

Regulation of Macrophage Growth and Antiviral Activity by Interferon- γ

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Abstract. Interferons, in addition to their antiviral activity, induce a multiplicity of effects on different cell types. Interferon (IFN)- γ exerts a unique regulatory effect on cells of the mononuclear phagocyte lineage. To investigate whether the antiviral and antiproliferative effects of IFN- γ in macrophages can be genetically dissociated, and whether IFN- α and IFN- γ use the same cellular signals and/or effector mechanisms to achieve their biologic effects, we have derived a series of somatic cell genetic variants resistant to the antiproliferative and/or antiviral activities of IFN- γ . Two different classes of variants were found: those resistant to the antiproliferative and antiviral effects of IFN- γ against vesicular stomatitis virus (VSV) and those resistant to the antiproliferative effect, but protected against VSV and encephalomyocarditis virus (EMCV) lysis by IFN- γ . In addition, a third class of mutants was obtained that was susceptible to the growth inhibitory activity, but resistant to the antiviral activity of

IFN- γ . Analysis of these mutants has provided several insights regarding the regulatory mechanisms of IFN- γ and IFN- α on the murine macrophage cell lines. The antiproliferative activity of IFN- γ on these cells, in contrast to that of IFN- α , is mediated by a cAMP-independent pathway. The antiproliferative and antiviral activities of IFN- γ were genetically dissociated. Variants were obtained that are growth resistant but antivirally protected, or are growth inhibited but not antivirally protected against VSV or EMCV. The genetic analysis indicated that IFN- α and IFN- γ regulate the induction of the dsRNA-dependent P1/eIF-2 α protein kinase and 2',5'-oligoadenylate synthetase enzymatic activities via different pathways. Finally, a unique macrophage mutant was obtained that was protected by IFN- γ against infection by VSV, but not EMCV, suggesting that antiviral mechanisms involved in protection against these different types of RNA viruses must be distinct at some level.

INTERFERONS (IFNs)¹ are defined as molecules that induce an antiviral state, but in addition are known to exert a multiplicity of effects on a variety of different cell types, including the inhibition of cell proliferation (17, 24, 35, 47). Of the three classes of IFN known, IFN- α and IFN- β share a common cell receptor while IFN- γ binds to a distinct cell receptor (5). Two unique interferon-induced enzyme activities have been implicated in the antiviral effects of IFN- α and IFN- β , namely, the P1/eukaryotic initiation factor-2 α (P1/eIF-2 α) protein kinase and 2',5'-oligoadenylate synthetase (2',5'-(A)_n synthetase) systems (reviewed in 28, 36), but it is unknown whether similar mechanisms are used by IFN- γ . While IFN- α and IFN- γ elicit common cellular responses in many cell types, IFN- γ has specific effects on cells of the mononuclear phagocyte lineage. IFN- γ can increase Fc receptor-mediated phagocytosis (38), induce the expression of major histocompatibility

complex class I and class II antigens (23, 46), and activate macrophages to produce a respiratory burst after endocytosis of bacteria or parasites (34). Electrophoretic analysis of polypeptides derived from IFN- γ -treated human fibroblast lines revealed that at least 12 unique proteins are induced by IFN- γ (50). The molecular mechanisms of the antiproliferative and antiviral effects of interferon, or the unique activation of macrophages by IFN- γ remain largely unknown.

One approach to understanding the mechanisms by which IFNs, and in particular IFN- γ , act to regulate mononuclear phagocyte function is a somatic cell genetic analysis using cloned macrophage cell lines. In previous studies we observed that macrophage variants in cAMP metabolism of the J774.2 murine macrophage cell line were 50-fold less sensitive to the antiproliferative effects of IFN- α than the parental clone (43). Conversely, a role for cAMP was confirmed by selecting for IFN- α -resistant mutants, five of which were shown to have defective adenylate cyclase, providing genetic evidence that IFN- α uses a cAMP-dependent signal transduction mechanism in mediating the antiproliferative effects in at least one type of cell (33). The antiviral state was fully

1. *Abbreviations used in this paper:* eIF-2, eukaryotic initiation factor-2; EMCV, encephalomyocarditis virus; Gⁱ, growth inhibited; G^r, growth resistant; IFN, interferon; 2',5'-(A)_n, 2',5'-oligoadenylate; VP, antivirally protected; V^s, susceptible to viral lysis.

induced by IFN- α in the adenylate cyclase mutants, thus demonstrating that it was possible genetically to dissociate the antiviral and antiproliferative effects of IFN- α .

