected and the drugs are assayed for their ability to limit the replication of virus and the spread to adjacent cells during several rounds of replication. Synergistic antiviral activity was also detected when IFN pretreatment followed by ACV post-treatment was examined by this method (Fig. 2). IFN alone showed antiviral action with an ED₅₀ of 120 IU/ml. ACV inhibited virus replication with an ED₅₀ of 2.59 µM. When pretreatment with 50 IU/ml of IFN was followed by ACV post-treatment at various concentrations, the ED₅₀ for ACV decreased to 0.28 µM, while with 25 IU/ml of IFN the ED₅₀ for ACV was 0.8 µM. Combinations of 12 IU/ml of IFN with ACV resulted in only a slight reduction in number of plaques when compared with ACV alone (data not shown). Plaque size did not vary with different treatments.

Measurement of Cytotoxicity of Antiviral Combinations

Stromal fibroblasts were treated simultaneously with combinations of IFN and ACV at doses as high as 1000 IU/ml and 5 µM, respectively. No inhibition of cell growth or cytotoxic effects were detected at either 3 or 7 days after treatment commenced.

Discussion

Synergistic antiviral activity was detected when human corneal stromal cells were treated with human IFN-alpha A2 and ACV. This enhanced antiviral activity was demonstrated in cell cultures isolated from seven different pairs of human corneas (age range 35 to 80 years). This finding indicated that the responsiveness to combined treatment was proba-
bly a general phenomenon in this cell type. Potentiation of antiviral activity was observed over the entire range of IFN (200 IU/ml to 6 IU/ml) and ACV (5 μM to 0.15 μM) tested, occurring consistently even when IFN demonstrated no detectable antiviral activity of its own. Similar variability in responsiveness to the antiviral action of IFN alone was also seen in Vero cells (data not shown). The absence of antiviral activity by IFN was not due to an inactivation of the IFN since activity was retained against encephalomyocarditis virus, a virus known for its sensitivity to the action of IFN.

Continuous treatment with combinations of doses of IFN and ACV greater than or equal to those which showed complete protection from viral CPE did not diminish the growth or viability of corneal stromal cells. Baba et al.²⁶ and Levin and Leary.¹⁷ similarly found no anticcullary activities of combinations of ACV and natural human IFN-alpha in human foreskin fibroblasts or embryonic fibroblasts, respectively. When present at high concentrations (usually 1000 IU/ml or higher), IFN has been shown to decrease growth of a variety of cell types, but rarely is it responsible for overt toxic effects on cells (see ref. 27 for review). At lower titers IFN is usually not growth-inhibitory. Although ACV generally has little or no cellular toxicity at concentrations demonstrating antiviral activity, limited cellular effects have been detected at higher doses; for example, in the Chang conjunctival cell line 100 μM ACV resulted in only a 23% increase in generation time without detectable toxicity.²⁸ The absence of toxic effects resulting from treatment with combinations of IFN and ACV which were shown to possess synergistic antiviral activity indicates that such combined treatment should not result in increased toxicity or inhibition of wound healing in the eye.

The treatment of stromal cells with combinations of ACV and IFN after infection in culture reduced the amount of virus produced when compared to ACV or IFN treatment alone. However, pretreatment of cells with IFN prior to infection and subsequent treatment with ACV or ACV plus IFN after infection resulted in a still greater inhibitory effect. Both of these conditions of treatment could be interpreted as reflecting situations present in the herpes-infected cornea. In the cornea only a fraction of the susceptible cells are usually infected at any time. From our results one would expect that combined treatment would reduce virus production from those infected cells. Generally, however, a greater portion of the cells of the cornea are uninfected. Treatment of these cells with IFN before they become infected followed by ACV or IFN plus ACV would limit virus replication in and the spread of virus from these cells if subsequently infected. While the exact conditions of treatment present in the cornea cannot be reproduced in cell culture, the detection of synergistic anti-HSV activity in cells of corneal origin indicates that the therapeutic efficacy of combined treatment demonstrated against herpetic keratitis may reflect an enhanced activity present at the cellular level within the cornea.

Key words: herpes simplex virus, interferon, acyclovir, keratocytes

Acknowledgment

Human corneas were provided by the Wisconsin Lion’s Eye Bank, Medical College of Wisconsin, Milwaukee, Wisconsin. We wish to thank Hoffmann LaRoche, Nutley, New Jersey for the IFN and Burroughs Wellcome Company, Research Triangle Park, North Carolina for the ACV used in these studies.

References


