Antiviral activities of hybrids of two major human leukocyte interferons

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ABSTRACT

Four hybrid human leukocyte interferon (LeIF or IFN-α) genes have been constructed by in vitro recombination of LeIF-A (IFN-α2) and LeIF-D (IFN-α1) genes at common restriction endonuclease sites located within their coding regions. These hybrid genes have been expressed in E. coli under trp promoter control. The interferons produced [LeIF-AD (BglII), -AD (PvuII), -DA (BglII), -DA (PvuII)] have antiviral properties distinct from the parental molecules LeIF-A and -D, varying considerably in their abilities to inhibit plaque formation by different viruses in a range of mammalian cells. All six of the cloned LeIFs exhibit the heat stability, pH 2 stability and antigenic specificity of natural leukocyte interferons.

INTRODUCTION

The molecular cloning of DNA sequences coding for human interferons in bacteria has revealed the existence of multiple molecular species of leukocyte interferon (LeIF, IFN-α) (refs. 1-5) and several of these species have been found to have distinct antiviral activity in various mammalian cell cultures (6, 7). Two of the 12 or more species of leukocyte interferon so far identified appear to comprise over 60 percent of the interferons present after induction of the human myeloblast cell line KG-1 (4). These two inter-ferons are designated LeIF-A and LeIF-D (4) or IFN-α$_2$ and IFN-α$_1$ (3), respectively. LeIF-A and LeIF-D consist of 165 and 166 amino acid residues (LeIF-A has a deletion of a.a. 44) and differ in 29 amino acid positions. Common BglII and Pvu II restriction endonuclease sites occur in these interferon genes between positions corresponding to amino acid residues 61 and 62, and 91 and 92 respectively. Cleaving the genes with either BglII or PvuII and recombining the two gene fragments has allowed construction of four hybrid interferon genes (8). In the studies reported here those genes were expressed in E. coli under trp promoter control to yield the hybrid interferons designated LeIF-AD (Bgl), LeIF-AD (Pvu), LeIF-DA (Bgl) and LeIF-DA (Pvu), and correspond to the IFN α-2 (B) α-1, IFN α-2 (P) α-1, IFN

α-1 (B) α-2, and IFN α-1 (P) α-2 described by Streuli and co-workers (8).

Since interferons have been found to confer some protection in heterologous species against viral infections both in animals and cell cultures (9–12), we have measured the antiviral activity of these hybrid interferons in various mammalian cell cultures. These bacterial–derived interferons have unique antiviral effects in different virus–cell systems. The results show that the hybrid interferons have properties unlike those of the parent interferons or mixtures of the parent interferons, and demonstrate that new more potent interferons may be produced via recombinant DNA technology.

MATERIALS AND METHODS

Cells and Viruses. Continuous cell cultures of human amnion cells (WISH), mouse fibroblasts (L–929), hamster kidney (BHK) and bovine kidney cells (MDBK) were obtained from the American Type Culture Collection, Rockville, MD. HeLa cells, monkey kidney cells (Vero), and rabbit kidney cells (RK–13) were purchased from Flow Laboratories, Inglewood, CA. A primary rabbit kidney line was obtained from MA Bioproducts, Walkersville, MD. All cell lines were maintained in minimal essential medium (MEM) and 5 percent fetal calf serum from Grand Island Biologicals, Grand Island, NY.

Vesicular Stomatitis (VS) Virus (Indiana strain) was purchased from American Type Culture Collection and encephalomyocarditis (EMC) virus was grown and stored as described previously (13). The Herpes Simplex Virus, Type 1 (HSV–1) used in this study was a fresh clinical isolate kindly provided by Dr. Richard Glogau, San Francisco, and was passaged once in WISH cells prior to use on Vero cells.

