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Potentiation of Interferon Activity by Mixed Preparations of Fibroblast and Immune Interferon

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Mixed preparations of fibroblast and immune interferons interacted with cells synergistically to cause the development of a much greater level of protection than expected on the basis of their separate activities. This increased level of protection was 5- to 20-fold greater than expected on the basis of a simple additive effect of the interferons. The potentiating factor copurified with both fibroblast interferon and immune interferon as they were partially purified. The potentiation was not an artifact of a more rapid development of immune interferon-induced antiviral resistance in the presence of fibroblast interferon. The results were consistent with the hypothesis that fibroblast and immune interferons mutually potentiate each other, thus supporting the supposition that they have different modes of action.

There is strong evidence that the interferon system has three important functions in the host: an antiviral function, an antitumor function, and an immunoregulatory function (1). An understanding of these functions of interferon is complicated by the fact that both the human interferon system and the mouse interferon system are complex and can each be provisionally subdivided into at least three antigenically distinct types, which are named for their cellular sources (3, 7-9, 12, 20). Fibroblast and leukocyte interferons are usually induced by viruses or synthetic polyribonucleotides, whereas immune interferons are usually induced in sensitized lymphocytes by a specific antigen or in unsensitized lymphocytes by T-cell mitogens (6, 11, 16, 18, 19).

In this work, the interaction of two of these interferons was studied. Our observations suggest that when cell monolayers are treated with mixtures of fibroblast and immune interferons, a great enhancement or potentiation of antiviral protection occurs. The level of protection observed for such mixed preparations was 5- to 20-fold greater than expected on the basis of the separate activities of these interferons. Thus, this increased antiviral activity is not merely an additive effect of the interferons, but represents a synergistic amplification of interferon-mediated protection against virus infection. The potentiating factor(s) copurifies with the fibroblast and immune interferons.

MATERIALS AND METHODS

Cells and viruses. Mouse L cells (clone 929) were employed in all experiments and assays. Cells were routinely passaged every 2 to 3 days in sealed 32-ounce (946-ml) bottles and maintained in Eagle minimal essential medium (Hanks base; Grand Island Biological Co.) supplemented with 10% fetal calf serum (Flow Laboratories) at 37°C. For yield reduction experiments and plaque assays, cells were plated at 10^6 cells per dish on standard (Falcon Plastics) and Contur (Lux) 35-mm plastic petri dishes, respectively. They were maintained in Eagle minimal essential medium (Earle base; Grand Island Biological Co.) supplemented with 10% fetal calf serum at 37°C in a humidified, 4% CO_2 atmosphere.

Mengovirus was employed for all experiments and assays. For yield reduction experiments, 10 plaque-forming units (PFU) per cell was incubated with L-cell monolayers for 45 min. After a double wash to remove unabsorbed virus, the cultures were incubated for 24 h before progeny virus was harvested and quantitated by plaque assay. For plaque assay experiments, after a 45-min absorption period, these monolayers were overlaid with a starch (Sigma Chemical Co.) overlay as previously described (5). After 24 h of incubation, the monolayers were stained with 7 to 10 drops of a 0.2% (wt/vol) neutral red (Sigma) preparation, and plaques were counted 2 to 3 h later.

Interferon production and titration. Mouse C243 cells were used for the production of fibroblast-type interferon as previously described (14). C243 cells were maintained in the same medium as described above for L cells. Monolayers were stimulated with Newcastle disease virus. The supernatant fluids were harvested at 24 h postinfection. The supernatant fluid was adjusted to pH 2 by the addition of HCl. After 5 days of treatment at 4°C, the supernatant fluid was adjusted to pH 7.2 with NaOH and stored at −20°C. This unpurified fibroblast interferon preparation had a specific activity of 10^3 U/mg of protein. Partially purified mouse L-cell fibroblast interferon having a specific activity of 10^7 U/mg of protein was supplied by the Antiviral Substances Program, National Insti-

tute of Allergy and Infectious Diseases (13).

