Antiviral Research, 19 (1992) 149–159

AVR 00577

Biological activities of recombinant murine interferons alpha 1 and alpha 4: large difference in antiproliferative effect

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(Received 13 December 1991; accepted 8 May 1992)

Summary

The mature forms of two recombinant murine interferons alpha, alpha 1 and alpha 4, have been expressed in vitro using an established transcription and translation system. The relative specific antiviral activity, antiproliferative activity and the natural killer cell stimulating activity of both subtypes were compared in vitro. While the antiviral and natural killer cell stimulating activities of the 2 subtypes were similar, the relative antiproliferative activities varied markedly. On the basis of equal molar inputs, MuIFN-α1 had less than 8% of the antiproliferative activity of MuIFN-α4. This data shows that a large functional difference exists between these two subtypes which are known to be expressed at different levels in mouse L-cells in vitro.

Murine interferon-alpha 1; Murine interferon-alpha 4; Antiviral activity; Antiproliferative activity; NK cell stimulation

Introduction

The interferons alpha (IFNs-α) are biological regulators present in all vertebrates. They are a family of closely related proteins capable of inducing a virus-resistant state in cells (Isaacs and Lindenmann, 1957; Lindenmann, 1981). In addition to the antiviral effect, the IFNs-α have numerous other biological

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effects including antiproliferative effects on normal and tumor cells in vitro and in vivo and immunomodulatory effects, such as the stimulation of the cytotoxic activity of natural killer (NK) cells, cytotoxic T-cells and of macrophages (Weissmann and Weber, 1986; Pestka et al., 1987). There are at least 14 different human interferon alpha (HuIFN-α) subtypes and 11 known murine interferon alpha (MuIFN-α) subtypes. However, the exact biological role of the different IFN-α subtypes is unclear.

The MuIFN-α subtypes are expressed at different levels in vitro in mouse L-cells, with MuIFN-α4 being the most highly expressed subtype (Kelley and Pitha, 1985b; Zwarthoff et al., 1985). Recent studies using site-directed mutagenesis and in vitro expression systems have shown that the specific antiviral activity of MuIFN-α4 is only some 2-fold greater than that of MuIFN-α1 on mouse L929 cells (Beilharz et al., 1991).

There have been numerous reports on the growth inhibitory actions of MuIFNs-α, β and γ on various murine cancer cell lines in vitro and in vivo (Brunda and Wright, 1986; Kuroki et al., 1987; Sayers et al., 1990; Yasui et al., 1990). Yet, little is known about the relative antiproliferative activities of the various MuIFNs-α subtypes.

The present study compares the relative specific activities of MuIFN-α1 and MuIFN-α4 for (1) antiviral effect, (2) NK cell stimulating effect and (3) antiproliferative effect. The antiviral effects were determined on murine L929 cells and J2E cells (Klinken et al., 1988) and the antiproliferative effects were studied on J2E cells. J2E cells are an erythroleukemic cell line generated by transforming erythroid precursor cells with the J2 retrovirus containing the *raf* and *myc* oncogenes (Klinken et al., 1988). These cells are able to terminally differentiate into mature, hemoglobin-synthesizing erythroid cells, when exposed to the hormone erythropoietin. For the biological effects studied, a significant difference in the antiproliferative specific activities between the two subtypes was observed.

Materials and Methods

The nucleotide sequences of MuIFN-α1 and MuIFN-α4 genomic clones have been published previously (Kelley and Pitha, 1985a). The genomic clones were gifts from Prof. P.M. Pitha (Johns Hopkins Oncology Center, Baltimore, USA). Sequenase Version II DNA sequencing kit was purchased from United States Biochemicals. Enzymes for in vitro transcriptions and translations and wheatgerm extract minus methionine were obtained from Promega-Biotec (Madison, WI, USA). All other enzymes were purchased from Pharmacia (Uppsala, Sweden) or Toyobo (Osaka, Japan). L-^{35}S-methionine (>1000 Ci/mmol) was obtained from NEN-DuPont, USA.