Interferon Assays. The antiviral activities of bacterial extracts were routinely determined by plaque reduction assays as follows. Serial half-$\log_{10}$ dilutions of samples were made directly in MEM containing 2 percent fetal calf serum. After removal of maintenance medium, 1 ml aliquots of the diluted samples were added to confluent monolayers of cell cultures in six-well Costar cluster dishes. The cell monolayers were incubated for 18–20 hrs at 37°C in 5 percent CO_2 atmosphere. Following incubation, the interferon was removed and an appropriate virus inoculum was added to each well. The virus was adsorbed for 1 hr, the inoculum removed, and the monolayer was covered with an overlay of 0.7 percent noble agar and minimal essential medium containing two percent fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml). The infected

cultures were incubated at 37°C for 24–48 hrs until plaque formation was observed. The cells were fixed by addition of 1 ml 10 percent formalin in 0.15 M NaCl, the agar removed, and the monolayer stained with crystal violet solution. The number of plaques per 35 mm well in infected control monolayers was between 30 and 50 and the results recorded are the average of duplicate plates.

Cytopathic effect (CPE) inhibition assays were performed in 96–well microtiter trays as described previously (14, 15). Values determined by this method are expressed as units relative to the NIH leukocyte standard G–023–901–527.

Construction of Plasmids. The construction of a plasmid, pLeIF A25, which directs the synthesis of mature LeIF A in E. coli has been described previously (2). The plasmid pLeIF D3 which directs the synthesis of mature LeIF–D was constructed in a similar manner (P. Gray, D. Goeddel, unpublished results) using the LeIF–D cloned cDNA sequence (4). The constructions of the four hybrid plasmids pLeIF AD (BglII), pLeIF AD (PvuII), pLeIF DA (BglII), and pLeIF DA (PvuII) are outlined in Figure 1. Restriction endonucleases were purchased from New England Biolabs and used according to vendor's directions. DNA restriction fragments were purified by electrophoresis through 6 percent polyacrylamide gels and recovered by electroelution (2). Ligation reactions were performed for 4 hours at 20°C in 20 μl of 20 mM Tris–HCl (pH 7.5), 10 mM dithiothreitol, 10 mM $MgCl_2$, 0.5 mM ATP using 100 units of T4 DNA ligase (New England Biolabs) and approximately 0.2 pmoles of each restriction fragment. Conditions for the transformation of E. coli 294 and purification of plasmid DNA have been described previously (16). Restriction mapping and DNA sequence analysis by the method of Maxam and Gilbert (17) were performed on the ligation junctions of the recombinant plasmids to confirm the predicted constructions.

Interferon Preparations. E. coli 294 harboring the leukocyte interferon expression plasmids were grown in 10 ml of M9 salts (18) containing 0.2 percent glucose, 0.5 percent casamino acids and 5 μg/ml tetracycline to an A_{550} of 1.0. Cells were harvested by centrifugation and resuspended in 1 ml of 15 percent sucrose, 50 mM Tris–HCl (pH 8.0), 50 mM EDTA. One mg of lysozyme was added and, after 5 minutes at 0°C, the cells were disrupted by sonication. After centrifugation, the supernatants were collected and used directly in plaque reduction assays or CPE inhibition assays. All interferon preparations had specific

activities of between $2x10^3$ and $4x10^5$ units/mg protein as determined
in the microtiter assay on WISH cells challenged with VS virus.

   In Vitro Synthesis of Interferons. The _in vitro_ coupled transcription-
translation system of Zarucki-Schulz _et al_. (19) with the following
modifications (M. Brawner and S.R. Jascunas, personal communication) was
used to synthesize ^{35}S-labelled interferons. The enzyme fractions were
prepared as described by Kaltschmidt _et al_. (20) except that pyruvate kinase
and _E. coli_ RNA polymerase were purchased from Sigma Chemical Co. Each
reaction included 5 μg of plasmid DNA and was performed in 25 μl total
volume containing 33 μM unlabeled methionine and 2 μM ^{35}S-methionine (1000
Ci/mmole; New England Nuclear). Incubations were at 37°C for 30 minutes.

   Five μl of each reaction mixture was analyzed by electrophoresis through
a 15 percent SDS-polyacrylamide gel (21). The ^{35}S-labelled interferon
bands were localized by fluorography (22), excised from the gel and
radioactivity quantitated by scintillation counting. CPE inhibition assays
(using VSV on both WISH and MDBK cells) were performed on 5 μl aliquots of
each reaction mixture.

   Characterization of Interferons. The antigenic specificities of the
various interferon preparations were determined by neutralization of
antiviral activities with rabbit anti-LeIF and anti-FIF antibodies supplied
by the Antiviral Substances Program of the National Institutes of Allergy
and Infectious Diseases (NIAID). The anti-LeIF preparation had a titer of
10,000/ml and the anti-FIF a titer of 1024/ml when reacted with 20 units of
their respective interferon standards in a standard CPE inhibition assay
using human WISH cells and VS virus.

