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Antiviral and Protein-Inducing Activities of Recombinant Human Leukocyte Interferons and Their Hybrids

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The antiviral activities of recombinant human leukocyte interferons IFN-αA and IFN-αD as well as five hybrids of these interferons against retroviruses, vesicular stomatitis virus, and encephalomyocarditis virus were studied in feline, human, and murine cells. Although these interferon species had widely different potencies, their activities against these viruses were, in general, proportional. The IFN-αA/D (*Bgl*) hybrid was the most potent species, and the IFN-αD/A (*Bgl*) hybrid was the least potent. However, the latter species did not interfere with the action of the former species. Like natural human leukocyte interferon, each of the seven species of recombinant interferons induced the synthesis of at least five proteins in human fibroblasts, whereas induction of only one such protein was readily detected in a feline fibroblast line in which these interferon species inhibited the replication of all three viruses.

Human leukocyte interferon (IFN-α) consists of many species that are encoded by individual members of the IFN-α multigene family. Some of these species have been expressed in bacteria. Extensive sequence homologies among the IFN-α genes have permitted the construction of hybrid recombinant molecules (reviewed in reference 6).

Although interferon action was originally considered to be species specific, this concept has been modified substantially. Some human IFN-α species exhibit high levels of activity on nonhuman cells. In some cases, their potency is higher in heterologous cells than in human cells. IFN-αA and IFN-αD exhibit quantitatively different activities on cells from different organisms, and the hybrids of these two IFN-α species have their own characteristic spectra of activity that are distinct from those of the parental molecules (7, 9, 13, 14).

The biological action of the leukocyte interferon species and their hybrids has been tested predominantly by measuring their antiviral activity against vesicular stomatitis virus (VSV). Since different molecular mechanisms are involved in interferon mediated inhibition of replication of different viruses (reviewed in references 4 and 10), it is not known whether the conclusions drawn about the species specificity and the receptor interactions from these measurements with VSV would also be true for actions of interferon against other viruses. For example, the antiretroviral action of interferon is exerted at the level of viral assembly and release from the plasma membrane, whereas replication of exogenously infecting cytopathic viruses such as VSV or encephalomyocarditis virus (EMCV) is inhibited at the level of viral RNA and protein synthesis. Cell lines have been described in which interferon does not inhibit EMCV or VSV but inhibits the production of retroviruses (2, 3, 11). It has also been reported that in certain cell lines interferon can inhibit VSV without inhibiting EMCV, or vice versa (5, 11), indicating that these two viruses are inhibited by different mechanisms. It is not clear at which points of interferon action the differential antiviral pathways diverge from one another.

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In the studies reported here, we examined the actions of IFN-αA, IFN-αD, and hybrid interferons against retrovirus, VSV, and EMCV with human, feline, and murine cell lines. The purpose of these studies was to determine the relative effectiveness of several species of leukocyte interferon against these viruses in cells from three organisms.

In addition, treatment of cells with interferon results in induced synthesis of several proteins (8, 9). Although synthesis of these proteins parallels the establishment of the antiviral state in interferon-treated cells, their specific functions remain to be elucidated. Thus, we compared the ability of recombinant IFN-αA, IFN-αD, and their hybrids to induce specific proteins in human and feline cells. The results of these experiments are also presented here.

MATERIALS AND METHODS

Interferons. The natural human leukocyte interferon preparation used in these studies had a specific activity of 10^6 U/mg of protein. The recombinant interferons, IFN-αA, IFN-αD, and their hybrids were produced and purified as described previously (7). Some of the characteristics of the recombinant interferon species used in these studies are summarized (see Table 1).

Cells. Human RD-114 cells and human GM2767 fibroblasts were grown in minimum essential medium containing 10% fetal bovine serum. Other cells were grown in Dulbecco minimum essential medium containing 10% fetal bovine serum. The RD-114 line continuously produces RD-114 retrovirus (3). The murine NIH/MOL,C line is an NIH/3T3 line chronically infected with the Moloney strain of murine leukemia virus (11), and FlF3 is a feline fibroblast line chronically infected with a feline leukemia virus. (FeLV).

