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Anti-rhinoviral activity of recombinant and hybrid species of interferon alpha

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Summary

To define further differences in antiviral activity as well as to identify candidate interferons for study in the prevention of rhinovirus colds, the antiviral activities of nine species of recombinant interferon alpha (IFN-αA, IFN-αB, IFN-αC, IFN-αD, IFN-αJ, [Ser-116]IFN-αJ1, IFN-αK, IFN-αJ/C(*Fnu*4HI), and IFN-αA/D(*Bgl*II)) were evaluated against rhinovirus types 39 (RV 39) and 1A (RV 1A). WI-38 cells were exposed to various concentrations of each interferon and were then infected with RV 39, RV 1A, or VSV. Efficacy was determined by protection from cytopathic effect using a tetrazolium dye assay. The 50% inhibitory concentrations ranged from 4 ± 3 pg/ml for IFN-αC to >3000 pg/ml for IFN-αD against RV 39, and from 6 ± 4 pg/ml for IFN-αJ/C(*Fnu*4HI) to >3000 pg/ml for IFN-αD against RV 1A. IFN-αJ/C(*Fnu*4HI), [Ser-116]IFN-αJ1, and IFN-αC were the most active of the interferons, and were all more active than IFN-αA, against RV 39, RV 1A, and VSV. These interferons warrant further study against rhinoviruses and other viruses.

Interferons; Alpha interferons; Rhinovirus; Vesicular stomatitis virus

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Introduction

Natural and recombinant alpha interferons, administered locally to the nose as drops or spray, are effective in preventing experimental and natural rhinovirus colds (Hayden, 1986; Hayden, 1989; Sperber and Hayden, 1988). Nasal irritation, however, including mucosal friability, bleeding, erosions and ulcerations, occurs with increasing duration of administration (Hayden, 1986; Hayden, 1989; Sperber and Hayden, 1988). For interferons to have clinical usefulness in illnesses such as the common cold they must have a more favorable therapeutic index than previously studied recombinant alpha interferons. Whereas IFN-αA and IFN-α2 have been the most extensively evaluated interferons against rhinoviruses, numerous other species of interferon alpha exist which differ in amino acid sequence (Pestka, 1983; Pestka, 1986) and biologic properties (Greiner et al., 1986; Li et al., 1990; Ortaldo et al., 1983; Ortaldo et al., 1984; Rehberg et al., 1982; Sen et al., 1984).

We evaluated nine recombinant and hybrid species of alpha interferon for in vitro activity against rhinovirus types 1A and 39 in a tetrazolium dye microassay (Scudiero et al., 1988; Weislow et al., 1989) adapted to measure interferon protection of human diploid lung fibroblast cells from rhinovirus-induced cytopathic effect (CPE). Anti-rhinoviral activity was compared with activity against vesicular stomatitis virus and with recently reported (Sperber et al., 1992b) relative activity of the same interferons against human immunodeficiency virus type 1 (HIV-1). Since some of the alpha interferons differ by only a few amino acid residues, a secondary goal was the continuing study of interferon activity in the framework of sequence-function relationships.

Materials and Methods

Cells. Human diploid embryonic lung fibroblast cells (WI-38; purchased from Viromed Laboratories, Inc., Minnetonka, MN) grown in stationary flasks in Eagle's modified minimal essential medium (GIBCO, Gaithersburg, MD) plus 10% heat-inactivated fetal bovine serum, L-glutamine, vancomycin, gentamicin and amphotericin B were used (passage 19 to 24) to seed 96-well microtiter plates (Falcon, Becton and Dickinson, Lincoln Park, NJ).

Viruses. American Type Culture Collection strains were used for rhinovirus type 1A (RV 1A; approx. 100 $TCID_{50}$/well; ATCC No. 1110; passage history HeLa/10, WI-38/2, MRC-5/1), rhinovirus type 39 (RV 39; approx. 100 $TCID_{50}$; ATCC No. 1149; HeLa/19, WI-38/6, MRC-5/1) and vesicular stomatitis virus (VSV, Indiana strain; ATCC No. 158).