RESULTS
   Construction of plasmids containing hybrid leukocyte interferon genes.
The human leukocyte interferon genes LeIF-A (IFN-α2) and LeIF-D (IFN-α1)
code for mature interferon molecules of 165 and 166 amino acids respectively
(2-4). Overall these proteins differ from each other by 29 amino acids with
LeIF-A lacking amino acid 44 (asp) which has been found in all other
leukocyte interferon sequences determined to date (1-6). Both the LeIF-A
(2) and LeIF-D (Gray and Goeddel, unpublished results) genes have been
directly expressed in _E. coli_ under _trp_ promoter control to yield the
corresponding mature LeIF proteins. Because the LeIF-A and -D genes possess
common restriction endonuclease sites (4), it is possible to construct
genetic hybrids containing portions of each parental molecule. The

constructions of such hybrids utilizing a common BglII restriction site or a
PvuII restriction site are outlined in Figure 1.

The restriction fragments designated a through j were recombined by
ligation in the combinations shown (Fig. 1) to yield the four new hybrid
plasmids identified as pLeIF-AD (BglII), pLeIF-DA (BglII), pLeIF-AD (PvuII)
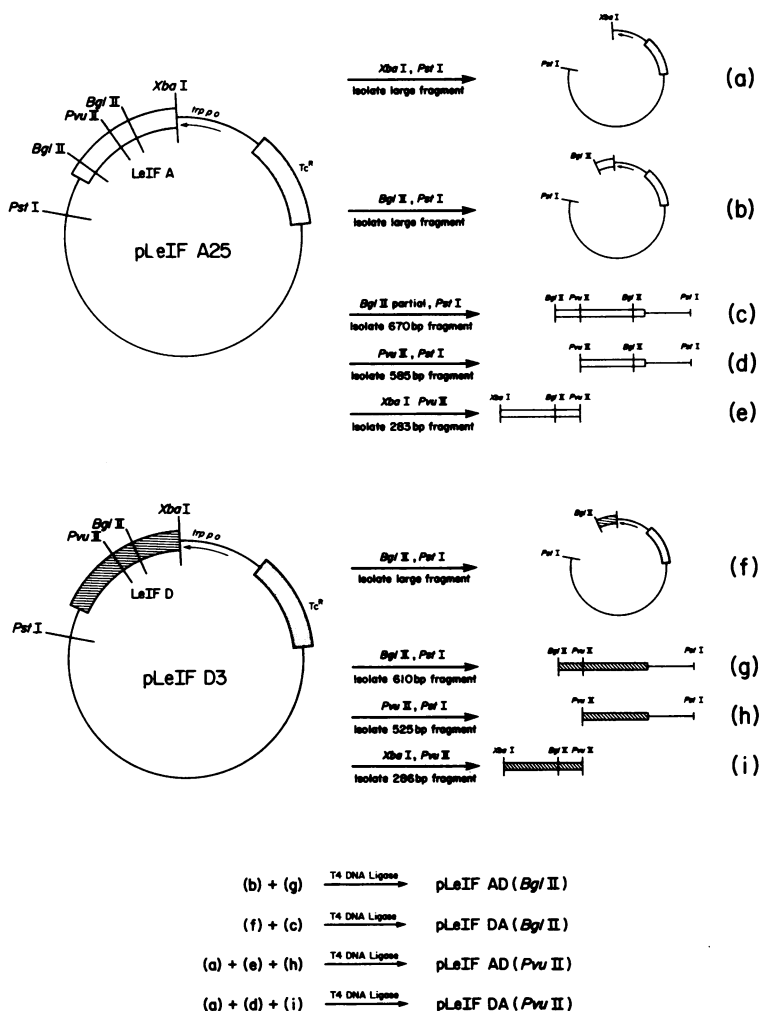

Fig. 1   Construction of plasmids for direct expression of human hybrid
         leukocyte interferons in E. coli. The isolation of the various
         DNA restriction fragments and the conditions for the ligation
         reactions are outlined in Materials and Methods.

and pLeIF-DA (PvuII). The hybrid genes were coupled with the same trp
expression system utilized for LeIF-A and -D (2) so that direct expression
of these unique leukocyte interferon genes would occur in E. coli.