Reverse transcriptase assay. Cell culture medium containing the virions was first clarified by centrifugation at 1,600 × *g* for 15 min. The supernatant was directly used for the enzyme assay, or in some cases the virus was pelleted first by centrifugation at 160,000 × *g* for 60 min before resuspension for assay. In addition to the virus, the assay mixture contained 5 μCi of [^{3}H]TTP (60 to 80 Ci/mmol; New England Nuclear Corp.) 0.01 absorbance of 260 nm units of (P-L Biochemicals, Inc.) as the template-primer, polyriboadenylic acid (deoxythymidylic acid)$_{12\text{-}18}$ 0.5 mM $MnCl_2$, 50 mM

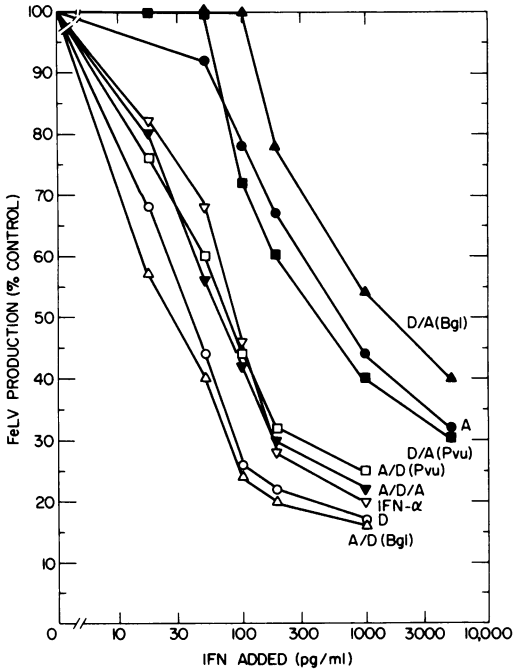

FIG. 1. Effects of increasing concentrations of human leukocyte interferons on FeLV production by feline FlF3 cells. Cells (5×10^5) were plated on 60-mm plates. The cells were treated with the indicated concentrations of interferon for 18 h. At this time, the medium was replaced by fresh medium containing the same concentration of interferon. After 4 h the medium was collected, and the quantity of FeLV released into the medium was determined by measuring reverse transcriptase activity. Results are expressed as a percentage of virus production by untreated cells. Symbols: ●, IFN-αA; △, IFN-αA/D (*Bgl*); □, IFN-αA/D (*Pvu*); ▲, IFN-αD/A (*Bgl*); ■, IFN-αD/A (*Pvu*); ▼, IFN-αA/D/A; ○, IFN-αD; ▽, natural IFN-α. For purposes of comparison of unfractionated natural human leukocyte interferon (IFN-α) with the purified species, it was arbitrarily assumed that the interferon in this preparation had a specific activity of 2×10^8 U/mg.

Tris-hydrochloride (pH 7.8), 1 mM dithiothreitol, 50 mM KCl, 0.2% Nonidet P-40, and 0.01% bovine serum albumin in a total volume of 0.1 ml. Incubations were performed at 37°C for 20 min. At the end of the reaction, radioactivity incorporated into trichloroacetic acid-insoluble material was measured.

VSV and EMCV yield reduction assay. Interferon-treated cells were infected with VSV or EMCV at a multiplicity of infection of 10. After 1 h of virus adsorption, cells were washed free of excess virus and then incubated overnight in culture medium. Virions were released from the cells by alternate freezing and thawing three times. Virus yield was measured by standard plaque assays on L929 cells.

Analysis of proteins induced by interferon. Confluent monolayers were incubated with or without interferon in a medium containing one-fourth the usual concentration of methionine and 20 μCi of [^{35}S]methionine per ml. Cell extracts were made, and supernatants obtained at 200,000 × *g* were prepared as described previously (9). Extracts containing equal amounts of labeled proteins were analyzed by sodium dodecyl sulfate gel electrophoresis on a 15% polyacrylamide gel. Labeled molecular weight markers were included in the same gel.