Spleen cell cultures used for the production of immune interferon were prepared by dissociating the spleens of 8- to 12-week-old female C57 BL/6 mice (Jackson Laboratories). The cultures were maintained in Eagle minimal essential medium (Microbiological Associates) supplemented with 5% fetal calf serum (Microbiological Associates), nonessential amino acids (Microbiological Associates), sodium pyruvate (Microbiological Associates), and 10 μM 2-mercaptoethanol. The cultures were stimulated with 0.5 μg of staphylococcal enterotoxin A as previously described (10) and incubated at 37°C on a rocker platform (Bellco) in a sealed box charged with a humidified atmosphere containing 10% CO_2 and 7% O_2. The staphylococcal enterotoxin A was provided by the Microbial Biochemistry Branch, Division of Microbiology, Food and Drug Administration, Cincinnati, Ohio. The supernatant fluids were harvested after 2 days of incubation with staphylococcal enterotoxin A, centrifuged at 650 × *g*, and stored at −70°C.

Fibroblast and immune interferon titers were determined by plaque reduction assay and are reported as National Institutes of Health reference units per milliliter. Monolayers of L929 cells were incubated for 12 h with the interferon samples, challenged with about 80 PFU of mengovirus per plate, and overlaid with starch as described above.

Column chromatography. An immune interferon partial purification procedure has been described previously (15). The immune interferon preparations were differentially precipitated by ammonium sulfate. The precipitates obtained as the ammonium sulfate concentration was raised from 55 to 80% saturation were pelleted, resuspended in distilled water, and dialyzed against phosphate-buffered saline. These preparations were fractionated on an Ultrogel AcA 34 (LKB Laboratories) gel filtration column (2.5 by 60 cm) washed with phosphate-buffered saline. Fractions were stored at −70°C until assayed for interferon and for potentiator activities.

RESULTS

Potentiation of antiviral activity by mixed preparations of immune and fibroblast interferons. Possible synergistic interactions of immune and fibroblast interferons were studied by contrasting the separate protective abilities of immune and fibroblast interferons with the combined protective ability of mixed preparations of immune and fibroblast interferons. L-cell monolayers were treated with the interferon samples for 12 h and then challenged with mengovirus at a multiplicity of 10 PFU/cell. Progeny virus yields were harvested at 24 h postinfection to allow completion of a one-step virus growth cycle. The virus yields were determined by plaque assay. Protection of interferon-treated monolayers was determined by dividing the virus yield of untreated monolayers by the virus yield of interferon-treated monolayers to obtain a fold inhibition value. Results of a representative experiment are shown in Table 1. Both immune interferon and fibroblast interferon provided protection against virus yield when employed separately; 3 U of immune interferon reduced virus yield by approximately 3-fold, whereas 26 U of fibroblast interferon reduced virus yield by 43-fold. More importantly, the level of protection observed when immune and fibroblast interferons were added in combination was 714-fold, much greater than predicted on the basis of their separate protective abilities. The virus yields were compared with a standard curve of virus yields generated concurrently for known amounts of fibroblast interferon in order to convert the observed virus yield to units of actual interferon activity. The virus

TABLE 1. *Potentiation of interferon activity by mixed preparations of immune interferon and fibroblast interferon*[a]

IF sample	Virus yield (PFU/ml)	Fold inhibition	IF titer (U/ml)		Fold potentiation[d]
			Actual[b]	Expected[c]	
No IF	$1.0 \times 10^9 \pm 0.1 \times 10^9$[e]				
Immune IF	$3.8 \times 10^8 \pm 0.4 \times 10^8$	2.6	3		
Fibroblast IF	$2.3 \times 10^7 \pm 0.1 \times 10^7$	43	26		
Immune IF + fibroblast IF	$1.4 \times 10^6 \pm 0.03 \times 10^6$	714	320	29	11

[a] Mouse L-cell monolayers were treated for 12 h with growth medium (no interferon [IF]), immune interferon, fibroblast interferon, and immune interferon and fibroblast interferon in combination. The monolayers were challenged with mengovirus at a multiplicity of infection of 10 PFU/cell, and virus yields were harvested 24 h later.

[b] Actual interferon titers were determined by comparison of virus yield with a standard yield reduction curve of interferon activity determined concurrently.

[c] Expected interferon titer was determined by adding the actual titers of the interferons present in the mixed interferon preparation.

[d] The potentiation factor was determined by dividing the actual interferon titer by the expected interferon titer.