A schematic representation of the resulting hybrid interferons is
depicted in Figure 2. Each hybrid differs from the parental molecule by
13 or 16 amino acids depending on whether the N-terminal or C-terminal
fragment was derived from LeIF-A or -D. The number of amino acid residues
from each terminus is as follows:

	N-terminus		C-terminus	
	LeIF-A	LeIF-D	LeIF-A	LeIF-D
LeIF-AD (Bgl)	61	--	--	104
LeIF-AD (Pvu)	91	--	--	74
LeIF-DA (Bgl)	--	62	104	--
LeIF-DA (Pvu)	--	92	74	--

Thus, the LeIF-AD hybrid interferons are one amino acid shorter due to the
deletion at position 44 in LeIF-A mentioned earlier.

Determination of relative specific activities of interferons. The
comparison of relative amounts of interferons produced in bacteria and
their antiviral activities (2, 3, 6, 7) can be complicated by variations
in rates of synthesis, in stability of the polypeptides, or in specific
activities in different assay systems. In order to determine relative
specific activities of the various interferons, equal amounts of plasmid

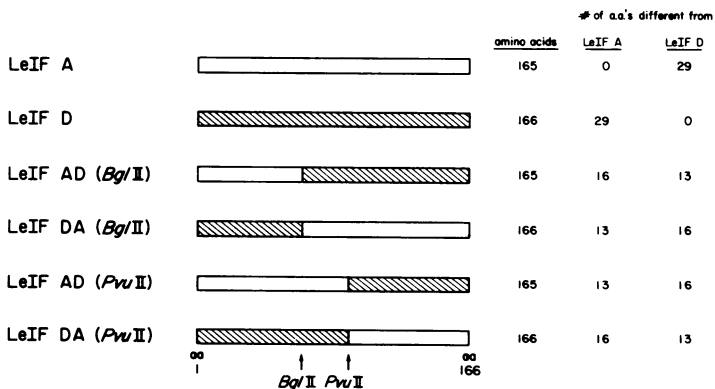|		# of a.a.'s different from	
	amino acids	LeIF A	LeIF D
LeIF A	165	0	29
LeIF D	166	29	0
LeIF AD (*Bgl*II)	165	16	13
LeIF DA (*Bgl*II)	166	13	16
LeIF AD (*Pvu*II)	165	13	16
LeIF DA (*Pvu*II)	166	16	13

aa 1 — *Bgl*II *Pvu*II — aa 166

Fig. 2   Representative diagram of the parental and hybrid leukocyte
interferons showing the interferons produced and their
differences from the parental molecules.

DNA containing the parental and hybrid gene sequences were transcribed and translated in a coupled in vitro system containing ^{35}S–methionine. The resulting proteins were assayed for activity on both human (WISH) and bovine (MDBK) cell lines. Equivalent amounts were also electrophoresed through SDS–polyacrylamide gels and the radiolabelled IFN bands were counted. These data are shown in Table 1. The specific activities determined on WISH cells varied significantly with LeIF–AD (Pvu) having the highest value and LeIF–DA (Bgl) the lowest. In sharp contrast, the specific activities on MDBK cells were essentially the same for all the interferons, since CPE inhibition assays typically vary over a 2–3 fold range.

Antiviral properties of interferons in different cell lines. The relative antiviral effects of the parental interferons and hybrids made from them are presented in Table 2. These interferon titers were obtained

TABLE 1. Relative Specific Activities of Interferons Produced by In vitro Synthesis from Plasmid DNA

LeIF	IFN band cpm(x10^{-2})	Activity on:		Specific Activity		Specific Activity Ratios
		WISH	MDBK	WISH	MDBK	MDBK/WISH
		units		unitsx10^3/cpm (pct.)		
A	155	60	96	3.87 (100)	6.19 (100)	1.6
D	87	5	40	0.57 (15)	4.60 (74)	8.0
AD (Bgl)	108	80	65	7.41 (190)	6.02 (97)	0.8
AD (Pvu)	111	120	32	10.8 (280)	2.88 (47)	0.3
DA (Bgl)	121	<0.5	80	<0.04 (<1)	6.61 (110)	>160
DA (Pvu)	65	<0.5	45	<0.08 (<2)	6.92 (110)	>90

The different plasmids were employed in a cell–free transcription–translation system as described in Materials and Methods. The units of interferon were determined in a standard CPE inhibition assay using VS virus and the NIH LeIF standard.

