Antiviral Research, 8 (1987) 209–221
Elsevier

AVR 00268

Recombinant interferons alpha and gamma: comparative antiviral activity and synergistic interaction in encephalomyocarditis virus infection of mice*

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(Received 17 July 1987; accepted 5 October 1987)

Summary

The antiviral properties of 2 recombinant DNA-produced interferons, a human hybrid interferon alpha that is active in mice and a murine interferon gamma, were examined in the treatment of mice infected with encephalomyocarditis virus. A single dose of interferon alpha induced a protective state in mice more rapidly than did interferon gamma, but the activity of the latter was more long lasting. When interferon and virus were administered 6 h apart, either intraperitoneally or intravenously, interferons alpha and gamma were equally effective. However, this was not the case when the routes of treatment and infection were different. Interferon alpha showed somewhat reduced activity when the route of administration (intravenous) was different from the route of virus challenge (intraperitoneal) while interferon gamma showed very little activity when tested in this manner. When interferons alpha and gamma were administered in combination to mice, a marked synergistic antiviral effect was observed.

Antiviral; EMC virus; Interferon; Synergy

* First presented at the NATO Advanced Study Course/FEBS Advanced Course on Antiviral Drug Development: A Multidisciplinary Approach, Il Ciocco, Lucca, Italy, May 1987.

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Introduction

Originally called immune or type II interferon, interferon-gamma (IFN-γ) was first distinguished from leukocyte interferon, interferon-alpha (IFN-α), by the nature of the stimulus necessary to elicit its production, the cell type from which it derived and by its relative instability at low pH. Subsequent studies have highlighted a number of other differences between these interferons which may be of importance when considering the use of these molecules for chemotherapy in man. In particular, IFN-γ has greater antiproliferative activity, relative to antiviral activity, than IFN-α (Blalock et al., 1980) while IFN-α induces an antiviral state in cells more rapidly than IFN-γ (Dianzani et al., 1978). In addition, Branca and Baglioni (1981) have shown that the two interferons bind to distinct receptors at the cell surface while other studies suggest that they may exert an antiviral action through different pathways in the virus-infected cell. For example, Samuel and Knutson (1983) have shown that while both IFN-α and IFN-γ induce a resistance in human cells to challenge with vesicular stomatitis virus (VSV), treatment with IFN-α, but not with IFN-γ, results in the induction of a protein kinase activity that phosphorylates eIF-2 and the ribosome-associated protein P_1. Moreover, the antiviral activity of IFN-α is also apparent in the inhibition of viral protein synthesis in VSV-infected cells (Masters and Samuel, 1983) although a similar effect is not seen following treatment with IFN-γ (Ulker and Samuel, 1985).

There have been many reports of the synergistic in vitro interactions of purified preparations of α and γ interferons, either natural or recombinant. Synergistic antiproliferative activity of human interferons (Fleischmann et al., 1984a,b; Czarniecki et al., 1984; Denz et al., 1985; Oleszak and Stewart, 1985) and murine interferons (Brunda and Wright, 1986) and synergistic immunomodulatory activity (Weigent et al., 1983) have been reported. Using either crude or purified preparations of murine interferons, Fleischmann and Fleischmann (1984) and Fleischmann et al. (1984b) have shown IFN-γ to exert a synergistic antiviral effect when used in combination with IFN-α (or -β) in mouse cells infected with mengovirus. Synergy between IFN-α (or -β) and IFN-γ has also been shown using purified human interferons in herpes simplex virus (HSV) (Czarniecki et al., 1984), mengovirus (Fleischmann et al., 1984a) and VSV (Oleszak and Stewart, 1985) infected cell cultures.

On the other hand, there have been no reports demonstrating synergistic antiviral interaction between 2 interferons in the systemic treatment of virus infections in vivo although such activity might be predicted from in vitro observations. In contrast, the potentiation of the in vivo anti-tumour activity of murine α or β interferon by murine γ interferon has been shown (Fleischmann et al., 1980; De Clercq et al., 1982; Koren et al., 1986). Furthermore, a synergistic antiviral interaction in vivo between a single interferon and a nucleoside antiviral compound has been reported (Fraser-Smith et al., 1984a,b, 1985; Cerruti et al., 1985; Connell et al., 1985).