[e] Mean ± standard deviation.

yields by monolayers treated with a combined total of 29 U of mixed interferons (3 U of immune interferon plus 26 U of fibroblast interferon) were at a level expected for monolayers treated with more than 300 U of interferon. Thus, an 11-fold potentiation of interferon activity occurred. More than 12 separate immune interferon preparations were examined and shown to have potentiating abilities of from 5- to 20-fold. No potentiating activity was observed when fibroblast interferon was mixed with supernatant fractions from unstimulated spleen cells.

The potentiating effect is a nonadditive, synergistic effect that may not even be simply multiplicative. For a multiplicative effect, the anticipated level of virus yield by monolayers treated with mixed interferons for the experiment shown in Table 1 would have been less than 120-fold (2.6-fold times 43-fold). The actual yield was reduced by 714-fold, sixfold lower than would be predicted by a simple multiplicative effect.

Effect of partial purification of fibroblast interferon on potentiation. At least four possibilities exist as a basis for the potentiating effect: (i) a potentiator of immune interferon is present in the fibroblast interferon preparation; (ii) a potentiator of fibroblast interferon is present in the immune interferon preparation; (iii) fibroblast interferon potentiates immune interferon by accelerating the kinetics of establishment of the immune interferon antiviral state; or (iv) the two interferons mutually potentiate each other. The first possibility was tested by comparing the potentiating activity when an immune interferon preparation was mixed with either unpurified fibroblast interferon (10^3 U/mg of protein) or partially purified fibroblast interferon (10^7 U/mg of protein). If the potentiation phenomenon was due to a factor which was separable from interferon and which was present in the unpurified fibroblast preparation, a marked reduction in the potentiating ability relative to the interferon titer of the partially purified interferon preparation might have been anticipated. Table 2 presents the results of such an experiment. The immune interferon preparation reduced the virus yield by about 5-fold, whereas the unpurified and partially purified fibroblast interferons reduced the virus yields by 13- and 14-fold, respectively. Mixed preparations of immune interferon plus either unpurified or partially purified fibroblast interferon reduced virus yields by 345- and 370-fold, respectively. Both the mixed immune interferon-unpurified fibroblast interferon preparation and the mixed immune interferon-partially purified fibroblast interferon preparation exhibited a fivefold potentiation of antiviral protection. Thus, the potentiating abilities of the mixed immune and fibroblast interferons were not affected by a 10,000-fold purification of the fibroblast interferon. Therefore, it was unlikely that the potentiating phenomenon was due to a factor easily separable from fibroblast interferon.

Effect of partial purification of immune interferon on potentiation. Partially purified immune interferon was tested for its potentiating ability in order to determine whether the potentiation phenomenon was due to a factor separable from immune interferon. The immune interferon was purified as previously described (15). Briefly, the immune interferon was purified 200-fold by differential ammonium sulfate precipitation and Ultrogel AcA 34 gel filtration chromatography. The potentiation capability of this partially purified immune interferon was compared with that of unpurified immune interferon. The results of a representative experiment are presented in Table 3. Approximately equal amounts of unpurified and partially purified immune interferon (6 and 5 U, respectively) were found to potentiate 20 U of fibroblast interferon

TABLE 2. *Effect of partial purification of fibroblast interferon on potentiation*[a]

IF sample	Virus yield (PFU/ml)	Fold inhibition	IF titer (U/ml)		Fold potentiation
			Actual	Expected	
No IF	$7.7 \times 10^8 \pm 0.3 \times 10^8$[b]				
Immune IF	$1.6 \times 10^8 \pm 0.2 \times 10^8$	4.8	9		
Purified fibroblast IF	$5.5 \times 10^7 \pm 0.2 \times 10^7$	14	17		
Fibroblast IF	$5.9 \times 10^7 \pm 0.3 \times 10^7$	13	16		
Immune IF + purified fibroblast IF	$2.1 \times 10^6 \pm 0.2 \times 10^6$	370	130	26	5
Immune IF + fibroblast IF	$2.2 \times 10^6 \pm 0.7 \times 10^6$	345	130	25	5

[a] Mouse L-cell monolayers were treated for 12 h with growth medium (no interferon [IF]), immune interferon, 10,000-fold-purified fibroblast interferon, unpurified fibroblast interferon, immune interferon plus purified fibroblast interferon in combination, and immune interferon plus unpurified fibroblast interferon in combination. The monolayers were challenged with mengovirus at a multiplicity of infection of 10 PFU/cell, and virus yields were harvested 24 h later.