The cpm for LeIF–D, –AD (Bgl) and –AD (Pvu) were obtained by multiplying the actual value by 6/7 to account for the additional methionine in the carboxy half of LeIF–D.

The values in parentheses are the specific activities as the percent of that of LeIF–A.

TABLE 2.   Relative Antiviral Activities Induced by Leukocyte Interferon
Preparations

Cell Line	INTERFERON TITERS ($\log_{10}$)						
	A	D	AD(Bgl)	AD(Pvu)	DA(Bgl)	DA(Pvu)	A+D
WISH (Human)	6.3	5.4	6.6	6.8	4.3	4.6	6.0
HeLa (Human)	4.3	3.9	6.6	--	4.1	5.5	3.1
BHK (Hamster)	4.8	4.1	4.6	5.2	6.9	7.6	6.2
L-929 (Mouse)	2.4	3.6	6.6	5.2	2.9	3.6	5.4
MDBK (Bovine)	6.5	6.3	6.5	6.2	6.5	6.5	6.4
Primary Rabbit Kidney	3.8	4.1	5.6	--	5.6	7.1	5.7
RK-13 (Rabbit)	4.8	4.4	3.9	4.0	5.4	5.8	4.4
Vero (Monkey)	5.5	5.4	6.1	5.7	4.9	4.6	3.2

Interferon titers are expressed as the $\log_{10}$ dilution of an interferon
preparation required to reduce VS virus plaque formation by 50 percent.
All values are adjusted to reflect the differences in MDBK cell specific
activities given in Table 1.

by determining the dilution of bacterial interferon preparations (see
Materials and Methods) needed to cause a 50 percent reduction of VS virus
plaque number in the different types of cell cultures. To correct for the
fact that different absolute amounts of interferons were present in each
bacterial preparation, the titers have been adjusted to reflect the
relative specific activities determined for MDBK cells (Table 1). The
adjusted values shown in Table 2 therefore are those expected if all the
bacterial preparations had equivalent interferon concentrations. The
validity of such a correction is demonstrated by the fact that the
antiviral titers of the various IFN preparations on WISH cells (Table 2)
are consistent with their specific activities determined on the same cells
(Table 1).

The LeIF-AD hybrids have significantly greater activities than either
parental molecule on mouse L-929 cells, human HeLa cells, and primary
rabbit kidney cells. The LeIF-DA hybrids are particularly active on
hamster (BHK) and rabbit cells. The most striking difference between the
various interferons is their ability to induce an antiviral state against
VS virus in the mouse L-929 cells. LeIF-AD (Bgl) has at least one
thousand times more activity on L-cells than LeIF-A, -D, -DA (Bgl) or -DA

(Pvu).

The comparison of antiviral properties was extended by testing a
mixture of equal amounts of preparations of LeIF-A and LeIF-D on the
various cell lines. Interestingly, simply mixing these two interferons
does not always cause an antiviral activity which is a mean of the two
constituents (Table 2). In fact, lower activities are observed on HeLa
and Vero cells while in BHK, L-929, and Primary Rabbit Kidney cells the
combination of LeIF-A and -D gave higher titers than those obtained with
LeIF-A or -D alone.