TABLE 1. Characteristics of cloned IFN-αA, IFN-αD, and hybrid interferons

Interferon species	No. of amino acids different from		Sp act[a]
	IFN-αA	IFN-αD	
IFN-αA	0	29	3.2×10^8
IFN-αA/D (*Bgl*)	16	13	2.8×10^8
IFN-αA/D (*Pvu*)	13	16	3.8×10^8
IFN-αD/A (*Bgl*)	13	16	1.0×10^6
IFN-αD/A (*Pvu*)	16	13	2.7×10^6
IFN-αA/D/A	3	26	4.3×10^7
IFN-αD	29	0	4.4×10^6

[a] The specific activity is given in units per milligram as measured on human AG-1732 cells by a cytopathic inhibition assay with VSV. Data are from Rehberg et al. (7).

RESULTS

Antiviral actions of IFN-αA, IFN-αD, and their recombinants on feline FlF3 cells. The feline fibroblast line FlF3 is chronically infected with FeLV. We tested the effects of the human interferons on the production of FeLV by these cells. The dose-response curves are shown in Fig. 1. Since the interferon preparations were pure, the interferon concentrations were presented in picograms per milliliter instead of units per milliliter, except as noted. The specific activities of the different species of interferon in cells of different species are listed in Table 1. IFN-αA/D (*Bgl*) and IFN-αD were the two most active species (Fig. 1). IFN-αA/D (*Pvu*) and IFN-αA/D/A displayed similar ranges of activity as natural IFN-α. IFN-αA, IFN-αD/A (*Pvu*), and IFN-αD/A (*Bgl*) exhibited much less activity than the other species.

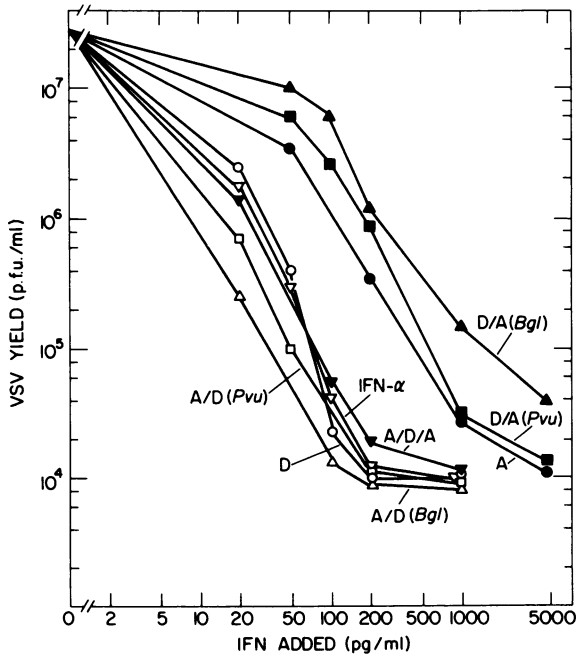

FIG. 2. Effect of increasing concentrations of different recombinant human leukocyte interferons on VSV replication in feline FlF3 cells. Cells were treated with interferon for 18 h and then infected with VSV at a multiplicity of infection of 10. Yields of virus produced after 16 h were measured. Symbols are the same as those described in the legend to Fig. 1.

TABLE 2. Anti-VSV effects of a mixture of IFN-αA/D and IFN-αD/A in feline FlF3 cells

Interferon concn (pg/ml)		VSV yield (PFU/ml)
IFN-αA/D (*Bgl*)	IFN-αD/A (*Bgl*)	
		5.0×10^6
50		4.7×10^4
150		5.5×10^3
	100	6.0×10^6
	150	8.0×10^6
50	100	4.5×10^4
20		5.7×10^5
200		6.5×10^3
	200	5.3×10^6
	220	7.0×10^6
20	200	2.8×10^5

To compare the antiretroviral potencies of these seven interferon species to their classical antiviral potencies, the degree of inhibition of VSV replication was tested in the same cells in response to various concentrations of these interferons. The anti-VSV activity (Fig. 2) of these interferon species was parallel to their antiretroviral potencies (Fig. 1). IFN-αA/D (*Bgl*) was the most potent species in the feline cells, both by anti-VSV and anti-FeLV action, whereas IFN-αD/A (*Bgl*) was the least potent species.

