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Comparative activities of combinations of acyclovir, vidarabine or its 5′-monophosphate, and cloned human interferons against herpes simplex virus type 2 in human and mouse fibroblast cultures

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Summary

Human interferon-αA/D (Bgl), an alpha-hybrid cloned interferon, displays activity in both human and mouse cell lines. We measured the effects of this interferon in double and triple combinations with acyclovir, vidarabine or its 5′-monophosphate against herpes simplex virus type 2 in mouse and human fibroblasts. A 75% cytopathic effect reduction assay employing a modified checkerboard technique was used. Results in human fibroblasts were compared with those obtained when recombinant human interferon-α_2 was substituted for the hybrid. Combinations of the hybrid interferon and nucleoside antiviral agents evoked comparable synergistic isobolograms and fractional inhibitory concentration indices in human and mouse cells versus herpes simplex virus type 2. Similar interactions were found when human interferon-α_2 was substituted. Uninfected cells treated with the tested combinations showed no toxicity. These data suggest that combinations of recombinant human interferon-αA/D (Bgl) and nucleosides in mouse models of herpes infection deserve study.

acyclovir; antiviral combinations; herpes simplex virus; interferon; vidarabine; vidarabine monophosphate

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Introduction

Although recombinant technology has spurred the development of human interferons (HuIFNs), inadequate amounts of species-specific preparations have greatly hampered studies of the antiviral activity of exogenous IFN in laboratory animals [14]. As a result, assessment of the potential therapeutic utility of HuIFNs in preclinical evaluations of human infection has not been as thorough as that of other candidate antiviral agents. Investigators at the Roche Institute of Molecular Biology have developed a human hybrid HuIFN consisting of the N-terminal sequence of subtype A spliced to the C-terminal of subtype D at the Bgl site [13]. Recombinant human hybrid interferon-αA/D (Bgl) (HuIFN-αA/D) crosses species barriers, and shows activity in both human and mouse cell lines [7]. For the first time, enough biologically pure IFN is available for testing in mouse cells and models.

Several human recombinant HuIFN trials have involved therapy of herpes infections. Double-blinded, placebo-controlled trials of recombinant human HuIFN-α_2 (HuIFN-α_2) have been done in this center and elsewhere. This preparation does not significantly shorten the course of a genital herpes relapse, even when treatment begins within 12 h of onset. Prophylactic administration does not prevent recurrences (Crane, L., manuscript in preparation; [8]). Although HuIFN monotherapy might be ineffectual, cell culture data suggest that HuIFN may augment the antiviral actions of nucleoside drugs in vivo. Combinations of nucleosides and HuIFNs generally evoke synergistic or additive effects against herpes simplex virus type 2 (HSV-2) in human cell cultures [4,10,15]. Recently, we have measured synergy with combinations of vidarabine (ara-A), acyclovir (ACV), and crude mixed preparation of murine IFN-α/β versus HSV-2 in primary mouse embryo fibroblasts (MEF). In further studies, synergy resulted in a mouse model of genital herpes treated with a combination of ACV and Poly IC(LC), an IFN inducer administered because mouse IFNs were not readily available for in vivo use [5]. If HuIFN-αA/D exhibits synergy with nucleosides in vitro, further studies using mouse models may allow testing of this hypothesis. In this study, we explored interactions of nucleoside antivirals and HuIFN-αA/D alone, and in double and triple combinations versus HSV-2 in human foreskin (FS) and MEF cultures, using a cytopathic effect (CPE) reduction assay. Results were compared with similar combinations using HuIFN-α_2 in FS.

Materials and Methods

Nucleosides tested in these studies included ACV, provided by Dannie H. King, Burroughs Wellcome Co., Research Triangle Park, NC, and ara-A or its 5'-monophosphate (ara-AMP), both donated by Edwin L. Marcus, Warner-Lambert/Parke-Davis, Ann Arbor, MI, along with pentostatin, an adenosine deaminase inhibitor. Patrick W. Trown, Hoffmann-La Roche, Nutley, NJ, donated human recombinant HuIFN-αA/D (Bgl). Schering Corporation, Bloomfield, NJ, supplied HuIFN-α_2. Cloned HuIFN-αA/D activity was established by calibration with NIH standard mouse IFN in mouse L-929 cells using vesicular stomatitis virus (VSV) as the challenge. The

HuIFN-α_2 was calibrated using NIH standard human IFN against VSV in FS. Activity was expressed as international units (IU) per ml [18].