Interferons. Recombinant and hybrid interferons were produced and purified as previously described (Li et al., 1990; Rehberg et al., 1982). The interferons tested were: IFN-αA, IFN-αB, IFN-αC, IFN-αD, IFN-αJ, [Ser-116]IFN-αJ1,

	1 10	20	30	40	50	60	70	80
IFN-αA	CDLPQTHSLG	SRRTLMLLAQ	MRKISLFSCL	KDRHDFGFPQ	EEF-GNQFQK	AETIPVLHEM	IQQIFNLFST	KDSSAAWDET
IFN-αD	....E....D	N.....	.SR..PS...	M.....	...D.....	.PA.S....L	.....T.	.....D
IFN-αA/D(*Bgl* II)	.....	.....	.....	.....	.....	.....	.....T.	.....D
IFN-αJ	.....R	N..A.I....	.GR..P....	....E.R..E	...D.H....	TQA.S.....	...T.....	E.....EQS
(Ser^{116})IFN-αJ1	.....R	N..A.I....	.GR..P....	....E.R..E	...D.H....	TQA.S.....	...T.....	E.....EQS
IFN-αC	.....	N..A.I..G.	.GR..P....	.....RI..	...D.....	.QA.S.....	...T.....	E.....EQS
IFN-αJ/C(*Fnu* 4HI)	.....R	N..A.I....	.GR..P....	....E.R..E	...D.H....	TQA.S.....	...T.....	E.....EQS
IFN-αB	.....	N..A.I....	..R..P....	.....E...	...DDK....	.QA.S.....	...T.....	.....L...
IFN-αK	.....	H...M.....	..R.....	.....R...	...D.....	..A.S....V	...T.....	....V....R

	81 90	100	110	120	130	140	150	160	166
IFN-αA	LLDKFYTELY	QQLNDLEACV	IQGVGVTETP	LMKEDSILAV	RKYFQRITLY	LKEKKYSPCA	WEVVRAEIMR	SFSLSTNLQE	SLRSKE
IFN-αD	.....C....	.....	M.EER.G...	..NV.....	K...R.....	.T.....	.....	.L.....	R..R..
IFN-αA/D(*Bgl* II)	.....C....	.....	M.EER.G...	..NV.....	K...R.....	.T.....	.....	.L.....	R..R..
IFN-αJ	..E..S....	.....	..E...E...	..N..F....	.....	.M.....	.....	...F....KK	G..R.D
(Ser^{116})IFN-αJ1	..E..S....	.....	..E...E...	..N..R....	.....	.M.....	.....	...F.....K	R..R.D
IFN-αC	..E..S....	.....	..E...E...	..N.....	.....	.I.R.....	.....	.L.F.....K	R..R.D
IFN-αJ/C(*Fnu* 4HI)	..E..S....	.....	..E...E...	..N.....	.....	.I.R.....	.....	.L.F.....K	R..R.D
IFN-αB	...E..I..D	VLC	D.E...I.S.	..Y.....	.....	.T.....S..	.....	.....I...K	R.K...
IFN-αK	....L.....	.....	M.E.W.GG..	..N.....	.....	.T.....	.....	...S.R....	R..R..

Fig. 1. The amino acid sequences of IFN-αA, IFN-αD, IFN-αA/D(*Bgl*II), IFN-αJ, [Ser-116]IFN-αJ1, IFN-αC, IFN-αJ/C(*Fnu*4HI), IFN-αB, and IFN-αK. The amino acids are indicated only when they differ from IFN-αA. Reproduced with permission from Sperber et al. (1992b).

IFN-αK, IFN-αJ/C(*Fnu*4HI) (IFN-αJ$_{1-75}$/C$_{76-166}$(*Fnu*4HI)), and IFN-αA/D(*Bgl*II) (IFN-αA$_{1-62}$/D$_{64-166}$(*Bgl*II)) (Fig. 1). Serial half $\log_{10}$ dilutions from 9 to 3000 pg protein/ml were prepared with medium as the diluent. For informational purposes, the equivalences of the letter designations with the numerical designations for the interferons (Pestka, 1986) are as follows: IFN-αA, [Lys-23]Hu-IFN-α_2; IFN-αB, [Val-98 Leu-99 Cys-100 Asp-101]Hu-IFN-α_8; IFN-αD, [Val-114]Hu-IFN-α_1; IFN-αJ, [Met-60]Hu-IFN-α_7; IFN-αJ1, [Met-60 Glu-159 Arg-161]Hu-IFN-α_7; IFN-αK, Hu-IFN-α_6.