Because IFN- γ is known to have unique effects in regulating macrophage function, as well as inducing the antiviral and antiproliferative effects on macrophage cell lines, we have attempted here to investigate two questions: (a) whether one can genetically dissociate the antiviral and antiproliferative effects of IFN- γ , as in the case of IFN- α ; and (b) whether IFN- α and IFN- γ use the same cellular signals and/or effector mechanisms to achieve these biological effects.

Materials and Methods

Cells

The cloned cell line, J774.2, was originally established from a mouse reticulum cell sarcoma derived by P. Ralph et al. (39) and shown to have a macrophage-like phenotype (4, 32). J774.2 and variant clones were maintained in DME, supplemented with 20% heat-inactivated horse serum, 0.1 mM nonessential amino acids (NEAA), 10% NCTC 109 conditioned media (Microbiological Associates, Walkersville, MD), penicillin, and streptomycin at 37°C in a humid incubator containing 7% CO₂.

The mouse hepatoma cell line, E₃, was grown in DME supplemented with 10% heat-inactivated FCS, 5% NCTC 109, and 1% NEAA. Primary rat embryo fibroblasts were cultured in DME supplemented with 10% FCS. DME, horse and fetal calf serum, NEAA, penicillin, and streptomycin were from Gibco Laboratories, Grand Island, NY.

Viruses

The Indiana serotype of vesicular stomatitis virus (VSV) was prepared in chicken embryo fibroblasts, clarified by centrifugation (1,000 g, 10 min), and had a titer of 3.0×10^8 pfu/ml. The stock was divided into small portions, frozen at -70°C, and diluted into regular macrophage cell culture medium before use. Encephalomyocarditis virus (EMCV) was obtained from J. Paucker (University of Pennsylvania, Philadelphia, PA) and had a titer of 5×10^7 pfu/ml.

Interferon

Recombinant *Escherichia coli*-derived murine IFN- γ (Genentech Inc., San Francisco, CA) had a specific activity of 1.24×10^7 U/mg. Murine IFN- α was obtained from Lee Biomolecular Company (catalogue No. 22061; San Diego, CA) and had a specific activity of 2.7×10^6 U/mg. Interferon activity was assayed by the inhibition of cytopathic effects of EMCV on L cells in microtiter wells. Interferon activity was routinely titered once a month to determine if any loss of activity had occurred.

Cloning of Macrophage Lines

Macrophage cell lines and variants were cloned in Sea plaque agarose (FMC Corporation, Rockland, ME) on a rat embryo fibroblast or E₃ feeder layer. A 1:20 dilution of a confluent layer of feeder cells was plated 18 h before cloning in a 60-mm tissue culture dish (Falcon Labware, Oxnard, CA). 16 h later the medium was aspirated off and 4 ml of medium plus agarose (final concentration 0.45% [wt/vol]) was allowed to solidify at 4°C for 10 min. Macrophage cell suspension was diluted 1:10 with medium plus agarose and 1 ml of cell suspension was layered on top of the solidified agarose. After 10 min at 4°C the cells were placed in a 37°C incubator at 7% CO₂. Macrophage colonies were picked 7-9 d later.

Selection of IFN-resistant Variants

J774.2 cells were cloned in 4,000 U/ml of IFN- γ without prior mutagenesis. The clones were then grown in increasing concentrations of IFN- γ from 500 to 4,000 U/ml over a 3-4-wk period. Clones able to grow in the presence of 4,000 U/ml of IFN- γ were allowed to grow in cell culture in IFN- γ -free medium for a month and were then retested for their ability to grow in IFN- γ . A spontaneous mutant was derived from J774.2 cells which were maintained in culture for 1.5 yr, that was growth inhibited by IFN- γ , although susceptible to viral lysis (J774.2-7 = G³/V⁵). After the discovery that the culture was not as responsive vis-à-vis the antiviral activity of IFN- γ , the cells were subcloned for isolated colonies and retested for their phenotype.