The relative merits of interferons α and γ as antiviral or antitumour therapeutic agents in man remain unclear. However, recombinant DNA technology has now

made available in pure form quantities of several interferon types, facilitating more extensive in vivo studies. The cloning and expression of the gene coding for mouse IFN-γ (rMuIFN-γ) (Gray and Goeddel, 1983) has presented the opportunity to study this interferon in experimental virus infections in mice and to compare its activity with that of an α interferon. Since quantities of murine IFN-α were not available to us, we have used a hybrid human IFN-α (rHuIFN-αA/D) (Weck et al., 1981) that has been shown to be active in mice (Weck et al., 1981, 1982; Kramer et al., 1983). We used encephalomyocarditis (EMC) virus in our experiments because previous studies have shown it to be particularly responsive to the antiviral effect of interferon in infected mice (Weck et al., 1982; Kramer et al., 1983; Sim et al., unpublished). Here we show significant differences between the 2 interferons with respect to both the kinetics of induction and regression of the antiviral state in vivo and their efficacy by 2 routes of administration in EMC virus infections of mice. We present evidence to show that these molecules exhibit a synergistic interaction in vivo that may possibly be exploited in the treatment of virus disease.

Materials and Methods

Interferons

rHuIFN-αA/D, specific activity 3.0×10^7 Units/mg, was prepared at the Roche Research Center using monoclonal antibody purification methods as described elsewhere (Rehberg et al., 1982). rMuIFN-γ, specific activity 2.8×10^6 U/mg, was supplied by Genentech Inc., S. San Francisco, California. Interferons were assayed in a VSV cytopathic inhibition assay in L-929 cells as previously described (Kramer et al., 1983). Activities of rHuIFN-αA/D were expressed as units per millilitre in terms of the National Institutes of Health mouse reference interferon (G-002-904-511). This same standard, a mixture of mouse interferons α and β, was found to be inappropriate for standardization of activities of rMuIFN-γ. Using an internal standard of rMuIFN-γ, the sensitivity of L-929 cells to this interferon was seen to change with passage, independently of their sensitivity to the NIH α/β interferon. Therefore, activities of rMuIFN-γ were expressed in terms of a laboratory standard.

Cell culture studies

L-929 cells were cultured in Eagle's Minimum Essential Medium with Earle's salts, supplemented with L-glutamine, 10% foetal bovine serum and gentamycin. Confluent monolayers were transferred to maintenance medium (as above except with 2% serum), treated with interferon, or mock treated, and further incubated for the indicated time. All cultures were then infected by removing the interferon inoculum, washing the monolayer and adding EMC virus, 3 p.f.u./cell, and incubating for a further 8 h. Cells were scraped-off into the medium and the virus released by 3 cycles of freezing and thawing. Virus yields (50% tissue culture infectious dose – $TCID_{50}$) were determined by titrations in L-929 cells in 96-well plates.

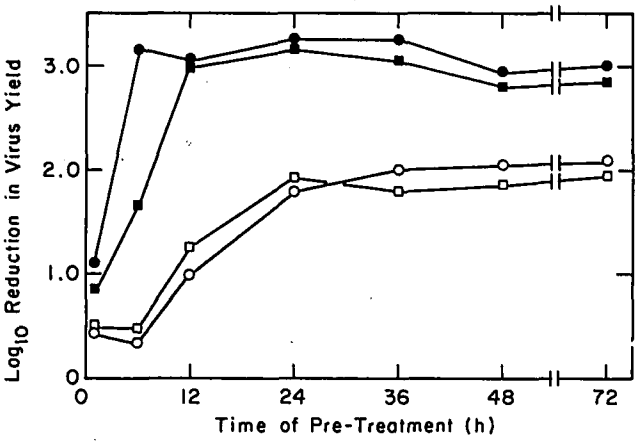

Fig. 1. Induction of resistance to EMC virus infection in L cells. Cultures were treated with 100 U/ml (●) or 1 U/ml (○) of rHuIFN-αA/D, or with 100 U/ml (■) or 1 U/ml (□) rMuIFN-γ and further incubated for the indicated time. Interferon-treated cultures were infected with EMC virus and the virus yield compared to that of mock-treated, infected cultures. Each point is the mean of the yields from 3 replicate cultures.