Gene manipulation and expression

The details of the methods used to: (1) alter the genomic clones of MuIFN-α1 and MuIFN-α4 by site-directed mutagenesis to allow subsequent expression of the mature forms of each protein, (2) subclone the altered genomic clones into expression vectors and (3) express each protein in a ^{35}S-quantifiable form in vitro in either rabbit reticulocyte lysate (RRL) or wheatgerm lysate, have been detailed elsewhere (Tymms and McInnes, 1988; Beilharz et al., 1991).

Antiviral assays

The antiviral activities of the in vitro expressed MuIFNs-α were assessed on mouse L929 cell monolayers in a cytopathic effect (CPE) reduction bioassay (Jilbert et al., 1986) with murine encephalomyocarditis virus (EMCV) as the challenge virus. The activities of the MuIFNs-α were calibrated against a mixed MuIFNs-α reference standard (Lee Biomolecular, San Diego, CA, USA; 4.5×10^5 IU/ml, 5.5×10^5 IU/mg). Relative specific antiviral activities were compared on the basis of ^{35}S input cpm. In order to check for the effect of variable ^{35}S incorporation into the IFN proteins, the bioassays were performed in two ways: (1) equal ^{35}S cpm from each preparation were used and the volume variations compensated for by the addition of RNA-free RRL or wheatgerm lysate and (2) equal volumes of each preparation were used and the activities were subsequently normalized on input ^{35}S cpm. In both cases the number of methionines in each protein was taken into account and results were the same with both approaches. The antiviral activity of each subtype was then expressed as a percentage of MuIFN-α1 activity, based on a minimum of 3 independent determinations.

Qualitative estimation of the antiviral activities of MuIFNs-α1 and -α4 were also performed on J2E cells (Klinken et al., 1988). J2E cells were seeded at 1×10^5 cells/ml and grown for 24 h at 37°C in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), in an atmosphere of 5% (v/v) CO_2 in air. The mixed IFNs-α, MuIFN-α1 and MuIFN-α4 were added to 1 ml of cells to a final concentration of 10, 30 and 100 IU/ml (as determined by antiviral assay on L929 cells). The IFNs were incubated with the cells for 4 h and EMCV was then added to a concentration of $10^3 \times TCID_{50}$. Controls of cell only and cell and wheat germ lysate only were included. The cell numbers and their viability were determined every 24 h thereafter by a visual trypan blue exclusion assay using a hemocytometer, performed in duplicate for each time point.

NK cell stimulation assays

Single cell suspensions from the spleens of 8-week-old C3H/HeJ mice, housed under minimal disease conditions, were prepared as previously described (Allan et al., 1982) and resuspended at 2×10^7 cells/ml in culture

medium [RPMI-1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 40 mg/ml gentamicin, 100 U/ml penicillin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol and 10% FCS]. One ml of cells was added to the wells of 24-well tissue culture trays (Costar, Cambridge, MA, USA) and an equal volume of IFN diluted in culture medium was added. After incubating for 4 h at 37°C in 10% CO_2 in air, the cells were recovered, washed 3 times with RPMI-1640 containing 20 mM HEPES and 2% FCS, and finally resuspended in culture medium. Viable cell counts were performed by trypan blue exclusion and the cell concentrations were adjusted to 1×10^7 cells/ml. NK cell activity of these IFN-activated spleen cells (effectors) was then assayed in a standard 4-h ^{51}Cr-release assay against YAC-1 cells (target cells), using 1×10^4 target cells and $0.125–1.0 \times 10^6$ effectors per well, each sample in triplicate (Dawkins and Shellam, 1979). Controls of media only and RRL only were included to indicate basal levels. Data are presented as % specific cytotoxicity, calculated from 3 independent assays as follows:

$$\% \text{ specific cytotoxicity} = \frac{\text{test cpm} - \text{spontaneous release cpm}}{\text{maximum release cpm} - \text{spontaneous release cpm}} \times 100$$

Maximum release cpm represents the radioactivity released from target cells incubated with 1% Triton X-100. Spontaneous release cpm is the background release of ^{51}Cr from target cells incubated in the absence of effectors. Test cpm represents ^{51}Cr release into wells containing effector and target cell populations. As was the case with the antiviral assays, appropriate normalisations based on both input cpm and equal RRL volumes were undertaken.