Antiviral activities of individual agents and combinations were measured by a CPE reduction assay, as reported from our laboratory [5]. Pools of HSV-2 strain MS, passed in continuous lines of FS, usually reached titers of $1.0 \times 10^{7.0}$ to $1.0 \times 10^{8.0}$ 50% tissue culture infective doses ($TCID_{50}$) per ml in FS and $1.0 \times 10^{6.0}$ to $1.0 \times 10^{7.0}$ $TCID_{50}$ per ml in MEF. We have previously described the preparation of MEF tissue cultures [5]. In these experiments, 12–14-day-old ICR-Swiss embryos (Harlan–Sprague–Dawley, Inc., Indianapolis, IN) were used. Eagle's minimal essential medium containing L-glutamine and supplemented with 10% fetal calf serum was used for cell growth or freezing of virus aliquots (–70°C). Medium enriched with 2% fetal calf serum was used for cell maintenance and antiviral assays. Cell lines were cultured for mycoplasma bi-weekly; only mycoplasma-free cells were used.

Assays were done in 96-well (8 rows, 12 wells per row) flat-bottom microtiter plates (Gibco, Grand Island, NY) sealed with pressure-sensitive film (Falcon Plastics, Division of BioQuest, Oxnard, CA). Each well was seeded with 2.0×10^4 cells and incubated at 37°C until confluent. Medium was drained from microtiter plates, and the first seven rows inoculated with 0.1 ml of medium containing 100–200 $TCID_{50}$ of HSV-2 in medium. The first row was designated the virus control; the eighth row was designated the cell control. After sealing the plates, virus was adsorbed for 2 h at 37°C. Two-fold dilutions of antiviral agents were prepared in medium. Because ara-A and ara-AMP are rapidly deaminated to arabinosyl hypoxanthine, 0.5% pentostatin was added [16]. Six wells, each containing 0.1 ml, were tested per dilution. Sealed plates were incubated for 72 h at 37°C. Then, the monolayers were fixed with 10% formalin and stained with crystal violet. Cytopathic effect was scored from 0 to 4+ with a stereoscopic microscope. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of drug in microgram per milliliter or HuIFN in IU per milliliter protecting 75% of the cells from infection. Assays of HuIFN antiviral activity differed in that HuIFN was incubated with cells for 24 h before virus adsorption. Recombinant HuIFN-α_2 was tested only in FS because in preliminary experiments, this HuIFN had no antiviral activity against HSV-2 in mouse cells, even at concentrations of 30 000 IU/ml. Other strains of HSV-2 were also screened and demonstrated similar results.

We tested double and triple combinations of antiviral agents using Berenbaum's abbreviated checkerboard technique [3,5]. Stock solutions of antiviral agents in combinations were prepared in medium in fixed ratios of MICs. The following ratios were explored for each pair of antiviral agents: 0.42 : 1.58, 0.73 : 1.27, 1 : 1, 1.27 : 0.73, and 1.58 : 0.42 to determine the lowest concentrations of drugs needed for each ratio to reduce CPE by 75%. Similarly, nine ratios were evaluated for triple combinations: 0.8 : 0.8 : 0.4, 0.8 : 0.4 : 0.8, 0.4 : 0.8 : 0.8, 0.6 : 0.6 : 0.8, 0.6 : 0.8 : 0.6, 0.8 : 0.6 : 0.6, 0.4 : 0.4 : 1.2, 0.4 : 1.2 : 0.4, and 1.2 : 0.4 : 0.4. Four 2-fold dilutions of these solutions were made; additionally, the stocks were then diluted 3 : 4 and four 2-fold dilutions made. These dilution series tested twice as many points than that reported by Allen and co-workers [1]. Each stock solution and dilution thereof was tested in six wells as before. Concurrently assayed controls included MICs of individual antiviral agents,

and untreated cell and virus controls. All experiments were done at least twice. Two- and three-dimensional isobolograms were generated by computer. Actual FICs of single antiviral agents and FIC indices of combinations were computed as previously reported [1,3,5]. The FIC index equaled $FIC_x + FIC_y + FIC_z$. The FIC of an individual drug equaled (MIC_{xc}/MIC_x), where MIC_{xc} was the concentration of drug x in the combination. Synergy was defined as an FIC index $\leqslant 0.5$. Indices ranging from 0.5 to 0.9 were suggestive of synergy, those equalling 1.0 were additive, and an index from 1.1 to 1.9 was deemed indifferent. Antagonism was defined as an FIC index $\geqslant 2.0$ [1,5].