In vivo studies

Swiss albino (CD_1) mice (18 – 20 g females, 10 animals per group) were housed under controlled conditions. Animals were treated with interferon diluted in Dulbecco's phosphate buffered saline containing 0.5% bovine serum albumin (PBS/BSA) and infected by the indicated route with 10 × 50 percent lethal dose (LD_{50}) of EMC virus prepared in L-929 cells. Control groups of animals received vehicle alone as appropriate. Mice were checked daily for 21 days and deaths recorded. LD_{50} values (virus titrations) and 50% protective dose (PD_{50}) values (interferon titrations) were determined by the method of Reed and Muench (1938).

Results

Kinetics of interferon action

Dianzani et al. (1978) have shown, using human or mouse interferons in homologous cells, that IFN-α induces an antiviral state in those cells more rapidly than IFN-γ. We examined the ability of rHuIFN-αA/D and rMuIFN-γ to induce and maintain an antiviral state in mouse L-cells. At different times from 72–1 h before infection, cultures were treated with either 100 or 1 U/ml of either interferon type and incubated at 37°C until time 0. All cultures were then washed to remove any residual interferon and infected with EMC virus. The yields of virus, relative to mock-treated controls, 8 h after infection are shown in Fig. 1. When cultures were treated with 100 U/ml rHuIFN-αA/D, the yield of EMC virus was slightly reduced in those treated with interferon for 1 h and maximum reduction in virus yield was obtained after 6 h of interferon pretreatment. Cultures that had been maintained in the presence of rHuIFN-αA/D for 6–72 h prior to infection

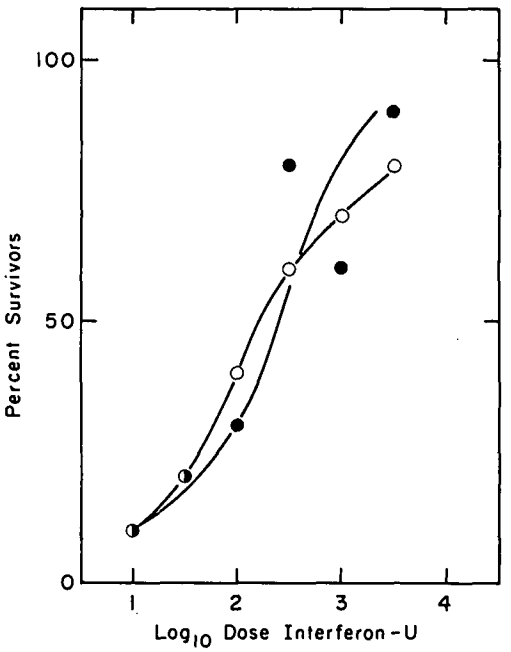

Fig. 2. Comparison of the protective efficacy of rHuIFN-αA/D and rMuIFN-γ in EMC virus infected mice. Groups of 10 mice were treated i.p. with rHuIFN-αA/D (○) or with rMuIFN-γ (●) and infected 6 h later with EMC virus.

exhibited a similar resistance to infection. In contrast, maximum reduction in EMC virus yield from cultures treated with 100 U/ml rMuIFN-γ was only observed in those pretreated for at least 12 h. At the low dose of 1 U/ml, the difference between the 2 interferon types was not apparent and maximum reduction in virus yield was observed only after 24 h interferon pretreatment.

In order to determine whether rHuIFN-αA/D and rMuIFN-γ also differed in their ability to induce a protective antiviral state in vivo, we first performed dose titrations of each agent (0.5 $\log_{10}$ dilutions of interferon), treating animals intraperitoneally (i.p.) 6 h before infection with EMC virus by the same route. The results (Fig. 2) show that the interferons afforded protection to mice from virus infection

TABLE 1

Dose of interferon, administered as a single treatment, required to protect 50% of mice from a lethal infection with EMC virus.