Antiproliferative assays

J2E cells were seeded at 1×10^5 cells/ml and grown to early logarithmic phase (24 h) at 37°C in DMEM supplemented with 10% FCS in an atmosphere of 5% (v/v) CO_2 in air. The MuIFNs-α were then added to 1 ml of cells to the required final concentration and cell numbers were estimated 24, 41 and 72 h after IFN addition by trypan blue or eosin exclusion. Control wheatgerm lysate was added to the samples containing the mixed MuIFNs-α standard to account for any growth inhibitory activity of the wheatgerm extract in which the MuIFNs-α1 and MuIFN-α4 were synthesized. The stability of the IFNs after 24 and 48 h of incubation was assessed by performing CPE reduction assays, as described above, on the supernatants.

Results

1. Antiviral assays

Antiviral assays were performed on two cell lines. Quantitative assays to determine the relative specific antiviral activity of MuIFN-α 1 and MuIFN-α4 were performed on L929 cells while qualitative assays were performed on J2E cells.

(a) Antiviral assays on L929 cells

Using mouse L929 fibroblasts no significant difference in the antiviral specific activities of the in vitro synthesized MuIFN-α4 and -α1 were observed. From 7 independent determinations the antiviral specific activity for MuIFN-α4 was 122% of the specific activity of MuIFN-α1. The IFNs-α which had been titrated in these antiviral assays on L929 cells were subsequently used in antiproliferative and NK cell stimulation assays.

(b) Antiviral assays on J2E cells

Antiviral assays were also performed on J2E cells. Following a lag period, the J2E cells grew exponentially from 24 to 72 h after seeding. For all antiviral assays the cells were seeded and grown overnight to ensure that they were

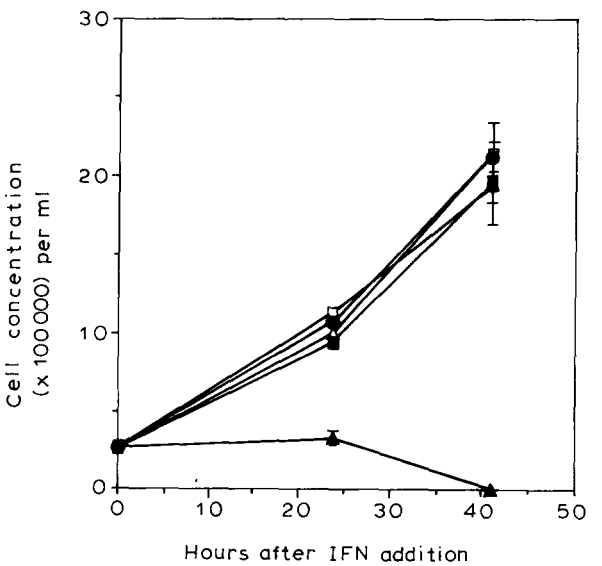

Fig. 1. Antiviral activities of MuIFN-α1, MuIFN-α4 and mixed MuIFNs-α on J2E erythroleukemia cells. The J2E cells were seeded at 10^5 cells/ml, grown overnight, then exposed to 10 IU/ml of each of the MuIFNs-α and challenged with EMCV 4 h later. The growth of J2E cells was measured 41 h after IFN addition, under the following conditions: (1) in the absence of IFNs-α and EMCV (●), (2) in the presence of EMCV alone (▲), (3) in the presence of mixed MuIFNs-α and EMCV (△), (4) in the presence of MuIFN-α1 and EMCV (□) and (5) in the presence of MuIFN-α4 and EMCV (■).

growing exponentially before exposure to MuIFNs-α or virus. The ability of 10 IU/ml of MuIFNs-α1, -α4 and mixed MuIFNs-α to protect J2E cells is shown in Fig. 1. In the absence of IFNs, J2E cells and J2E cells with wheatgerm lysate added were susceptible to the CPE of EMCV – within 24 h the number of viable cells was reduced to 40% of the controls, and at 41 h no viable cells were detected. In contrast, the presence of 10 IU/ml of mixed MuIFNs-α, MuIFN-α1 or MuIFN-α4 successfully inhibited the CPE of EMCV and the J2E cells grew to the same levels as the cell controls. Taking the relative specific activities into account, the antiviral activities of recombinant MuIFNs-α1 and -α4 do not differ significantly on J2E cells. This result is similar to that observed with L929 cells.

