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Potentiating effect of murine interferon-γ-containing lymphokine preparations on the antiviral and antiproliferative effects of murine interferon-α/β: identification of the potentiation factor as murine interferon-γ itself

W. Robert Fleischmann, Jr. and Christina M. Fleischmann

Department of Microbiology, The University of Texas Medical Branch, Galveston, TX 77550, U.S.A.

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Summary

Mixed preparations of murine interferon-γ (MuIFN-γ) and murine interferon-α/β (MuIFN-α/β) have been shown to induce more than additive levels of antiviral protection, when compared to those induced by these interferons given separately. MuIFN-γ preparations contain many lymphokines and several of these as well as MuIFN-γ itself may participate in this potentiation. In the present study, natural as well as three recombinant DNA-derived MuIFN-γ's, in combination with antibody to MuIFN-γ have been employed to examine the precise role of MuIFN-γ. The antiviral effect was examined with a single cycle virus yield reduction assay and the antiproliferative effect with a colony formation inhibition assay. Recombinant DNA-derived MuIFN-γ was as effective as natural MuIFN-γ at participating in the potentiation of both the antiviral and antiproliferative activities. Antibody to MuIFN-γ effectively blocked the potentiation of both the antiviral and the antiproliferative activities of natural and recombinant DNA-derived MuIFN-γ's. Since the recombinant DNA-derived preparations from *E. colie* can be assumed not to contain mammalian proteins other than MuIFN-γ, the data conclusively demonstrate that the potentiation factor in MuIFN-γ preparations is MuIFN-γ itself.

interferon; potentiation

Introduction

Interferons are natural body proteins, induced by a variety of stimuli, which have been shown to have antiviral, antiproliferative, and immunoregulatory properties [1]. Three types of interferons have been recognized: α, β and γ [14]. Murine interferons α

(MuIFN-α) and β (MuIFN-β) are produced as a natural mixture (MuIFN-α/β) by infecting cultured fibroblastoid cell lines (L929 or C243) with virus [10]. Murine interferon-γ (MuIFN-γ) is produced by lymphocytes after stimulation with T-cell mitogens [7,9,16]. In mice as well as in man IFN-α and IFN-β have many similar biological properties and have been called type I interferons to differentiate them from IFN-γ or type II interferon.

A synergistic enhancement or potentiation of biological activity was originally observed in the mouse system by combining preparations of MuIFN-γ with preparations of MuIFN-α/β [4,17]. Initial studies investigated the potentiation of the antiviral action of interferon; subsequent studies have extended the observation to interferons' in vivo antitumor activity [2,5], their in vitro antiproliferative activity [3], their activating effects on natural killer cells [15], and their bone marrow suppressive activity [11].

Potentiation has been suggested to be the result of a mutually synergistic interaction between the interacting interferons [3], rather than to effects of other non-interferon molecules present in the preparation. However, due to difficulty in obtaining pure preparations of MuIFN-γ, definitive proof for the participation of this interferon in the potantiation phenomenon has not been available. This paper describes studies employing MuIFN-γ's produced by recombinant DNA technology in combination with antibody to MuIFN-γ which demonstrate that the potentiating factor in MuIFN-γ preparations is MuIFN-γ itself.

Methods and Materials

Cells

Mouse L-cells (clone 929) were grown at 37°C in a humidified, 4% $C0_2$ environment in Eagle's minimal essential medium (Earle's base) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamycin (11 μg/ml). The cells were routinely passaged every 2 or 3 days. For experiments and assays, cells were plated in either 96 well microtiter plates ($10^{4.78}$ cells/well) or 35 mm diam. plastic tissue culture dishes ($10^{6.08}$ cells/plate).

Interferons

MuIFN-α/β was produced by challenging mouse L-cells with Newcastle disease virus as previously described [6]. Previous studies have shown that 10 000-fold purification of MuIFN-α/β did not change its potentiation characteristics [4]. Therefore, crude MuIFN-α/β preparations ($10^{3.44}$ units/mg of protein) were employed for all experiments.

Natural MuIFN-γ was prepared in dissociated C57BL/6-mouse spleen cells stimulated in culture with the T-cell mitogen staphylococcal enterotoxin A (obtained from the Microbial Biochemistry Branch, Division of Microbiology, Food and Drug Administration, Cincinnati, OH) as previously described [12]. Previous studies have shown that 200-fold purification of MuIFN-γ did not change its potentiation characteristics [4]. Therefore, in the present study, crude preparations ($10^{1.68}$ units/mg of protein) were employed as the natural MuIFN-γ.