Experimental plan. When the monolayers in the microtiter plates had achieved 100% confluency, the media was removed and replaced with 0.15 ml of fresh media containing 3% heat-inactivated fetal bovine serum and interferon in triplicate or quadruplicate portions to yield the final concentrations above. The plates were gently agitated and then allowed to incubate for 24 h at 36°C in an atmosphere of 5% CO_2 and 95% relative humidity. RV 1A, RV 39, or VSV in 0.05 ml portions was added to the appropriate wells, gently agitated and incubated at 33°C (36°C for VSV). Cell controls and virus controls were included on each plate. After 6 days, the plates were read microscopically for CPE and subsequently processed for the XTT assay as described below.

Microscopic evaluation of CPE. Prior to the addition of XTT the monolayers were examined microscopically for CPE and scored on a scale of 0%, 25%, 50%, 75% or 100%. The % protection was determined for each interferon concentration (100 – mean % CPE) and compared with the % protection as

TABLE 1

Absorbance values for WI-38 cells treated with XTT-PMS and read at 4 and 24 h. Cells were either uninfected or were infected with RV 39, RV 1A or VSV

Virus	XTT incubation	Cell control	Virus control	Cell control minus virus control
RV 39	4 h	0.74 ± 0.16	0.57 ± 0.08	0.17 ± 0.10
	24 h	1.52 ± 0.27	0.74 ± 0.10	0.77 ± 0.32
RV 1A	4 h	0.75 ± 0.17	0.54 ± 0.16	0.21 ± 0.03
	24 h	1.63 ± 0.44	0.55 ± 0.11	1.08 ± 0.33
VSV	4 h	0.78 ± 0.18	0.48 ± 0.08	0.28 ± 0.12
	24 h	1.67 ± 0.45	0.51 ± 0.09	1.16 ± 0.36

Values represent means ± S.D. of at least 4 experiments, each performed in at least triplicate. Culture duration was 6 days; XTT incubation was at 36°C. Cell control refers to uninfected cell monolayers; Virus control refers to 100% CPE of monolayer; Final PMS concentration, 1.5 μg/ml; XTT concentration, 20 μg/ml; RV 39, rhinovirus type 39; RV 1A, rhinovirus type 1A; VSV, vesicular stomatitis virus.

determined by the XTT assay.

XTT assay for determination of antiviral activity. In the presence of the electron coupling agent phenazine methosulfate (PMS), viable cells will reduce the tetrazolium salt 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino)-carbonyl]-2H-tetrazolium hydroxide (XTT). For the XTT assay, a solution containing 0.025 mM PMS-XTT was prepared by adding 0.05 ml of 5 mM PMS (Sigma, St. Louis, MO, 1.53 mg/ml) per 10 ml of XTT (Polysciences, Inc., Warrington, PA; 1 mg/ml prepared in media without additives) and added in 0.05 ml aliquots to each well (Scudiero et al., 1988). The plates were gently agitated and incubated at 36°C, 5% CO_2 and 95% relative humidity. After 24 h the plates were examined microscopically for evidence of toxicity and, after gentle mixing, read at an absorbance of 450 nm on a V_{max} kinetic plate reader (Molecular Devices, Menlo Park, CA). Initially the plates were examined at 4 h but the 24 h incubation permitted greater sensitivity without increasing toxicity (Table 1) and was used for these assays.

Antiviral activity was determined for each interferon concentration by comparison of the mean optical density of the replicate wells as follows:

$$\text{Protection}_x = \frac{OD_x - OD_{vc}}{OD_{cc} - OD_{vc}} \times 100\%$$

where OD_x indicates the optical density for the infected wells at interferon concentration x, and vc and cc indicate virus control and cell control, respectively. Typical optical density values were 1.3–1.8 for the cell controls and 0.4–0.9 for the virus controls. The interferon concentration necessary for 50% protection (EC_{50}) was determined by the median-effect analysis of Chou