2. NK cell stimulation assays

The ability of MuIFN-α1 and MuIFN-α4 to stimulate NK cells to kill YAC-1 target cells was measured and compared at effector to target cell ratios ranging from 12.5:1 to 100:1. Fig. 2 represents the specific NK cell stimulation of the MuIFNs-α at various effector to target cell ratios. As with the antiviral assays there was no significant difference in the NK cell stimulating specific

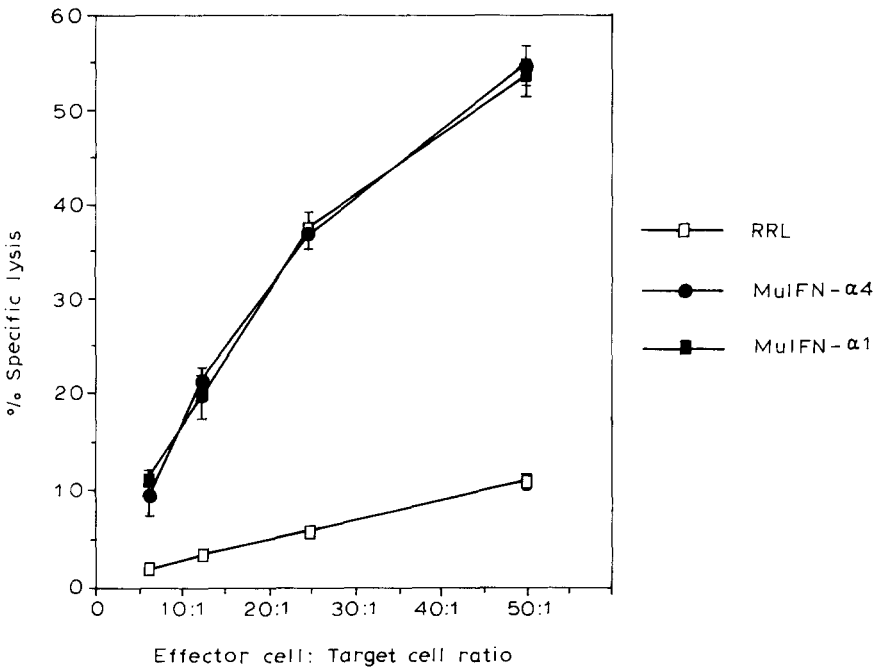

Fig. 2. NK cell stimulating activities of MuIFNs-α1, MuIFN-α4 and RRL. The ability of the MuIFN-α1, MuIFN-α4 and RRL to stimulate NK cells to lyse ^{51}Cr-labelled YAC-1 cells was measured over a range of effector to target cell ratios. Results are the means of triplicate determinations and error bars denote standard deviation.

activities of MuIFNs-α1 and -α4 over the range of effector to target cell ratios used.

3. Antiproliferative assays

To determine the effect of MuIFNs-α on the proliferation of J2E cells, cultures were established with various concentrations of mixed MuIFNs-α to 1000 IU/ml. The proliferation of J2E cells was inhibited in a dose-dependent manner in the presence of increasing concentrations of mixed MuIFNs-α until almost complete inhibition of growth occurred at 1000 IU/ml (Fig. 3).