It is not known which steps of interferon action determine the potency of different species. For example, it is possible that IFN-αD/A (*Bgl*) interacts less efficiently with the receptors on feline cells than does IFN-αA/D (*Bgl*). Alternatively, it is possible that they bind to receptors equally well but trigger various biochemical events differently. To differentiate between these possibilities, we examined the effect of concentrations of IFN-αD/A (*Bgl*) which did not inhibit VSV replication in FlF3 cells on the action of IFN-αA/D (*Bgl*),

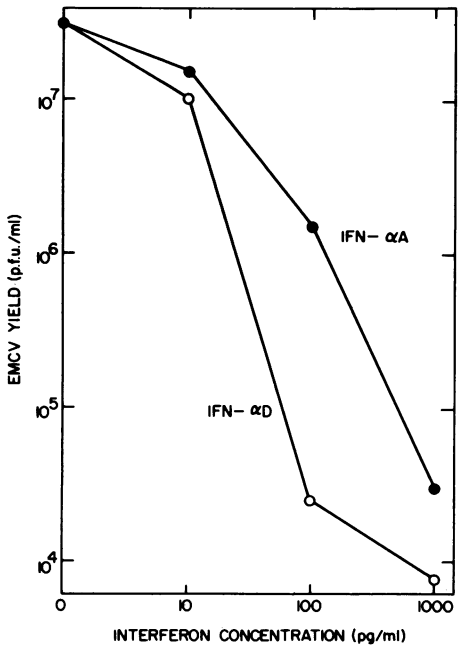

FIG. 3. Effects of IFN-αA and IFN-αD on EMCV replication in feline FlF3 cells. Experimental procedures were similar to those described in the legend to Fig. 2. Symbols: ●, IFN-αA; ○, IFN-αD.

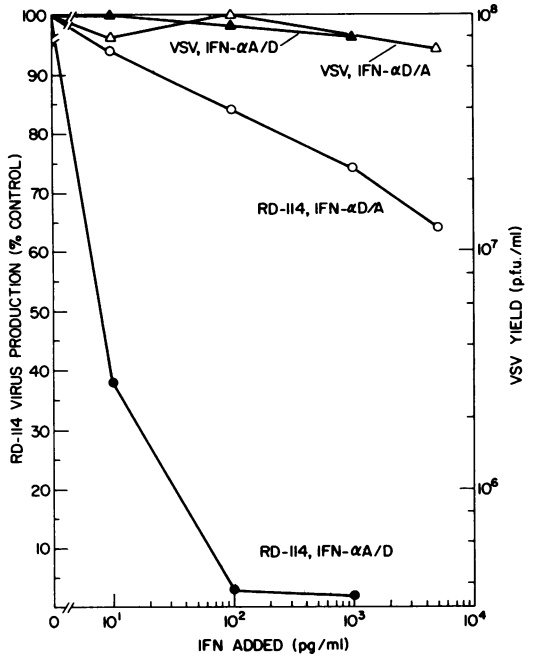

FIG. 4. Antiretroviral and anti-VSV actions of IFN-αA/D and IFN-αD/A in human RD-114 cells. Experimental protocols were similar to those described in the legends to Fig. 1 and 2, except that retrovirus production was measured by assaying reverse transcriptase activity in virus pellets. Symbols: ● and ▲, IFN-αA/D (*Bgl*); ○ and △, IFN-αD/A (*Bgl*). Circles denote RD-114 virus production, and triangles denote VSV yields.

which inhibited VSV replication strongly. A 10-fold excess of IFN-αD/A (*Bgl*) did not interfere with the action of IFN-αA/D (*Bgl*), suggesting that the affinity of IFN-αD/A (*Bgl*) for the interferon receptors is much lower than that of IFN-αA/D (*Bgl*) (Table 2).

The mechanisms of interferon-mediated inhibition of replication of VSV and EMCV are different. In fact, interferon can inhibit the replication of one of these viruses without inhibiting the other (11). In the experiment shown in Fig. 3, the effectiveness of recombinant IFN-αA and IFN-αD was tested against EMCV replication in FlF3 cells. IFN-αD was more potent than IFN-αA, and their relative potencies against EMCV and VSV were comparable.