Three recombinant DNA-derived MuIFN-γ preparations were generously provided by Drs. Patrick Gray, Ernst Rinderknecht, and Gene Burton (Genentech, Inc., San Francisco, CA). They were produced as previously described [8] in the transformed monkey cell line COS-7 (MuIFN-$\gamma_{R\text{-}COS}$, $10^{2.44}$ units/mg of protein), in Chinese hamster ovary cells (MuIFN-$\gamma_{R\text{-}CHO}$, $10^{3.44}$ units/mg of protein), and in *E. coli* (MuIFN-$\gamma_{R\text{-}Coli}$, $10^{6.66}$ units/mg of protein). Supernatant fluids from non-transfected, parental COS-7 and CHO cells were also provided.

All interferon preparations were titrated in our laboratory by a plaque reduction assay in microtiter plates with L-cells and vesicular stomatitis virus as previously described [3]. One laboratory unit/ml of interferon was defined as the concentration of interferon which reduced the number of virus plaques to 50% of the control level. All assay results are expressed in units/ml, corrected by comparison with a laboratory reference preparation of MuIFN-α/β, that had been calibrated on the W.H.O. international reference preparation G002-904-511 (N.I.H.). Specific activities are reported as units (as determined in our laboratory) per mg of protein (as reported to us by the laboratory of origin).

Antibody to MuIFN-γ [13] was generously provided by Dr. Howard Johnson (University of Texas Medical Branch). It had a neutralization titer of 10^2 against 10 units/ml of MuIFN-γ.

Virus yield reduction assays

L-cell monolayers were treated with growth medium or with serial dilutions of interferon in growth medium for 12 – 14 h. The interferon was removed and the cells were challenged with mengovirus (Franklin isolate) at a multiplicity of 10 plaque-forming units/cell. Virus was allowed to adsorb for 45 min. The cells were washed twice, overlaid with growth medium, and allowed to incubate for 24 h. Supernatant fluids were harvested and stored at –20°C until assayed for virus by plaque assay as previously described [4].

Assay of the antiproliferative effect of interferons

Colony inhibition assays were done with mouse B-16 melanoma cells as previously described [3]. Log phase B-16 cells were plated on 35 mm diam. culture dishes ($10^{2.48}$ cells/plate) and serial dilutions of interferon samples or mixtures were added. The cultures were incubated for 6 days and then stained with crystal violet (1% in 20/80, v/v methanol/water). Colonies were counted whereby a colony was defined as a group of 2 or more cells of identical morphology. This definition was chosen because it was based on a single replication event, and thus allowed for the most rigorous evaluation possible of the antiproliferative effectiveness of interferon [3]. For control plates the cloning efficiency thus determined was 60%, the number averaged 180 per plate, and the colonies contained on average 80–100 cells.

Colony counts were plotted against sample dilution, and 50% inhibition of cloning efficiency was taken as the titration end-point. Our laboratory reference preparations of MuIFN-α/β and MuIFN-γ were assigned an antiproliferative unitage on the basis of a series of tests conducted as described above. For this study one antiproliferative unit of MuIFN-α/β or MuIFN-γ corresponded to, respectively, 3000 and 18 antiviral

units. In each experiment the reference preparations were run in parallel with the samples and mixtures to be tested, thus allowing comparison of results between experiments.

Results and Conclusions

To determine whether natural and recombinant DNA-derived MuIFN-γ's could interact synergistically with MuIFN-α/β, the interferons were applied separately and in various combinations in single cycle virus yield reduction experiments. Table 1 presents the data from 3 such experiments in which one preparation of natural and 3 preparations of recombinant DNA-derived MuIFN-γ's (MuIFN-$\gamma_{R\text{-}COS}$, MuIFN-$\gamma_{R\text{-}CHO}$, and MuIFN-$\gamma_{R\text{-}Coli}$) were used. No significant differences in potentiation levels were seen between combinations of MuIFN-α/β with the preparation of natural MuIFN-γ and similar combinations containing either of the 3 recombinant DNA-derived MuIFN-γ's.