Interferon	Time of treatment (h) before virus challenge					
	1	6	12	24	36	48
rHuIFN-αA/D	17000[a]	870	2400	17000	>100000	>100000
rMuIFN-γ	>100000	1000	2200	4800	7600	>100000

[a]U/mouse.

in a dose dependent manner and, under these conditions, rHuIFN-αA/D and rMuIFN-γ showed similar in vivo activity. We next compared the 2 interferons in a similar experiment, administering a single treatment of interferon i.p., examining the effect of difference in time between interferon treatment and EMC virus infection. Dose response curves were constructed for each interferon at each pretreatment time and PD_{50} values were calculated (Table 1). As was observed in cell culture, treatment with rHuIFN-αA/D induced an antiviral state in mice more rapidly than treatment with rMuIFN-γ. Protection of mice was observed when they were challenged with EMC virus 1 h after treatment with rHuIFN-αA/D and mice were still protected to some extent if challenge was delayed until 24 h after interferon administration. The optimum time between treatment and challenge was 6 h. In contrast, efficacy following treatment with rMuIFN-γ was seen only after 6 h but the protection was maintained for up to 36 h. rHuIFN-αA/D and rMuIFN-γ appeared equally effective when administered 6 h before challenge and also at 12 h before infection, although they were both less effective at this time.

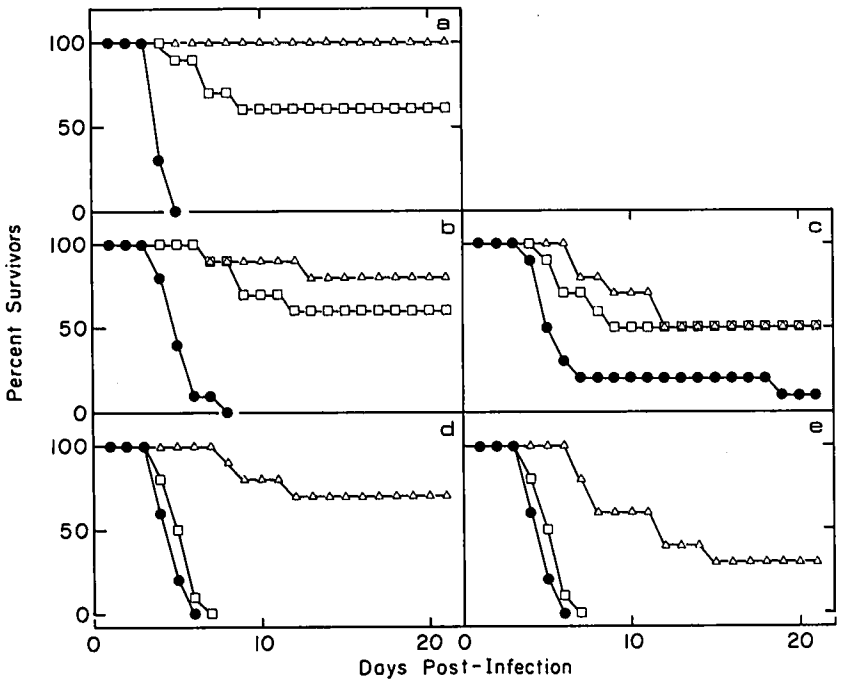

Fig. 3. Effect of route of administration on the interferon efficacy in EMC virus infected mice. Groups of 10 mice were treated with interferon either i.p. (panel a) or i.v. (panels b–e) and infected 6 h later with EMC virus either i.v. (panels b and c) or i.p. (panels a, d and e). Mice received either 32 000 U (△) or 320 U (□) of rHuIFN-αA/D (panels a, b and d) or rMuIFN-γ (panels c and e) or PBS/BSA alone (●).