Effects of Interferons on EMC Virus and Herpes Simplex Type 1

In order to assess to what extent the antiviral activity of the
various interferons is dependent on the particular virus challenge, their
activities in L-929 cells using EMC virus (Fig. 3A) and in Vero cells
using HSV-1 (Fig. 4A) were determined. In L-929 cells challenged with EMC
virus the relative potency of LeIF-A and LeIF-D are similar as compared to
challenge with VS virus, and LeIF-D is approximately 2.0 $\log_{10}$ more
active than LeIF-A against EMC plaque formation (Fig. 3). In contrast,
the relative activities of LeIF-AD (Bgl) and LeIF-DA (Bgl) against EMC
virus are the same as those measured with VS virus challenge although the
magnitude of the difference appears to be different. Infection of Vero
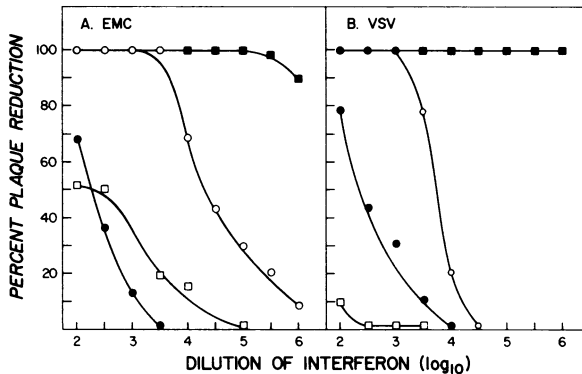

Fig. 3   Inhibition of EMC Virus (A) or VS Virus (B) plaque formation by
         LeIFs. Confluent monolayers of mouse L-929 cells were treated
         with various dilutions of interferon preparations and challenged
         with the appropriate virus. Following the determination of the
         number of plaque forming units, the percentage of plaque
         reduction caused by LeIF-A (●), LeIF-D (O), LeIF-AD (Bgl) (■)
         or LeIF-DA (Bgl) (□) was calculated. These curves have not been
         adjusted for the specific activities calculated in Table 1.

cells with HSV-1 showed that both LeIF-A and -D are less potent than
either of the two hybrids and that LeIF-DA (Bgl), which has low activity
against VS virus, is comparable in anti-HSV activity with LeIF-AD (Bgl)
(Fig. 4). Thus, the relative antiviral activities of the different cloned
leukocyte interferons vary with the type of virus employed. It should be
noted that the data presented in Figures 3 and 4 have not been adjusted to
reflect the relative specific activities determined in vitro (Table 1),
but rather are representative of the titration curves for the different
LeIFs.

Determination of the antigenic specificity, pH 2 stability, and heat
stability of LeIF-A, -D, -AD (Bgl) and -DA (Bgl) interferon preparations
demonstrated that these bacterial-derived interferons exhibit the
properties ascribed to naturally occurring leukocyte interferons.
Neutralization with a polyvalent anti-LeIF antibody revealed that
equivalent antiviral units (titrated on WISH cells) of LeIF-A and LeIF-AD
(Bgl) were neutralized at 1:480 and 1:960 dilutions, respectively,
and -D and -DA (Bgl) at a 1:60 dilution, in agreement with their relative
specific activities. Incubation with an anti-fibroblast interferon
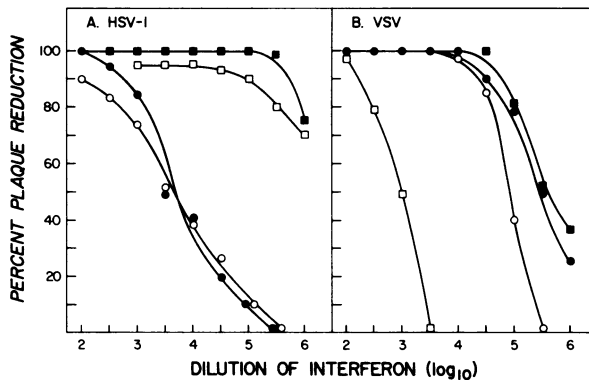

Fig. 4   Effects of Cloned Leukocyte Interferon Subtypes on HSV-1 (A) and
          VS Virus (B) Plaque Formation. Vero cells were pretreated with
          various dilutions of LeIF-A (●), LeIF-D (○), LeIF-AD (Bgl) (■)
          or LeIF-DA (Bgl) (□) for 18 hr prior to infection with HSV-1 or
          VS virus. Following incubation the number of plaque forming
          units was determined. The data are presented as the amount of
          plaque inhibition due to the presence of the different dilutions
          of interferon preparations but are not adjusted for the specific
          activity values given in Table 1.

antibody resulted in no loss of activity of any of these interferons. These interferons were stable to pH 2 treatment for 24 hrs and progressively lost activity when heated at 56°C for a similar period of time (data not shown).