TABLE 1

Potentiation of antiviral activity by mixing preparations of MuIFN-α/β with preparations either natural or recombinant DNA-derived MuIFN-γ

Experiment No.	Interferon Mixtures applied[a]	Antiviral titer (units/ml)		Potentiation factor[d]
		Observed[b]	Expected[c]	
1	IFN-α/β	36		
	IFN-γ_N	30		
	IFN-γ_N + IFN-α/β	506	66	7.7
	IFN-$\gamma_{R\text{-}COS}$	22		
	IFN-$\gamma_{R\text{-}COS}$ + IFNα/β	421	58	7.3
2	IFN-α/β	21		
	IFN-γ_N	11		
	IFN-γ_N + IFN-α/β	236	32	7.4
	IFN-$\gamma_{R\text{-}CHO}$	11		
	IFN-$\gamma_{R\text{-}CHO}$ + IFNα/β	223	32	7.0
3	IFN-α/β	21		
	IFN-γ_N	16		
	IFN-γ_N + IFN-α/β	177	37	4.8
	IFN-$\gamma_{R\text{-}Coli}$	13		
	IFN-$\gamma_{R\text{-}Coli}$ + IFNα/β	210	34	6.2

[a] IFN-α/β: natural MuIFN-α/β; IFN-γ_N: natural MuIFN-γ; IFN-$\gamma_{R\text{-}COS}$: recombinant DNA-derived MuIFN-γ produced in transfected COS cells; IFN-$\gamma_{R\text{-}CHO}$: recombinant DNA-derived MuIFN-γ produced in transfected Chinese hamster ovary cells; IFN-$\gamma_{R\text{-}coli}$: recombinant DNA-derived MuIFN-γ produced in *E. coli*.

[b] Determined by virus yield reduction assay, standardized on a reference preparation of MuIFN-α/β.

[c] Determined by adding the observed separate titers of the interferons present in the mixtures.

[d] Determined by dividing observed by expected titers.

Thus, the data suggest that the recombinant DNA-derived MuIFN-γ's were as effective as natural MuIFN-γ's in their participation in the potentiation of interferon's antiviral activity. Potentiation of antiproliferative activity was examined in a similar fashion.

The preparations of natural and recombinant DNA-derived MuIFN-γ's were employed separately and in combination with MuIFN-α/β in colony inhibition assays. In order to study potentiation in antiproliferative activity, serial dilutions of individual or mixed interferon preparations were applied in colony inhibition tests. The colony counts were plotted against dilution, and with 50% inhibition of cloning efficiency as an end-point (corresponding to one antiproliferative unit/ml), each individual sample or its dilution was assigned an 'observed' titer in antiproliferative units/ml. For mixtures of the two interferons 'expected' titers of any dilution could be calculated by simple addition of the observed titers of the individual ingredients. Potentiation was apparent from the fact that the observed titers of mixtures were several-fold higher than expected. Table 2 presents the data from a representative experiment in which preparations of natural MuIFN-γ and recombinant DNA-derived MuIFN-γ's from Chinese hamster ovary cells and from *E. coli* were used.

No significant differences in potentiation levels were seen between combinations of MuIFN-α/β with natural MuIFN-γ and similar combinations with the recombinant DNA-derived MuIFN-γ's. Thus, recombinant DNA-derived MuIFN-γ's appeared to be as effective as natural MuIFN-γ in their ability to potentiate the antiproliferative activity of MuIFN-α/β.

To more completely establish the role of IFN-γ in potentiation, natural and recombinant DNA-derived MuIFN-γ preparations were exposed to antibody raised against natural MuIFN-γ. These were then tested separately and in combination with MuIFN-α/β in single cycle virus yield reduction experiments. Table 3 presents the results of two representative experiments with two recombinant DNA-derived MuIFN-γ's (MuIFN-$\gamma_{R\text{-}CHO}$ and MuIFN-$\gamma_{R\text{-}Coli}$). It is clear from the data that the

TABLE 2

Potentiation of antiproliferative activity by mixing preparations of MuIFN-α/β and either natural or recombinant DNA-derived MuIFN-γ

Interferon mixtures applied[a]	Antiproliferative titer (units/ml)		Potentiation factor[d]
	Observed[b]	Expected[c]	
IFN-α/β	0.2		
IFN-γ_N	0.3		
IFN-γ_N + IFN-α/β	3.0	0.5	6.0
IFN-$\gamma_{R\text{-}CHO}$	0.3		
IFN-$\gamma_{R\text{-}CHO}$ + IFN-α/β	3.3	0.5	6.6
IFN-$\gamma_{R\text{-}Coli}$	0.4		
IFN-$\gamma_{R\text{-}Coli}$ + IFN-α/β	4.8	0.6	8.0

[a,c,d] See footnotes [a,c,d] to Table 1.