Antiretroviral actions of IFN-αA/D (*Bgl*) and IFN-α D/A (*Bgl*) in human and murine cells. We selected the most potent and the least potent recombinant human leukocyte interferon species to study further their antiretroviral potencies. Human RD-114 cells continuously produce the RD-114 retrovirus. Production of this virus is inhibited by both human leukocyte and immune interferons (3, 11a). IFN-αA/D (*Bgl*) was very efficient in inhibiting RD-114 virus production, whereas IFN-αD/A (*Bgl*) was much less active (Fig. 4). Natural human leukocyte or immune interferon does not inhibit VSV replication in these cells (3). Similarly, both of the recombinant human leukocyte interferons tested also failed to inhibit VSV replication in these cells.

In the murine NIH/MOL,C cells (11), IFN-αA/D (*Bgl*) inhibited both murine leukemia virus production and VSV replication, whereas IFN-αD/A (*Bgl*) did not inhibit either virus at up to 5,000 pg/ml (Fig. 5). These results are consistent with the ineffectiveness of IFN-αD/A (*Bgl*) on murine cells (7).

Induction of new proteins by the recombinant interferon species. Treatment of cells in culture with interferon results in the induction of synthesis of several new proteins. We investigated whether the same proteins are induced by natural leukocyte interferon and the cloned individual interferon species. Confluent cultures of human fibroblasts were treated with natural or with the different recombinant leukocyte interferon species. Untreated and treated cells were labeled with [^{35}S]methionine for 6 h starting from the time of addition of interferon, and the cell extracts were analyzed by gel electrophoresis as shown in Fig. 6. As reported earlier (8), five induced proteins can be identified in the extracts of leukocyte interferon-treated cells by this method of analysis. These proteins, designated by arrows in Fig. 6, have approximate molecular weights of 105,000, 88,000, 80,000, 67,000, and 56,000. In this experiment, induction of the 88,000-dalton protein was not very clear. IFN-αA, IFN-αD, and the hybrid interferons induced all of these proteins, indicating that different proteins are not induced by these different individual interferon species present in the natural leukocyte interferon preparation. Interestingly, there was one protein band at about 54,000 daltons which was present in cells treated with natural leukocyte interferon as well as in untreated cells, but it was not detected in cells treated with the other interferons. The significance of the inhibition of synthesis of this protein by the recombinant interferons is unclear.

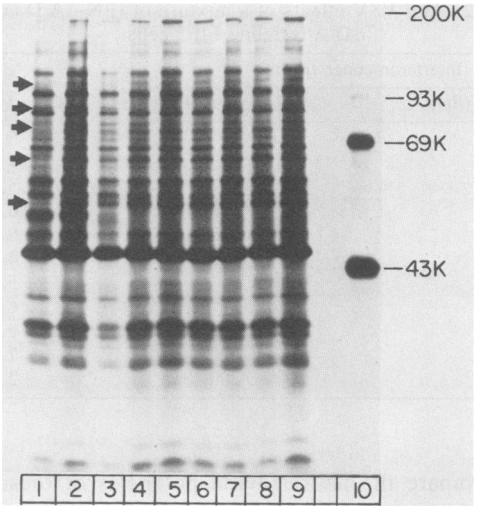

FIG. 6. Induction of new proteins by different interferon species in human GM2767 cells. In these experiments, interferon (400 U/ml) (as measured in human AG-1732 cells) was used, and the cells were labeled for 6 h from the time of addition of interferon. The numbers on the right are the molecular weights of marker proteins in kilodaltons. The arrows on the left indicate the induced proteins. Lanes: 1, no interferon; 2, natural leukocyte interferon; 3, IFN-αA; 4, IFN-αD; 5, IFN-αA/D (*Bgl*); 6, IFN-αA/D (*Pvu*); 7, IFN-αD/A (*Bgl*); 8, IFN-αD/A (*Pvu*); 9, IFN-αA/D/A; 10, molecular weight markers.