A dye uptake assay was used to assess cytotoxicity [11]. The lowest dilutions of each ratio or single drug exhibiting significant antiviral activity were tested. Six wells were assayed for each drug or combination. After a 72-h incubation with the test agent(s), the medium was drained from the plates, and 0.1 ml of 0.034% neutral red in phosphate-buffered saline added to each well. 2 h later, the dye was discarded and monolayers were washed twice with phosphate-buffered saline and drained. Color was absorbed by adding 0.25 ml of absolute ethanol–Sørensen citrate I buffer (1 : 1) to each well and incubating 30 min in the dark. Dye uptake per well was measured by absorbance at 540 nm using a model 3021 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Dye uptakes by drug-treated and control cells were compared. A significant decrease in dye uptake of treated cells (Student's *t*-test) was interpreted as cytotoxicity (confidence level = 95%).

Results and Discussion

Table 1 summarizes results of 75% CPE reduction assays for ara-A, ara-AMP, ACV, and cloned HuIFNs alone, and in double or triple combinations. In MEF, 4000 IU/ml of HuIFN-αA/D were required to reduce HSV-2 CPE by 75%. High levels of HuIFN-αA/D were also required in the human cell line. These findings have been observed with other isolates of HSV-2 in this laboratory (unpublished). All in vitro combinations tested in MEF showed synergy. Double and triple combinations exhibited synergy in FS, except the ara-A/ACV doublet which was suggestive of synergy. Irrespective of cell line, MICs of HuIFN-αA/D were lowered 4–18-fold and both HuIFN preparations yielded identical FIC indices in FS when combined with a nucleoside. Computer-generated isobolograms of antiviral doublets exhibited concave curves. Also, triplet isobolograms showed concave surfaces with 'sinking' towards the origin point, regardless of cell line (Fig. 1A–D). Addition of ara-AMP and ACV to herpes-infected FS pretreated with small amounts of HuIFN-αA/D greatly enhanced antiviral effects (Fig. 1D). All corresponding isobolograms of HuIFN-αA/D and HuIFN-α_2 triplets in FS were comparable. No single drug concentration or combination tested caused cytotoxicity.

Our data indicate that HuIFN-αA/D and selected nucleoside antiviral agents evoke similar synergistic activity in human and mouse cells versus HSV-2 and that comparable interactions occur when HuIFN-α_2 is substituted. These results may not necessarily predict in vivo effects. Cytopathic effect reduction assays may be useful to screen

TABLE 1

Activity of vidarabine (ara-A) or vidarabine 5'-monophosphate (ara-AMP), acyclovir (ACV), and human recombinant interferon-A/D (Bgl) (HuIFN-A/D) or recombinant human α_2 interferon (HuIFN-α_2) alone, and in combination against herpes simplex virus type 2 (strain MS) in either human foreskin fibroblast or primary mouse embryo (ICR-Swiss) fibroblast tissue cultures

Mouse embryo fibroblast					Human foreskin fibroblast					
MIC				FIC index[a] of combinations[b]	MIC					FIC index[a] of combinations[b]
g/ml			IU/ml		g/ml			IU/ml		
ara-A[b]	ara-AMP	ACV	HuIFN-A/D		ara-A	ara-AMP	ACV	HuIFN-A/D	HuIFN-α_2	
5.0	–	–	–	–	1.3	–	–	–	–	–
–	5.0	–	–	–	–	6.3	–	–	–	–
–	–	0.13	–	–	–	–	0.50	–	–	–
–	–	–	4000	–	–	–	–	625	–	–
–	–	–	–	–	–	–	–	–	313	–
1.3	–	0.031	–	0.5_S	0.26	–	0.20	–	–	0.6_{SS}
0.53	–	–	400	0.2_S	0.13	–	–	123	–	0.3_S
					0.13	–	–	–	62	0.3_S
–	0.99	0.026	–	0.4_S	–	1.3	0.099	–	–	0.4_S
–	1.3	–	1000	0.5_S	–	1.2	–	132	–	0.4_S
					–	1.3	–	–	62	0.4_S
–	–	0.020	590	0.3_S	–	–	0.063	78	–	0.3_S
					–	–	0.063	–	39	0.3_S
0.5	–	0.012	300	0.3_S	0.25	–	0.050	94	–	0.5_S
					0.13	–	0.025	–	23	0.2_S
–	0.28	0.037	225	0.4_S	–	0.63	0.025	47	–	0.2_S
					–	0.63	0.025	–	23	0.2_S

[a] Fractional inhibitory concentration (FIC) index of ≤ 0.5 indicates synergy; the lower the FIC index, the greater degree of synergy. The lowest of the 5 or 9 FIC indices determined is shown. All indices were < 1.0. Each assay was done at least twice.