Growth Curves

Cells in log phase were seeded (7.5×10^3 cells/0.15 ml) in 96-well plates (Linbro, Flow Laboratories, Hamden, CT), in the absence or presence of the various IFNs and cholera toxin. At the time intervals indicated 0.025 ml of tritiated thymidine at 0.04 mCi/ml was added per well (final concentration of 1 μ Ci/well). After a 45-min incubation 0.05 ml of 0.45% NP-40 in PBS was added per well for 5-10 min with moderate rotation on an orbital gyrator. Nuclei were harvested onto glass filter paper, dried, and counted with liquid scintillant for 1 min on a liquid scintillation counter. Results for each experimental point is the average of triplicate wells. Individual wells did not differ generally >1 SD from the mean. Preliminary experiments showed that the inhibition of thymidine incorporation by IFN- α and IFN- γ paralleled the actual growth inhibition as measured by counting the number of viable cells excluding trypan blue with a hemocytometer.

Viral Infection

Cells ($2-4 \times 10^5$) were plated in 2-cm² wells (Linbro) for 21 h in 1 ml of medium in the presence and absence of IFN. VSV or EMCV, at a multiplicity of infection of 0.1, was added and the cells cultured for an additional 18-24 h. Supernatants were collected and stored frozen at -70°C.

Viral Yield

Serial 10-fold dilutions (0.2 ml) of the above supernatant fluids were added to confluent Vero cell monolayers in 2-cm² wells. After 1-h adsorption at 37°C, 1 ml of warm medium containing 0.75% carboxyl methyl cellulose was added, and wells were incubated for an additional 36-48 h. The monolayers were then fixed for 10 min, stained with crystal violet, and viral plaques counted.

RNA Isolation

For the preparation of cytoplasmic RNA, cells were harvested, either by pipetting or scraping them off the dish, then washed twice with cold PBS, and resuspended in 0.45 ml of ice-cold buffer containing 10 mM Tris HCl, 1 mM EDTA, and 10 mM vanadyl nucleoside. Cells were lysed by adding 0.05 ml of 5% NP-40 (final concentration 0.5%), incubated 5 min on ice, and then another 0.05 ml was added for an additional 5 min. The nuclei were centrifuged at 1,500 rpm for 5 min and the supernatant was transferred into another tube. 2 vol of SDS buffer containing 200 mM Tris pH 9.0, 50 mM sodium chloride, 10 mM EDTA, and 0.5% SDS was added to a single volume of supernatant. The supernatant was deproteinized with phenol twice and then with chloroform isoamylalcohol. 0.1 vol of 3 M sodium acetate pH 5.2 and 2.5 vol of 95% ethanol was added and the RNA was precipitated overnight at -20°C.

RNA Analysis

RNA was analyzed by Northern blot analysis (14). The human 2',5'-(A)_n synthetase gene probe (clone 6-2) (42), was subcloned into pGEM for the production of riboprobes. Biodyne nylon blots were hybridized with T7 riboprobes. Cytoplasmic RNA was blotted onto Biodyne filters and pre-hybridized for 4 h at 59°C under the following conditions: 50% formamide, 6 \times SSC, 1% SDS, 0.1% Tween 20, 100 μ g/ml tRNA, and 10 mM DTT. Labeled RNA (10⁶ cpm/ml) was added to the hybridization solution for an additional 18 h. The filters were washed once with 2 \times SSC and 0.1% SDS for 30 min at room temperature, then with 2 \times SSC and 0.1% SDS at 65°C, and finally 0.2 \times SSC and 0.1% SDS at 65°C for 30 min.

Gel Electrophoresis

SDS (0.1%)-10% PAGE was performed according to Laemmli (27). Gels were run with molecular mass markers as follows: lysozyme (M_r = 14,300), β -lactoglobulin (M_r = 18,400), α -chymotrypsinogen (M_r = 25,700), albumin (M_r = 43,000), bovine serum albumin (M_r = 58,000), phosphorylase B (M_r = 97,400), and myosin (H chain) (M_r = 200,000). For analysis of proteins labeled with [³⁵S]methionine, the gel was impregnated with En³Hance, dried, and subjected to autoradiography at -70°C.

Assay of 2',5'-Oligoadenylate Synthetase Activity

2',5'-(A)_n synthetase activity was assayed by methods previously described (18). Cells were cultured for 20-21 h with or without 500 IU/ml of interferon- α or - γ . They were then extracted with 0.15% NP-40, clarified by centrifugation, and the supernatant fluid frozen at -70°C.