[b] Mean ± standard deviation.

to the same degree (18-fold potentiation). Thus, there was no separation of the potentiating factor from immune interferon despite a 200-fold purification of the immune interferon preparation.

Fibroblast interferon does not potentiate immune interferon by accelerating the kinetics of establishment of the antiviral state by immune interferon. It has been shown previously that the antiviral state induced by immune interferon develops more slowly than that induced by fibroblast interferon(s) (2). Development of the fibroblast interferon-induced antiviral state is complete by 12 h, whereas development of the immune interferon-induced antiviral state is not complete until about 24 h. Thus, it was possible that the apparent potentiating activity might have been due to the more rapid development of immune interferon antiviral protection in the mixed interferon preparations. Table 4 shows the results of an experiment designed to address this question. Immune and fibroblast interferons were incubated separately and in combination on cell monolayers for either 12 or 24 h before virus challenge. As expected from previous experiments, the level of actual protection induced by immune interferon did increase from 12 to 24 h from 4 to 13 U/ml. The level of actual protection induced by a mixture of immune and fibroblast interferons also increased from 12 to 24 h from 127 to 360 U. If the fibroblast interferon had caused the more rapid development of immune interferon antiviral protection in the mixed preparations, maximal antiviral protection should have developed by 12 h and remained constant thereafter. Since a late rise in antiviral activity occurred for both immune interferon alone and immune interferon mixed with fibroblast interferon, fibroblast interferon did not alter the kinetics of establishment of the immune interferon antiviral state. Furthermore, the level of potentiation increased from 12 to 24 h from 5- to 11-fold. Thus, fibroblast interferon did not poten-

TABLE 3. *Effect of partial purification of immune interferon on potentiation*[a]

IF sample	Virus yield (PFU/ml)	Fold inhibition	IF titer (U/ml)		Fold potentiation
			Actual	Expected	
No IF	$6.8 \times 10^8 \pm 0.00$[b]				
Immune IF	$3.6 \times 10^8 \pm 0.2 \times 10^8$	1.8	6		
Purified immune IF	$3.9 \times 10^8 \pm 0.2 \times 10^8$	1.8	5		
Fibroblast IF	$4.9 \times 10^7 \pm 1.5 \times 10^7$	14	20		
Immune IF + fibroblast IF	$1.3 \times 10^6 \pm 0.05 \times 10^6$	526	480	26	18
Purified immune IF + fibroblast IF	$1.4 \times 10^6 \pm 0.04 \times 10^6$	476	450	25	18

[a] Mouse L-cell monolayers were treated for 12 h with growth medium (no interferon [IF]), unpurified immune interferon, 200-fold-purified immune interferon from an Ultrogel filtration of immune interferon, fibroblast interferon, unpurified immune interferon plus fibroblast interferon in combination, and partially purified immune interferon plus fibroblast interferon in combination. The monolayers were challenged with mengovirus at a multiplicity of infection of 10 PFU/cell, and virus yields were harvested 24 h later.

[b] Mean ± standard deviation.

TABLE 4. *Effect of time of incubation of cells with mixed interferons on potentiation effect*[a]

IF sample	Time of incubation (h)	Virus yield (PFU/ml)	Fold inhibition	IF titer (U/ml)		Fold potentiation
				Actual	Expected	
No IF	12	$6.3 \times 10^8 \pm 0.9 \times 10^8$[b]				
Purified immune IF	12	$2.1 \times 10^8 \pm 0.03 \times 10^8$	3.0	4		
Fibroblast IF	12	$2.4 \times 10^7 \pm 0.1 \times 10^7$	26	20		
Purified immune IF + fibroblast IF	12	$2.5 \times 10^6 \pm 0.4 \times 10^6$	250	127	25	5
No IF	24	$8.7 \times 10^8 \pm 0.5 \times 10^8$				
Purified immune IF	24	$7.4 \times 10^7 \pm 0.8 \times 10^7$	12	13		
Fibroblast IF	24	$4.1 \times 10^7 \pm 0.7 \times 10^7$	21	20		
Purified immune IF + fibroblast IF	24	$1.0 \times 10^6 \pm 0.04 \times 10^6$	909	360	33	11

[a] Mouse L-cell monolayers were treated for 12 or 24 h with growth medium (no interferon [IF]), 200-fold-purified immune interferon, fibroblast interferon, and partially purified immune interferon plus fibroblast interferon in combination. The monolayers were challenged with mengovirus at a multiplicity of infection of 10 PFU/cell, and virus yields were harvested 24 h later.