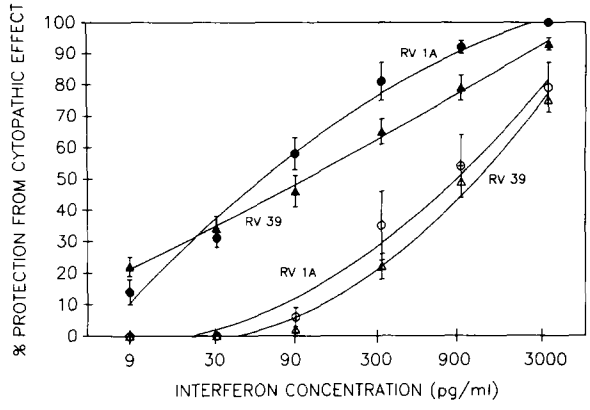

Fig. 2. Concentration-dependent protection from rhinovirus induced cytopathic effect by IFN-αA. WI-38 cells were infected with rhinovirus type 39 (triangle) or type 1A (circle). Cytopathic effect was determined by direct microscopic visualization (open symbols) or by use of a XTT-based dye microassay (filled symbols). Values represent mean of six independent experiments ± S.E.

and Chou (1987). Activity was determined from the mean of at least three experiments, each performed in triplicate or quadruplicate.

To ensure that the interferon under study was not toxic to the cells, cytotoxicity was assessed by comparing OD_{cc}, and OD_{cx} where OD_{cx} represents the optical density for the cytotoxicity controls at interferon concentration x.

Results

XTT assay and anti-rhinoviral activity of IFN-αA

The XTT assay was able to detect differences in anti-rhinoviral activity of IFN-αA over a wide range of interferon concentrations (Fig. 2). Cytotoxicity due to the addition of PMS-XTT was not observed. The EC_{50} (mean ± standard deviation) for IFN-αA was 85 ± 42 for RV 39 and 38 ± 17 pg/ml for RV 1A (Table 2). The concentration-dependent protection of WI-38 cells by IFN-αA against rhinovirus-induced CPE as determined by this assay correlated with the direct microscopic measurement of CPE (Fig. 2). The XTT assay however was better able to show differences in anti-rhinoviral activity at low interferon concentrations where the assay appeared to be more sensitive than direct microscopic observation.

Anti-rhinoviral activity of other alpha interferons

The XTT assay was able to demonstrate differences in anti-rhinoviral activity between the different species of interferon alpha. The mean EC_{50} for the interferons ranged from 4 to >3000 pg/ml against RV 39 and from 6 to >3000 pg/ml against RV 1A (Table 2). IFN-αC, IFN-αJ/C(*Fnu*4HI), and [Ser-

TABLE 2

Activity of recombinant and hybrid alpha interferons on WI-38 cells against RV 39, RV 1A and VSV

Interferon	50% Inhibitory concentration (pg/ml) vs. RV 39	RV 1A	VSV
IFN-αA	85 ± 42	38 ± 17	37 ± 29
IFN-αD	>3000	>3000	>3000
IFN-αA/D(*Bgl*II)	105 ± 78	58 ± 21	117 ± 30
IFN-αJ	19 ± 4	99 ± 13	199 ± 75
[Ser-116]IFN-αJ1	8 ± 10	8 ± 1	22 ± 12
IFN-αC	4 ± 3	11 ± 5	19 ± 6
IFN-αJ/C(*Fnu*4HI)	7 ± 3	6 ± 4	8 ± 5
IFN-αB	67 ± 14	90 ± 27	38 ± 19
IFN-αK	81 ± 41	30 ± 10	208 ± 58

Values represent means ± S.D.; RV 39, rhinovirus type 39; RV 1A, rhinovirus type 1A; VSV, vesicular stomatitis virus.

116]IF-αJ1 were the most active on a weight basis against the rhinoviruses. IFN-αD was substantially less active than the other interferons.

Anti-VSV activity of alpha interferons on WI-38 cells

The mean EC_{50} for the interferons against VSV ranged from 8 to >3000 pg/ml on WI-38 cells (Table 2). IFN-αJ/C(*Fnu*4HI), IFN-αC, and [Ser-116]IFN-αJ1 were the most active; IFN-αD was the least active. The anti-rhinoviral activities of the interferons relative to activities against VSV on WI-38 cells ranged from 0.1 to 2.3 for RV 39 and from 0.1 to 2.4 for RV 1A.

Cytotoxicity of alpha interferons

At the concentrations tested none of the interferons was significantly toxic to WI-38 cells as determined by analysis of non-infected, interferon-treated cells examined microscopically and in the XTT assay.