[b] Inferior suffix: S, synergy; SS, suggestive of synergy.

[c] Pentostatin was added to test system whenever ara-A or ara-AMP was evaluated.

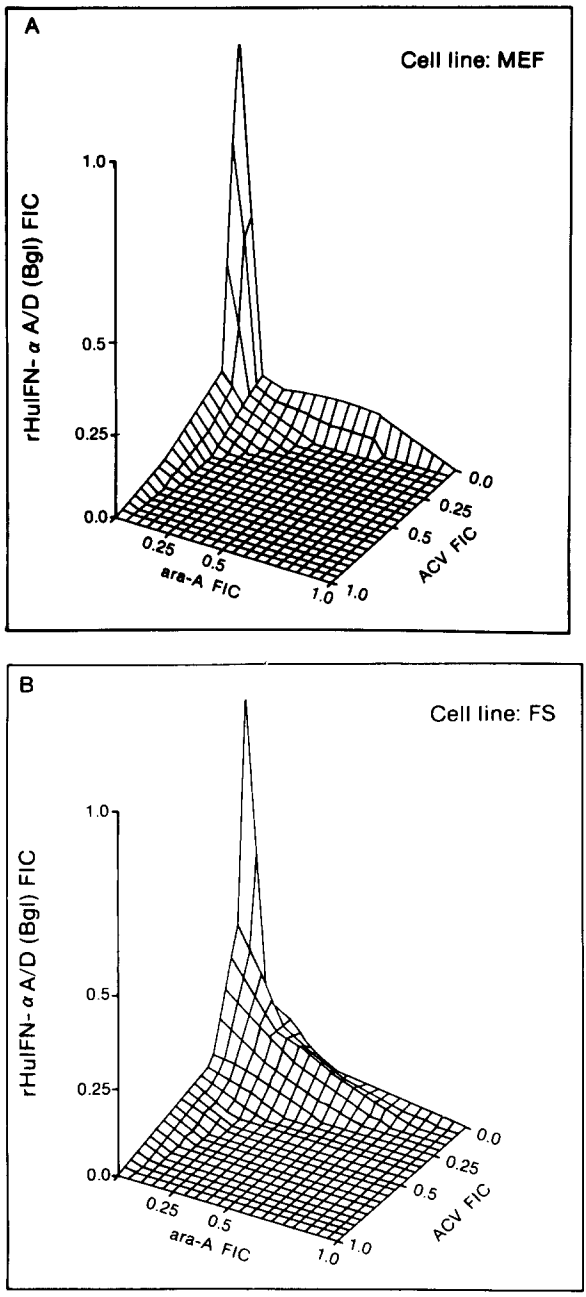
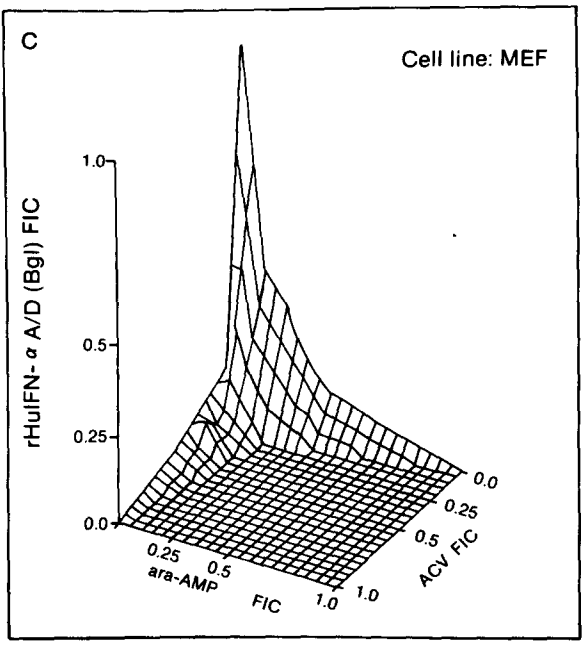
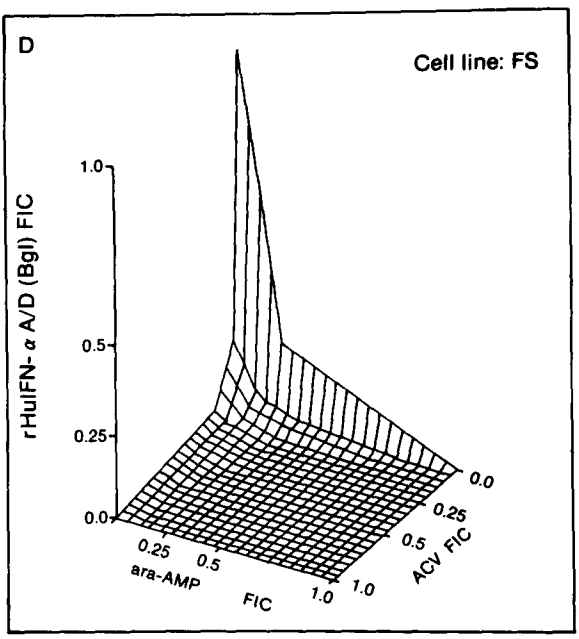