Effect of route of treatment on efficacy

The preliminary observations of Shalaby et al. (1985) suggested that rMuIFN-γ was ineffective when administered intravenously (i.v.) to mice that had been infected by the i.p. route. We examined the efficacy of interferons alpha and gamma given i.v. 6 h prior to EMC virus challenge. Virus was administered either by the same route as the interferon treatment or i.p. following i.v. interferon administration. Mice, both treated with rHuIFN-αA/D and infected by the i.p. route, were also included for comparison. Survival curves for representative groups of infected mice, both treated and control, are shown in Fig. 3. Survival data were obtained from titrations of each interferon at 7 concentrations in the range 10^2–10^5 U/mouse (data not shown) and PD_{50} values were calculated for each treatment regimen (Table 2). When the routes of treatment and of infection were both i.v. (Fig. 3b and c), the PD_{50} values showed rHuIFN-αA/D and rMuIFN-γ to be equally effective (Table 2). Moreover, rHuIFN-αA/D administered i.v. was only 4-fold less active than the same interferon given i.p. to mice that were then challenged with virus by the same route (Fig. 3a,b and Table 2). However, when interferon was given i.v. and the mice challenged by the i.p. route, there was a marked difference between the 2 interferons. Neither interferon showed a protective effect at a low dose of 320 U while at a high dose of 32 000 U rHuIFN-αA/D was more effective than rMuIFN-γ (Fig. 3d and e). The PD_{50} values revealed that rHuIFN-αA/D administered i.v. to mice challenged with virus by the i.p. route was only slightly less effective than when the route of treatment and infection (i.v.) were the same (Table 2). In contrast, the activity of rMuIFN-γ was markedly less when the routes of treatment and infection were different (Table 2).

Effect of treatment with a combination of IFN-α and -γ

Finally, in view of the differences in the antiviral properties of rHuIFN-αA/D and rMuIFN-γ seen here and the synergistic interaction in vitro reported by others for IFN-α and IFN-γ (Czarniecki et al., 1984; Fleischmann et al., 1984a), it was of interest to determine whether there was an interaction between the 2 interferons in vivo. In a preliminary experiment, groups of mice were treated i.p. with either rHuIFN-αA/D or rMuIFN-γ or with a 50:50 mixture (on a Unit basis) of both interferons and challenged 6 h later with EMC virus. From dose response curves, the PD_{50} values for rHuIFN-αA/D and for rMuIFN-γ were calculated to

TABLE 2

Efficacy of rHuIFN-αA/D and rMuIFN-γ when administered to mice by different routes.

Route of treatment	Route of infection	PD_{50} (U/mouse)	
		rHuIFN-αA/D	rMuIFN-γ
Intraperitoneal	Intraperitoneal	400	ND[a]
Intravenous	Intravenous	1800	1650
Intravenous	Intraperitoneal	2400	48 000

[a]ND = Not determined.

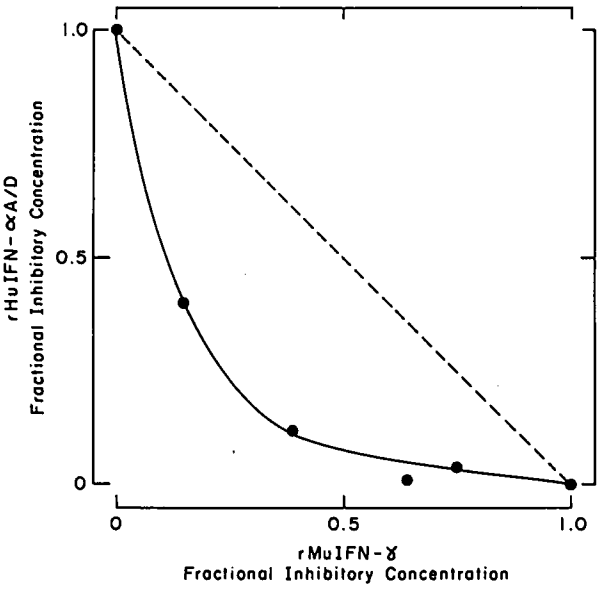

Fig. 4. Protection of mice from EMC virus infection by combinations of rHuIFN-αA/D and rMuIFN-γ. Groups of 10 animals were treated with interferon and infected 12 h later with EMC virus. PD_{50} values were determined for each interferon alone and for 4 doses of rHuIFN-αA/D in the presence of varying amounts of rMuIFN-γ and expressed as fractional inhibitory concentrations for each interferon in the combination. The expected plot, if the interaction between the interferons was additive, is shown as the dashed line.

be 500 U/mouse and 556 U/mouse respectively. From survival data of mice treated with the mixture of both interferons, the PD_{50} was determined to be 250 U/mouse, i.e., 125 U of rHuIFN-αA/D + 125 U of rMuIFN-γ. Thus it appeared that when mice were treated with both interferons in combination, the total Units of interferon required to protect 50% of animals was less than when treatment was with either interferon alone.