Discussion

Using CPE protection assays, we have been able to show significant differences in anti-rhinoviral activity between a number of species of interferon alpha. We adapted an XTT dye-based assay for use with human fibroblast cells and compared this assay with direct microscopic visualization of CPE. In both assays, dose-dependent protection from viral cytopathicity was observed, although the XTT-based assay appeared to be more sensitive, especially at lower interferon concentrations. There was less variability and subjectivity with the XTT method than with direct microscopic visualization which requires reader-dependent scoring of CPE. We were also able to verify the ability of the XTT assay to determine relative activity of the interferons by comparing the protective effects against VSV on WI-38 cells in the present study with anti-

VSV activity of the same interferons on WISH cells (Sperber et al., 1992b) by conventional CPE reduction methods used to determine specific activity (Familletti et al., 1981; Rubenstein et al., 1981). The same three interferons, in order, were most active against VSV in both assays.

The interferons differed by over 500-fold in their in vitro activity against rhinoviruses on WI-38 cells. IFN-αJ/C(*Fnu*4HI), IFN-αC, and [Ser-116]IFN-αJ1 were at least 10-fold more active than IFN-αA against RV 39, and at least 3-fold more active than IFN-αA against RV 1A. These results suggest that IFN-αJ/C(*Fnu*4HI), IFN-αC, and/or [Ser-116]IFN-αJ1 may have greater anti-rhinoviral activity than IFN-αA in vivo, although clinical protection from rhinovirus colds may not correlate solely with in vitro anti-rhinoviral activity (Sperber et al., 1989; Sperber and Hayden, 1989). Despite similar in vitro activity against RV 39 and RV 1A, IFN-α2b administered by nasal spray was more effective in preventing natural rhinovirus colds than nasal drops of [Ser-17]IFN-β (Hayden, 1989; Sperber et al., 1989; Sperber and Hayden, 1989). This difference, however, may have been due to the method of drug delivery or to the instability of IFN-β in nasal secretions (Hayden, 1989; Sperber et al., 1989; Sperber and Hayden, 1989). It is not known whether interferons with greater activity against rhinoviruses would have greater or less local toxicity than IFN-αA, but greater antiviral activity would theoretically permit the use of lower dosages, with potentially less toxicity and less direct immunogenicity. Additionally, other beneficial effects of interferons, such as normalization of middle ear pressures and Eustachian tube function observed during treatment of experimental RV 39 colds with [Ser-17]IFN-β (Sperber et al., 1992a), may be affected to varying degrees by different species of interferon, especially if these functions are independent of antiviral activity.

The interferons that were most active against RV 39 and RV 1A tended to be the most active against VSV on WI-38 cells. IFN-αJ/C(*Fnu*4HI), IFN-αC, and [Ser-116]IFN-αJ1 were the most active of the interferons against all three viruses. Analogous to their activity against RV 39 and RV 1A, each of these interferons was more active than IFN-αA against VSV. IFN-αD was 1/35th as active against RV 39, and about 1/80th as active against RV 1A and VSV on WI-38 cells than was IFNαA. The relative activities of the interferons against RV 39, RV 1A, and VSV are also consistent with recent observations of anti-HIV-1 activity of the same interferons. IFN-αJ/C(*Fnu*4HI) and [Ser-116]IFN-αJ1 were the most active against HIV-1 on MT-2 cells, each with 2- to 3-fold greater activity than IFN-αA; IFN-αD was the least active (Sperber et al., 1992b). Thus, as we observed for activity against HIV-1 (Sperber et al., 1992b), anti-rhinoviral activity of this series of recombinant and hybrid species of alpha interferon could not be readily predicted by their linear amino acid sequence. Rather, changes in anti-rhinoviral activity, anti-HIV activity, antiproliferative activity, and NK cell stimulatory activity, all can result from alterations in either half of the interferon molecule, suggesting that these functions are mediated by tertiary structure rather than any contiguous linear sequence of amino acids (Li et al., 1990; Ortaldo et al., 1983; Sen et al., 1984; Sperber et al.,

1992b).

This study extends the spectrum of viruses for which antiviral activity of this series of interferons has been determined. We have identified three interferons (IFN-αJ/C(*Fnu*4HI), IFN-αC, and [Ser-116]IFN-αJ1) with at least 3-fold greater activity than IFN-αA against both RV 39 and RV 1A. IFN-αJ/C(*Fnu*4HI) and [Ser-116]IFN-αJ1 also have greater activity than IFN-αA against HIV-1 on MT-2 cells, and against VSV on WISH, MDBK, and WI-38 cells. These modified interferons warrant further study against rhinoviruses and other viruses.

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