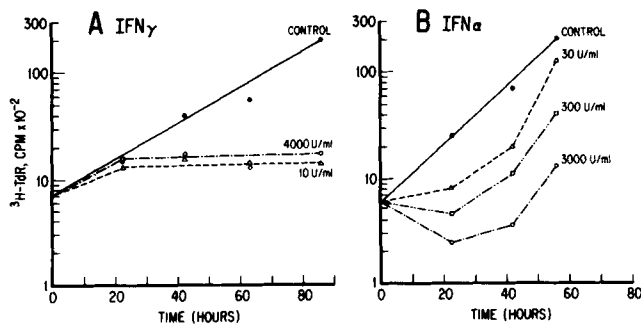


Figure 1. Growth of the parental line, J774.2, in IFN- γ and IFN- α . Cells were plated in medium alone (\bullet) or in varying concentrations of IFN- γ (A) or IFN- α (B). The results are presented as the average number of counts per minute (CPM) of tritiated thymidine ($^3\text{H-TdR}$) incorporated from triplicate wells.

Cell extracts were applied to polyI:polyC agarose columns (P. L. Biochemicals, Inc., Milwaukee, WI) and then equilibrated with 25 ml of 20 mM Hepes pH 7.5, 90 mM KCl, 8.5 mM magnesium acetate, 7 mM beta-mercaptoethanol, and 20% glycerol. The agarose column was then washed three times with 100 μl of cold reaction mixture containing the same buffer and 3 mM ATP, 0.2% creatine phosphate, 0.03% creatine phosphokinase, followed by the same mixture containing purified $^3\text{H-ATP}$. The column was incubated at 30°C for 15 h and then the supernatant was boiled for 3 min to destroy any nucleases. The 2',5'-(A)_n was separated from the unconverted ATP and DEAE columns by elution with 350 mM KCl at pH 7.5.

Assay of Double-stranded RNA-dependent PI/eIF-2 α Protein Kinase Activity

Cells were cultured for 21 h with or without IFN- γ (500 IU/ml) and then detergent extracts of cells were prepared as previously described (20). The reaction volume for the PI/eIF-2 α protein kinase assay was 0.05 ml and contained 100 mM KCl, 5.0 mM beta-mercaptoethanol, 10.0 mM magnesium chloride, 1 mM $^{32}\text{P-}\gamma\text{-ATP}$ at 10^4 cpm/pmol, 2 $\mu\text{g/ml}$ of double-stranded RNA (polyI:polyC), and 100 μg protein from cell extracts. Incubations were performed at 30°C for 30 min and phosphorylated proteins were analyzed by gel electrophoresis.

Results

Description of IFN Variants

The initial question we wished to address was whether the antiviral and antiproliferative effects of IFN- γ could be genetically dissociated, or were mediated by a common mechanism. IFN- γ exerts a powerful antiproliferative effect on the growth of cells of the J774.2 macrophage cell line (Fig. 1 A). Our approach to the question was to select variants of J774.2 genetically resistant to the antiproliferative effects of IFN- γ by cloning the cells in 4,000 IU/ml of IFN- γ without prior mutagenesis. The clones obtained were tested for their sensitivity to IFN- γ after growing 1 mo in IFN- γ -free medium, and five clones resistant to the antiproliferative effects of IFN- γ were isolated in two independent cloning experiments. The growth characteristics of some of these variants are illustrated in Fig. 2. Variants resistant to growth inhibition by IFN- γ were then analyzed for the induction of an antiviral state by IFN- γ as determined by reduction in yields of VSV (Table I). Two different classes of variants were found, based on their sensitivity to lysis by VSV after IFN- γ treatment: growth resistant to IFN- γ /protected against VSV lysis (G^r/V^p); and growth resistant to IFN- γ /susceptible to VSV lysis after IFN- γ treatment (G^r/V^s). G^r/V^p variants, represented by J7 γ R1, J7 γ R8, and J7 γ R10 were protected against VSV infection. G^r/V^s variants, represented by J7 γ R5 and J7 γ R7 were susceptible to VSV lysis. Of particular interest, one clone, J7 γ R5, that was susceptible to VSV was consistently protected by IFN- γ against the EMCV infection, and thus must have a functional IFN- γ receptor (Table II). A third class of variants, represented by a spontaneous mutant of J774.2 which arose after the cells were maintained for 1.5 yr in continuous culture, was susceptible to the antiproliferative effects of IFN- γ , but resistant to the antiviral effects. Cells growth inhibitable by IFN- γ yet susceptible to viral lysis (G^i/V^s) are represented by J774.2-7