In a second, more extensive experiment, mice were treated i.p. with either rHuIFN-αA/D, rMuIFN-γ or with both interferons in combination and infected i.p. 12 h later with EMC virus. For combination treatments, a sub-optimal dose of rHuIFN-αA/D was given to mice together with a range of doses (from 10–32 000 U) of rMuIFN-γ. From the survival results, that dose of rMuIFN-γ, together with the fixed, sub- optimal dose of rHuIFN-αA/D, required to protect 50% of mice was calculated. Four sub-optimal doses of rHuIFN-αA/D were used (10–320 U) in combination with rMuIFN-γ. PD_{50} values were also determined for each interferon used alone. In all 4 cases, the total number of interferon Units (rHuIFN-αA/D + rMuIFN-γ) in the combinations required to protect 50% of mice were less than the PD_{50} values for either interferon alone. The fractional inhibitory concentrations (FIC) for each interferon in the combination (PD_{50} of interferon A in combination/PD_{50} of interferon A alone) were calculated (Berenbaum, 1978) and the results analysed by plotting an isobol (Fig. 4). If the two interferons act in an

additive manner, then the data points would be expected to fall on a straight line connecting FIC values of 1.0 on the ordinate and abscissa. If the observed values fall to the left of such a line, the interaction is synergistic; values occurring to the right of the line indicate antagonism. Our results clearly show that there was a marked synergistic antiviral effect when the 2 interferons were administered in combination.

Discussion

Using highly purified preparations of recombinant proteins, we have found several differences in the pharmacological properties of rHuIFN-αA/D compared with rMuIFN- γ in virus-infected mice. Particularly striking were the differences in the kinetics of induction and maintenance of a protective antiviral state and the efficacy of the interferons when administered by a route other than the route of virus infection. It was important to show that both rHuIFN-αA/D and rMuIFN-γ protected mice in a dose-dependent manner when administered as a single treatment prior to infection (Fig. 2) since such responsiveness has enabled us to quantify the in vivo activity of these interferons and allowed us to make comparisons between the activities of the two proteins.

The protection of mice from virus challenge was observed as soon as 1 h after treatment with rHuIFN-αA/D and the protected state was maintained for 24 h although comparison of PD_{50} values clearly revealed that there was an optimum time between treatment and virus challenge (Table 1). In contrast, rMuIFN-γ was first seen to be protective only after 6 h had elapsed between treatment and virus challenge. However, the antiviral state induced by this interferon was still detectable in mice up to 36 h later. The more rapid induction of an antiviral state by rHuIFN-αA/D than by rMuIFN-γ in mice was also seen with high (100 U/ml), but not with low (1 U/ml), concentrations of interferon in cell culture (Fig. 1). The more prolonged antiviral state obtained in cell culture compared with that seen in animals may be the result, at least in part, of the absence of metabolism/elimination mechanisms that operate in vivo. However, pharmacokinetic studies in mice given interferon i.v. have shown the rate of clearance from the blood of rMuIFN-γ to be approximately 10-fold faster than of rHuIFN-αA/D (M. Palladino, personal communication). Therefore, the prolonged antiviral state induced in vivo by rMuIFN-γ does not appear simply to be the consequence of more favourable pharmacokinetic properties. Whether differences between interferons in the kinetics of antiviral activity also occur in man is not known but the observations of Barouki and colleagues are of interest in this regard. Examining the antiviral state in peripheral blood mononuclear cells of patients given a single intramuscular injection of IFN-α, Barouki et al. (1987) also found resistance to antiviral challenge was detectable within 1 h of interferon administration. Interestingly, they found that the antiviral state persisted for considerably longer (up to 144 h) than we observed in mice.