Wheatgerm lysate at dilutions less than 1 in 30 was found to be cytotoxic to the J2E cells. The specific antiviral activities of recombinant MuIFNs-α produced by this in vitro expression system ranges from 5000 to 15000 IU/ml. Hence, a concentration of 100 IU/ml of recombinant IFNs was used in the antiproliferative assays. The antiproliferative activities of 100 IU/ml recombinant MuIFN-α1 and MuIFN-α4 were then compared to that of 100 IU/ml of mixed MuIFNs-α. The results of 3 separate experiments are shown in Table 1. The mixed MuIFNs-α were the most potent inhibitors of J2E cell growth, reducing the cell numbers by 27% relative to control levels. In contrast, MuIFN-α1 was a poor inhibitor of proliferation (cell growth inhibited by only

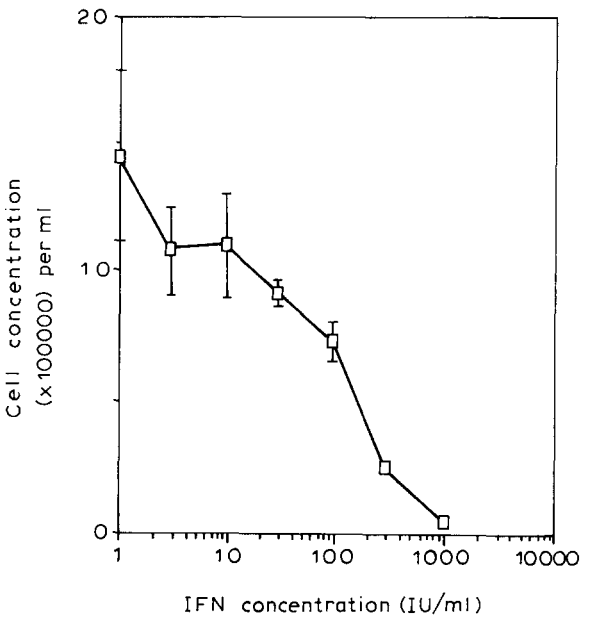

Fig. 3. Dose-response of J2E cells to mixed MuIFNs-α. J2E cells were seeded at 10^5 cells/ml and grown for 24 h at 37°C in 5% (v/v) CO_2 in air. Mixed MuIFNs-α at varying final concentrations were added in duplicate to 1 ml of cells in a 24-well plate. The cell numbers were determined 41 h after IFN addition. The results plotted are the means from duplicate determinations and error bars denote the standard deviations

TABLE 1

Antiproliferative activities of MuIFN-α1, MuIFN-α4 and mixed MuIFNs-α on J2E cells

	Inhibition of J2E cell proliferation (%)				
	Expt. 1	Expt. 2	Expt. 3	Mean ± standard deviation	Relative antiproliferative activities
Mixed MuIFNs-α	24%	29%	30%	27.7% ± 3.2	100
MuIFN-α1	0%	3%	0%	1% ± 1.7	4
MuIFN-α4	20%	20%	18%	19.3% ± 1.15	70

Cultures of J2E cells were established at 10^5 cells per ml overnight. The above MuIFNs were then added to a final concentration of 100 IU/ml and inhibition of growth was measured 41 h later. The relative antiproliferative activities were determined with the activity of the mixed MuIFNs-α set at 100%. The mean cell number ± standard deviation for the cell controls was 1.9 ± 0.3 × 10^6/ml.

1%), while MuIFN-α4 inhibited the cell growth significantly (19%). These experiments revealed that MuIFN-α4 had approximately 70% of the antiproliferative activity of the mixed MuIFNs-α while MuIFN-α1 possessed only about 4% of the mixed IFNs-α antiproliferative activity. From Table 1, the ratio of the antiproliferative activities of MuIFN-α1:MuIFN-α4 based on equal antiviral units was 4:70. However, when normalized on the basis of protein added, the ratio of the specific antiproliferative activities of MuIFN-α1:MuIFN-α4 was 8:100. There was no significant difference in level of antiviral units detected in the supernatants of the mixed MuIFNs-α, MuIFN-α1 and MuIFN-α4 after 24 and 48 h of incubation.