[b] Antiproliferative units/ml as defined by the 50% cloning efficiency inhibition end-point (see text).

TABLE 3

Neutralization of the potentiating activities of natural and recombinant DNA-derived MuIFN-γ preparations by antibody to natural MuIFN-γ

Experiment No.	Interferons Mixtures applied[a]	Antibody treatment	Antiviral titer (units/ml) Observed[b]	Expected[c]	Potentiation factor[d]
1	IFN-α/β	-	20		
	$IFN\text{-}\gamma_N$	-	25		
	$IFN\text{-}\gamma_N$ + IFN-α/β	-	354	45	7.9
	$IFN\text{-}\gamma_N$	+	<2		
	$IFN\text{-}\gamma_N$ + IFN-α/β	+	16	20	0.8
	$IFN\text{-}\gamma_{R\text{-}CHO}$	-	20		
	$IFN\text{-}\gamma_{R\text{-}CHO}$ + IFN-α/β	-	397	40	9.9
	$IFN\text{-}\gamma_{R\text{-}CHO}$	+	<2		
	$IFN\text{-}\gamma_{R\text{-}CHO}$ + IFN-α/β	+	10	20	0.5
2	IFN-α/β	-	14		
	$IFN\text{-}\gamma_N$	-	21		
	$IFN\text{-}\gamma_N$ + IFN-α/β	-	187	35	5.4
	$IFN\text{-}\gamma_N$	+	<3		
	$IFN\text{-}\gamma_N$ + IFN-α/β	+	10	14	0.7
	$IFN\text{-}\gamma_{R\text{-}Coli}$	-	27		
	$IFN\text{-}\gamma_{R\text{-}Coli}$ + IFN-α/β	-	265	41	6.5
	$IFN\text{-}\gamma_{R\text{-}Coli}$	+	<3		
	$IFN\text{-}\gamma_{R\text{-}Coli}$ + IFN-α/β	+	10	14	0.7

[a,b,c,d] See footnotes to Table 1.

antibody inactivated both the natural and the recombinant DNA-derived MuIFN-γ's. Further, the level of protection observed following treatment with the combination of MuIFN-α/β and the antibody-inactivated natural or recombinant DNA-derived MuIFN-γ preparations was equal to or less than that observed after treatment with MuIFN-α/β alone. Thus, potentiation was blocked by antibody to MuIFN-γ.

In conclusion, in this study three preparations of recombinant DNA-derived MuIFN-γ (produced in CHO cells, COS cells, and *E. coli*) have been shown to participate as well as their natural MuIFN-γ counterparts in potentiation of antiviral and antiproliferative activities of MuIFN-α/β. The presence of potentiating activity in each of these cloned MuIFN-γ preparations coming from 3 different cell sources makes it highly unlikely that molecules other than MuIFN-γ are involved, since this would imply that in each case 2 pieces of cDNA (one for MuIFN-γ and one for a potentiation factor) had been cotransfected. Further, the presence of potentiating activity in preparations obtained from *E. coli* as well as from transfected mammalian cells makes it highly unlikely that a non-specific factor produced by mammalian cells was responsible for potentiation. This possibility is made even more unlikely by the observation that supernatant fluids from non-transfected, parental CHO and COS cells did not contain potentiating activity (data not shown).

Antibody to MuIFN-γ blocked potentiation in antiviral assays, even of MuIFN-γ from *E. coli*. The antibody preparation was raised against a partially purified, natural

MuIFN-γ preparation. However, it is highly unlikely that it contained antibody to *E. coli*-specific proteins which would be responsible for potentiation. It was not possible to obtain antibody in sufficient quantity and of sufficient purity to study potentiation in cell growth inhibition assays.

The data presented provide conclusive evidence that the MuIFN-γ molecule itself is responsible for potentiating activity present in the MuIFN-γ preparations in antiviral assays. They also provide suggestive evidence that the same is true for potentiation observed in cell growth inhibition assays. Recent studies on the potentiation of natural killer cell activation by interferon have employed recombinant DNA-derived human IFN-γ to demonstrate the role of IFN-γ in that system [15]. Taken together, these observations with diverse systems suggest that the presence of IFN-γ itself is required together with either IFN-α or IFN-β for potentiation of all reported interferon activities to occur.

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