At the time of optimum (6 h) and near optimum (12 h) protection in vivo, rHuIFN-αA/D and rMuIFN-γ appeared to be equally effective on a unit basis (Ta-

ble 1). This was seen to be the case in several experiments although the calculated PD_{50} values varied in a 2- to 4-fold range. This variability may reflect differences in the responsiveness of mice from experiment to experiment and/or errors derived from in vitro determinations of interferon activity that are inherent in the assay. Shalaby et al. (1985) have also demonstrated rMuIFN-γ to be effective in mice against an EMC virus challenge and found no difference in the protective efficacy of rMuIFN-γ or rHuIFN-α2/α1 (the same human hybrid was used in our studies). On the other hand, using multiple dose treatment regimens in EMC virus infected mice similar to those of Shalaby et al., we have shown that rHuIFN-αA/D was approximately 5-fold more effective, on a unit basis, than rMuIFN-γ (Connell et al., 1986). We have constructed our own internal laboratory standard for the assay of rMuIFN-γ to permit us to make comparisons between experiments. However, in the absence of a recognised standard for this interferon, it is possible that others may find differences in potency between IFN-α and IFN-γ in vivo as a result of using a different cell culture unit for rMuIFN-γ activity. Meaningful comparisons of results between groups of workers is not possible, therefore, until a standard for rMuIFN-γ is established.

In marked contrast to the results of Shalaby et al. (1985), we have shown that rMuIFN-γ expresses antiviral activity when administered i.v. (Fig. 3). The failure of Shalaby et al. to show rMuIFN-γ to be effective by this route was probably a consequence of administering interferon i.v. to mice that were infected i.p. Thus, our results show that while rMuIFN-γ was markedly active given i.v. when the route of treatment and virus infection was the same, the protein was barely active when the routes of treatment and infection were different. In contrast, rHuIFN-αA/D showed significant antiviral activity when given by the i.v. route whether infection was by i.v. or i.p. routes. When the routes of treatment and infection were the same, either i.p. or i.v., rHuIFN-αA/D and rMuIFN-γ were both effective and we were unable to discern any differences in their potencies. The reasons for failure of rMuIFN-γ to protect mice when given i.v. against i.p. virus challenge are not understood at present. However, it is interesting to note that rMuIFN-γ, but not rHuIFN-αA/D, was toxic to mice when repeatedly administered over a period of 10 days by the i.p. route whilst it was not toxic at equivalent doses when administered i.v. (G.A. Truitt, personal communication) suggesting that the expression of other properties of this molecule is also highly dependent on the route of administration.

IFN-α and IFN-γ have been reported to show synergistic antiviral action in vitro (Czarniecki et al., 1984; Fleischmann and Fleischmann, 1984; Fleischmann et al., 1984a,b; Oleszak and Stewart, 1985) but we are unaware of any reports showing such synergy in vivo. Neumann-Haefelin et al. (1985) have briefly reported the use of IFN-α and IFN-γ in combination in the treatment of ocular HSV-1 infection of primates and claim evidence of a synergistic interaction between the 2 interferons. However, the data are, in our view, insufficient to support this claim. Our results (Fig. 4) clearly demonstrate such an interaction in EMC virus-infected mice. Furthermore, synergy between these 2 interferons is not restricted to either single dose treatments or to EMC virus infections only since we have also observed such in-

teractions in the treatment of mice infected with either EMC virus or HSV, adminstering the interferons in multiple treatment regimens (Connell et al., 1986). A number of mechanisms have been determined to be the basis for synergy between anti-bacterial compounds, particularly that the 2 drugs act sequentially in the inhibition of a common pathway (e.g., trimethoprim and sulfamethoxazole) or that one compound interferes with the production or action of a bacterial enzyme that would normally metabolize and inactivate the second (e.g., clavulanic acid with penicillins or cephalosporins). While the mechanism for the synergistic interaction of rHuIFN-αA/D and rMuIFN-γ is not known, we may speculate that the 2 interferons, each binding to their respective receptors, then induce mechanisms of antiviral action that are complementary but different.

Finally, while the in vivo studies reported here have confirmed some of the observations made previously by others using cell culture systems, we have also identified differences between interferons α and γ that would not be apparent in cell culture or could not be assumed, from in vitro studies, to be the case in vivo. Further studies are required to investigate more extensively the comparative antiviral activities of interferons α and γ in vivo, and particularly in man.

Acknowledgement

We thank Dr P. Trown for his advice and encouragement. We also gratefully acknowledge the support of many colleagues at Roche who prepared the rHuIFN-αA/D and who assisted in some of the experiments. We also thank Dr M. Palladino and colleagues at Genentech for the generous provision of rMuIFN-γ.

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