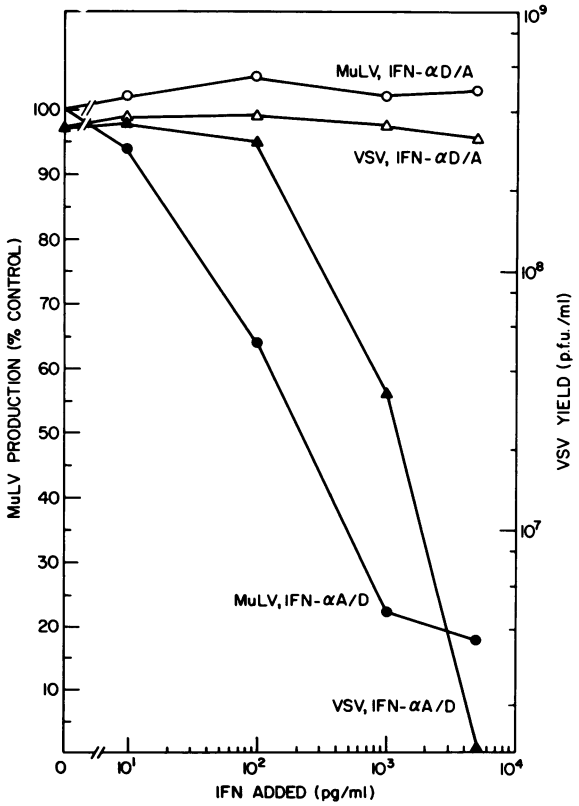

FIG. 5. Antiretroviral and anti-VSV actions of IFN-αA/D (*Bgl*) and IFN-αD/A (*Bgl*) in murine NIH/MOL,C cells. Experiments were similar to those described in the legends to Fig. 1 and 2. Symbols: ● and ▲, IFN-αA/D (*Bgl*); ○ and △, IFN-αD/A (*Bgl*). Circles denote murine leukemia virus production, and triangles denote VSV yields.

In a similar experiment, we investigated whether these interferon species induce similar proteins in the feline fibroblast line. FlF3 cells were treated with IFN-α, IFN-αA, IFN-αD, and the hybrid interferons for 6 h. Treated and untreated cells were labeled with [^{35}S]methionine from 0 to 6 h, and the proteins were analyzed (Fig. 7). In the same gel,

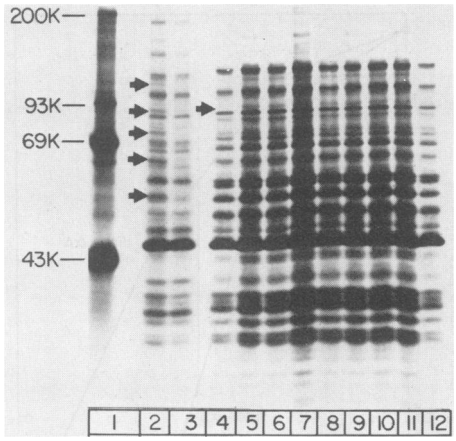

FIG. 7. Induction of new proteins by different interferon species in feline FlF3 cells. The feline cells were treated with 1,000 pg of different interferon species per ml and labeled for 6 h from the time of addition of interferon. The numbers on the left denote the molecular weights of marker proteins in kilodaltons. The arrows indicate the induced proteins. Lanes: 1, molecular weight markers; 2, human GM2767 cells labeled between 5 and 8 h after treatment with natural human leukocyte interferon began; 3, untreated GM2767 cells labeled for 3 h. Lanes 4 through 12 show results with FlF3 cells: 4, no interferon; 5, natural leukocyte interferon (IFN-α); 6, IFN-αD; 7, IFN-αA; 8, IFN-αA/D (*Bgl*); 9, IFN-αA/D (*Pvu*); 10, IFN-αD/A (*Bgl*); 11, IFN-αD/A (*Pvu*); 12, IFN-αA/D/A.

proteins from untreated and interferon-treated human fibroblasts, which were labeled between 5 and 8 h after the beginning of interferon treatment, were analyzed. All of the five induced proteins were distinctly present in interferon-treated human fibroblasts. In contrast, in the feline cells, only one interferon-inducible protein was detected. This protein exhibited an approximate molecular weight of 88,000.

DISCUSSION

Antiviral activities of cloned species of human leukocyte interferon and their hybrids have been tested previously primarily with VSV as the challenge virus (7, 9, 13, 14) and by the cytopathic effect inhibition assay. In this report, we used yield reduction assays to determine the dose-response curves. Because the yield reduction assay is more sensitive and quantitative, detailed comparisons of the antiviral activities of the various species of interferon were possible. In the feline cells, the seven cloned interferon species showed the same pattern of anti-VSV potencies irrespective of whether yield reduction assays or cytopathic assays were used.