DISCUSSION

Whether the hybrid human leukocyte interferons are formed by recombination at the BglII or PvuII sites in the genes for the parental interferon sub-types, LeIF-A and -D, does not significantly affect antiviral activity when assayed in a range of mammalian cell lines. Therefore the amino and carboxy termini, not the 62 to 92 amino acid region between the BglII and PvuII sites, would appear to be the major determinants of the specificity of the antiviral activity in the cell cultures examined as suggested by Streuli et al. (8). It may be noted that the middle portion in question appears to be relatively remote from the two disulfide bonds formed between the cysteines at positions 1 and 98, and 29 and 138 (23, 24). Moreover, there are only three amino acid differences between LeIF-A and -D in this central region (4).

The various leukocyte interferon sub-types and hybrids considered here were all expressed under trp promoter control in E. coli in essentially identical manner. As a result, identical levels of expression might be expected, particularly considering the close structural similarity of the various interferons. The use of a cell-free transcription-translation system revealed that the actual amounts of protein made from purified plasmid DNA varied slightly more than two-fold (Table 1). Comparison with earlier data for a range of mammalian cell types (7) needs to take account of these differences. In the case of LeIF-A, activity in mouse cells might also be affected by the presence of bacterial proteins (24). The relative specific activities of the hybrid interferons also differed significantly depending on which type of mammalian cells were used to assay biological activity (Tables 1 and 2). Taking into consideration the variability of standard CPE reduction assays (_50 percent), it is apparent that all the interferons have essentially the same specific activity when measured on MDBK cells. In contrast, in L-929 cells LeIF-AD (Bgl) has very pronounced activity which greatly exceeds the activity of either parental sub-type and the other hybrid, LeIF-DA (Bgl), regardless of the infecting virus (Fig. 3). The ratio of activities of LeIF-AD (Bgl) on human WISH, bovine MDBK, and L-929 cells is 1:1:1 (Table 2) which is

nearly identical to the 1:1:1.7 ratio reported for IFN α-2 (B) α-1 (8).
The activity of LeIF-AD (Bgl) is somewhat greater against VS virus, in
L-929 cells, than EMC virus and the difference between LeIF-AD (Bgl) and
-DA (Bgl) is greater for VS virus than EMC virus in L-929 cells (Fig. 3).
The relative specific activity of LeIF-A and LeIF-D against the two
viruses is similar with LeIF-D being more potent than LeIF-A against both
viruses. The two types of hybrid interferon have antiviral activity
against HSV-1 in Vero cells comparable to that of LeIF-A, -D and -AD
against VS virus in this cell but the parental interferons, LeIF-A and -D
have equal and much lower antiviral activity against HSV-1 in Vero cells
(Fig. 4).

The different antiviral activities of the materials here described
against different viruses suggests that the different LeIFs cause
induction of various antiviral mechanisms which have distinct effects
dependent on the challenge virus used. Several intracellular biochemical
events accompany interferon treatment, notably induction of a protein
kinase capable of phosphorylating the α-subunit of eIF-2 and a
2',5'-oligoadenylate (2'5'A) synthetase the product of which activates a
specific endoribonuclease (RNase F) (26). Thus different mechanisms might
be of particular importance for antiviral effects against different
viruses. This notion is supported by several observations.
Undifferentiated mouse embryonal carcinoma cells are protected against
picornaviruses (EMC and Mengo virus) but not VS, influenza or Sindbis
virus (27). In this case the protein kinase activity is not induced in
these undifferentiated cells (28) implying that effects on eIF-2 are not
involved in antiviral effects against the picornaviruses. In mouse NIH
3T3 cells even high concentrations of mouse interferon do not inhibit
replication of EMC or VS virus but Moloney murine leukemia virus
production is inhibited (29, 30). Epstein et al. (30) have reported that
RNase F is not produced in the NIH 3T3 cells by interferon treatment but
it is in interferon sensitive mouse L-929 cells, indicating that the ribo-
nuclease pathway is necessary for antiviral activity against both EMC and
VS virus in mouse cells. That different interferons might induce different
biochemical pathways is apparent from analysis of the interferon resistant
mouse leukemia cell, L1210R, in which an antiviral state is induced by
immune interferon but not fibroblast interferon (31). Our results,
presented here, support the notion that the various leukocyte interferon
sub-types cause differential expression of the various pathways known to

be affected by interferon treatment. Those pathways are under
investigation using the various hybrid leukocyte interferons described in
this report.

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