Fig. 1. Computer-generated, three-dimensional isobolograms of in vitro anti-HSV-2 activity of vidarabine (ara-A) or vidarabine monophosphate (ara-AMP), acyclovir (ACV), and recombinant human interferon-αA/D (Bgl), using an abbreviated checkerboard technique. Mouse embryo fibroblasts (1A and 1C) or human foreskin fibroblasts (1B and 1D) were infected with HSV-2, strain MS, and treated with compounds →





to determine concentrations resulting in 75% CPE inhibition. Extrapolated surfaces were generated from 27 points, and confirmed in three separate experiments. Synergistic interactions were seen with both combinations in both cell lines.

promising combinations, but animal studies must be done to confirm observations of synergy. Animal models of herpes infection are generally treated after virus inoculation, paralleling clinical experience. In these assays, nucleosides are added 2 h after virus adsorption (HuIFN, 24 h before adsorption), well before new virus production. Lastly, cytotoxicity assays cannot predict adverse drug interactions occurring at the target organ level.

Other investigators have shown that 1 IU of HuIFN-αA/D results in a 50% reduction in HSV-2 CPE [7]. The MICs of HuIFN-αA/D reported here are higher. However, we used MEF cultures instead of mouse L cells, and employed an HSV-2 inoculum that was 1000-fold greater. The addition of nucleoside(s) lowered the fractional MIC of HuIFN-αA/D from 4000 IU/ml to levels as low as 225 IU/ml. Do these high HuIFN levels (i.e., 225–4000 IU/ml) match the 'clinical situation'? Serum levels of IFN, induced by non-toxic doses of poly IC(LC) having antiviral effects in HSV-2-infected mice, range from 2000 to 25 000 IU/ml [5,12]. Serum levels of exogenous IFNs of 3000 IU/ml are non-toxic in human neonates and adults [2,17].

Patients with multiple-antibiotic resistant bacterial infections have been treated with synergistic triple antimicrobial combinations. This approach has proved useful where MICs of active antimicrobials are marginal, or therapeutic/toxic ratios are narrow [19]. In vitro studies of triple antiviral agents have been done for the same reasons [5,9]. Vidarabine and IFNs exhibit narrow therapeutic/toxic ratios in animals, including man. These observations prompted us to explore three-drug combinations. If therapeutic effects can be produced by combinations using minute amounts of antiviral agents, toxicity may be reduced or eliminated.

The results suggest that HuIFN-αA/D alone, and in double or triple combination with nucleoside antiviral agents should be tested in mouse models of HSV-2 infection. Preliminary data in a mouse model of neonatal HSV-2 support these in vitro findings [6]. Further experiments may define the role of HuIFN in human virus infections. Interferon therapy of human herpes has been disappointing. Its role in antiherpes therapy may be adjunctive, enhancing antiviral activity of other agents. Combination therapy with HuIFN and a nucleoside may overcome the shortcomings of single-drug treatment of herpes, and warrants continued investigation in mouse models.

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