It is known that the mechanisms of the antiretroviral activity of interferon is quite different from those mechanisms producing inhibition of cytopathic viruses. One purpose of the present study was to determine whether the antiretroviral activities of these interferon molecules parallel their anti-VSV actions in different cell lines. In feline cells, the dose-response curves of the antiretroviral action and the anti-VSV action of the seven interferon species correlated with each other. IFN-αA/D (*Bgl*) was the most active species, whereas IFN-αD/A (*Bgl*) was the least active. A change of only three amino acids of IFN-αA increased the potency against both FeLV and VSV by about 10-fold (see IFN-αA/D/A; Fig. 1 and 2). The only interferon species whose anti-VSV activity was apparently not parallel to anti-FeLV activity in FlF3 cells was IFN-αD. Although it was less active against VSV than IFN-α, IFN-αA/D (*Pvu*), and IFN-αA/D/A, it was more active against FeLV than the latter three species of interferon. The basis of this difference remains to be elucidated.

In the feline cell line, we tested the effects of only IFN-αA and IFN-αD on EMCV replication. The results indicated that like the antiretroviral action, the anti-EMCV action of these two species correlates with their anti-VSV actions. It remains to be seen whether the same holds true for the hybrid species.

In the human RD-114 cells and the murine NIH/MOL,C cells, the antiretroviral and the anti-VSV actions of only IFN-αA/D (*Bgl*) and IFN-αD/A (*Bgl*) were tested. In the murine cells, IFN-αD/A (*Bgl*) was not active at any of the concentrations tested and the anti-VSV and anti-murine leukemia virus effects of IFN-αA/D (*Bgl*) had similar dose-response characteristics. In RD-114 cells, IFN-αA/D (*Bgl*) was 1,000-fold more active against retrovirus production than IFN-αD/A (*Bgl*). We previously reported that natural human leukocyte or immune interferon does not inhibit VSV or EMCV replication in these cells (3). Consistent with these observations, we found that neither IFN-αA/D (*Bgl*) nor IFN-αD/A (*Bgl*) inhibited VSV replication in these cells.

The above results indicate that the specific interactions of leukocyte interferon with cells, which determine the efficacy of different interferon molecules in cells of different species, are the same for the antiretroviral and the classical antiviral effects of interferon, although the molecular mechanisms of the two classes of effects are entirely different. It is conceivable that the efficiency of an interferon molecule is determined at the level of interaction with the cell surface receptors, and the same receptor interaction triggers both classes of antiviral effects on these cells. Our results also demonstrated that, although IFN-αD/A (*Bgl*) exhibited poor activity compared with IFN-αA/D (*Bgl*), it did not interfere with the action of IFN-αA/D on feline cells, even when present in a 10-fold mass excess.

At least five proteins are induced by treatment of human fibroblasts with leukocyte interferon (1, 8, 15). These proteins are also induced by IFN-β or IFN-γ, although the kinetics of their induction in response to IFN-α and IFN-γ are different (G. C. Sen and B. Y. Rubin, Virology, in press). The functional roles of these induced proteins are unclear, but their synthesis parallels the antiviral properties of interferon, and some of these proteins may be responsible for enzyme activities induced by interferon. Our results demonstrated that all of these proteins are induced by both IFN-αA, IFN-αD, and their hybrids.

Moreover, we observed that synthesis of the 67,000-dalton and 56,000-dalton proteins was transient in IFN-αA- or IFN-αD-treated fibroblasts, as it is in cells treated with natural leukocyte interferon (8; data not shown). Induction of some of these proteins can also be demonstrated in cell lines other than fibroblasts, such as Daudi and HeLa cells. However, we could not detect any induced proteins in human RD-114 cells in which interferon does not inhibit VSV or EMCV replication (11a). In the feline FlF3 cells, we detected the induction of only the 88,000-dalton protein (Fig. 7), although these cells were responsive to each species of interferon tested. It is possible that other proteins are induced but are not detectable by one-dimensional gel electrophoresis or that the other proteins are not related to these antiviral effects in feline cells.

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