Discussion

In 1985, Zwarthoff et al. isolated and cloned 5 MuIFN-α genes and determined their levels of expression in mouse L-cells induced with Sendai virus, using S1 nuclease protection assays. MuIFN-α4 was the most highly expressed IFN-α subtype. The levels of MuIFN-α4 mRNA were at least 10-fold higher than that of MuIFNs-α1, -α2, -α5 and -α6. Similar studies using mouse L-cells induced with Newcastle disease virus (Kelley and Pitha, 1985a, 1985b) showed that MuIFN-α4 mRNA was expressed at 5- to 15-fold higher levels than MuIFNs-α2, -α5, -α6 and -α7, while MuIFN-α1 mRNA expression was not detected. Bisat et al. (1988) compared the levels of expression of MuIFNs-α1, -α4 and -α6 and observed that their levels of expression were cell-type dependent. However, irrespective of the inducer used, MuIFN-α4 mRNA was consistently expressed at higher levels than either MuIFNs-α1 or -α6 (10–50-fold higher in L-cells, 2-fold in FDC-1 cells).

The in vitro assays performed in this study confirmed previous antiviral data for MuIFNs-α1 and -α4 (Beilharz et al., 1991) in which there is no significant difference in the antiviral specific activities of MuIFNs-α1 and -α4. It was observed that within the limits of the antiviral bioassay, MuIFN-α4 was at

most 2-fold more active than MuIFN-α1 on mouse L929 cells. Similarly the NK cell stimulations of the two subtypes were very similar at all the effector to target cell ratios studied when equal protein amounts were compared. However, the antiproliferative activities were significantly different and this difference is not due to any difference in their stability.

The detailed comparisons of the antiproliferative activities were performed on J2E cells. The cells were ideal for use in this study because they were sensitive to both the antiproliferative and the antiviral activities of a standard preparation of MuIFNs-α which comprises a mixture of MuIFN-α subtypes (Figs. 3 and 1, respectively). This enabled a direct comparison of the relative antiproliferative and antiviral activities of the two MuIFNs, -α1 and -α4, on a single cell line.

There was no difference in the antiviral activities of MuIFNs-α1, -α4 and mixed MuIFNs-α on J2E cells (Fig. 1). In direct contrast to their approximately equal antiviral activities, the antiproliferative activities of the two MuIFN-α subtypes were markedly different (Table 1). Based on equal protein inputs, MuIFN-α4 inhibited the proliferation of J2E cells to a much greater extent than did MuIFN-α1. MuIFN-α1 was estimated to have only 8% of the antiproliferative capacity of MuIFN-α4.

In the HuIFNs-α system, the biological activities of cloned HuIFNs-α have been extensively studied. Fish et al. (1983) investigated the antiproliferative and antiviral activities of 5 different cloned HuIFN-α subtypes. While the subtypes had distinct biological properties, there was no correlation between the antiviral and antiproliferative activities of a particular HuIFN-α subtype. Others have noted a lack of NK cell stimulating activity in HuIFN-αJ (Ortaldo et al., 1984; Langer et al., 1986; Li et al., 1990), although it has the ability to bind to NK cells.

Similarly the present study has shown that there exists large functional differences between the two MuIFN-α subtypes studied. However, this functional difference cannot be due to differences in the IFN-α receptors of the different cells used in the bioassays, as was proposed by Li et al. (1990), because both antiviral and antiproliferative activities of MuIFNs-α1 and -α4 were performed on J2E cells. This large functional difference in antiproliferative activities must result from differences in their amino acid sequences. MuIFN-α4 and MuIFN-α1 have a total of 24 amino acid differences. However, only a limited number of such amino acid differences is considered biochemically important and lies within the three important domains implicated by Fish et al. (1989) for HuIFNs-α: the amino acids at positions 10, 17, 19, 20, 25, 97, 133. There is also a deletion of amino acid 103–107 from the MuIFN-α4 sequence. The effect of the amino acid 103–107 deletion and the individual amino acid differences on antiproliferative activity is the subject of ongoing structure/function studies in this laboratory.

Acknowledgements

This study was supported in part by a grant from the Australian National Health and Medical Research Council (NH&MRC), 890162 (M.W.B. and C.M.L.), and the following grants to S.P.K.: NH&MRC (910171), Australian Research Council, and University of Western Australia Medical Faculty Endowment.

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