[b] Mean ± standard deviation.

tiate the immune interferon simply by speeding up the kinetics of establishment of the immune interferon-induced antiviral state.

DISCUSSION

Mixed preparations of fibroblast and immune interferons provided a greater level of antiviral protection than expected on the basis of their separate protective effects. This potentiation was a synergistic amplification of the antiviral state, which resulted in a 5- to 20-fold increase in the protective capability of the mixed interferon preparations. Furthermore, the potentiation effect appeared to be greater than a simple multiplicative effect. Four likely explanations for this potentiation effect can be postulated.

First, a component of the fibroblast interferon preparation could cause potentiation. To test this possible explanation, the relative potentiation capabilities of unpurified and 10,000-fold-purified fibroblast interferons were measured. The potentiation levels observed for the two interferon preparations were identical. If a separate molecular entity present in the fibroblast interferon preparation had been responsible for the potentiation effect, it would have had to have been quantitatively copurified with fibroblast interferon. Since the fibroblast interferon preparation was purified 10,000-fold, this explanation of potentiation is considered unlikely.

Second, a component of the immune interferon preparation could cause potentiation. To test this possibility, the relative potentiation capabilities of unpurified and 200-fold-purified immune interferons were measured. Potentiation was the same for both 200-fold-purified and unpurified immune interferon. Since the potentiating activity quantitatively copurified with the immune interferon, there is no evidence that a separate molecular entity present in the immune interferon preparation was responsible for the potentiation effect. However, since the purification was dependent on molecular weight differences in the proteins, more work is necessary to determine the relationship of potentiator and immune interferon.

Third, fibroblast interferon could cause potentiation by accelerating the kinetics of establishment of the immune interferon-induced antiviral state. Establishment of the antiviral state occurs more slowly in response to immune interferon than to fibroblast interferon treatment (2). The possibility existed that the immune interferon-induced antiviral state developed more rapidly when the immune interferon was coincubated with the more rapidly acting fibroblast interferon. However, this was shown not to be the case. The protective effect of immune interferon when added alone did increase with time, but so did the protective and the potentiation effects when immune and fibroblast interferons were added together. Thus, fibroblast interferon did not potentiate immune interferon by speeding up the kinetics of establishment of the immune interferon-induced antiviral state.

Fourth, the two types of interferon could potentiate each other. Although there is no evidence to directly support this theory, the present inability to separate a potentiating factor from the two interferons causes this explanation to be the most attractive. This explanation suggests that the mechanisms by which fibroblast interferon and immune interferon block virus replication are distinct and that they can act synergistically.

Some immune interferon preparations have been shown to contain an inhibitor of interferon (W. R. Fleishmann, Jr., J. Georgiades, H. M. Johnson, F. Dianzani, and S. Baron, Bacteriol. Proc., p. 247, 1978). The potentiation phenomenon was not an artifact of this inhibitor effect. The inhibitor was not produced by staphylococcal enterotoxin A-stimulated mouse spleen cells until day 3 of stimulation. The immune interferon preparations used in this study were harvested after 2 days of mitogen stimulation and thus contained no inhibitor activity.

A phenomenon related to potentiation occurs when cultured cells pretreated with a low level of interferon develop antiviral activity faster and to a greater level when subsequently exposed to a high level of the same interferon than they would if exposed initially only to a high level of interferon (priming) (4, 17). The potentiation described in this paper appears to be different from priming, since (i) for potentiation the fibroblast and immune interferons were added simultaneously without the required pretreatment for the priming phenomenon, and (ii) potentiation required two different types of interferon, whereas priming occurs with two additions of the same interferon.

Although the potentiating factor has not yet been identified, the available evidence indicates that it copurified with the interferons. If the two types of interferons do potentiate each other, then these results suggest that the local production of multiple types of interferon may play a significant role in the activation of cells by interferon. Whatever the mechanism of potentiation, additional studies are necessary to define the role which the potentiation effect plays in influencing the progress of viral disease.

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