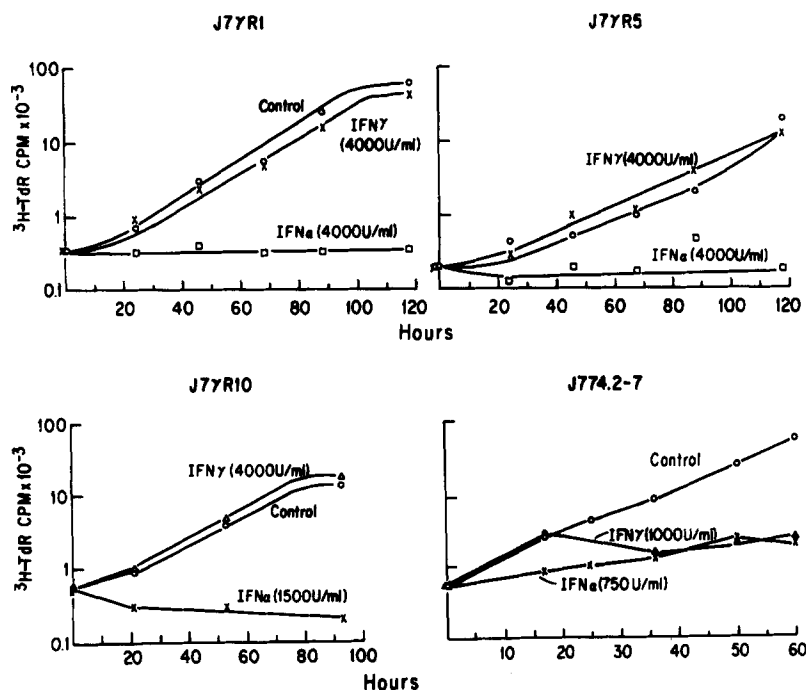


Figure 2. Effect of IFN- γ and IFN- α on the growth of variant clones. Cells were plated in medium alone (\circ) or in IFN- γ and IFN- α . Results of a representative experiment, which was reproduced at least five times, are presented. Each point represents the average counts per minute (CPM) of tritiated thymidine ($^3\text{H-TdR}$) incorporated from triplicate wells.

Table I. Reduction of Viral Yields in Parental and Variant Clones

Clone	Reduction of VSV yields (pfu/ml)	
	IFN- γ treatment	Reduction of pfu/ml
	<i>IU/ml</i>	
J774.2	10	10 ³
	100	10 ⁴
	1,000	10 ⁴
J7 γ R1	10	10 ⁴
	100	10 ⁴
	1,000	10 ⁴
J7 γ R5	10	<10 ¹
	100	<10 ¹
	1,000	<10 ¹
J7 γ R7	10	<10 ¹
	100	10 ¹
	1,000	10 ¹
J7 γ R8	10	10 ³
	100	10 ³
	1,000	nt
J7 γ R10	10	10 ²
	100	10 ³
	1,000	10 ³

Cell clones were incubated for 21 h in the presence or absence of the concentration of IFN- γ indicated. Each concentration was assayed in duplicate wells. Cells were then infected with VSV for 24 h after which the supernatants were assayed for viral yields. The results are presented as the reduction of viral yields when comparing a clone treated with IFN- γ with the same clone growing in medium alone. The titer of virus for the J774.2 and variant clones growing in medium alone was 1-5 \times 10⁷ pfu/ml. nt, not tested.

(Table II). The existence of mutants of these diverse phenotypes provides firm genetic evidence that the antiviral and antiproliferative effects of IFN- γ can be dissociated (summarized in Table III). Thus, there must be a divergence of the intracellular responses to IFN- γ beyond the receptor level allowing the antiviral and antiproliferative effects to be independently regulated within the cells.

Mechanism of Inhibition of Viral Replication by IFN- γ

The molecular mechanisms responsible for the antiviral effects of IFNs are not fully understood in any system, least of all for IFN- γ . With the possibility that our somatic cell mutants could provide insight into this important issue, the mutants defective in their antiviral response to IFN- γ were analyzed for the two known biochemical markers previously implicated in the antiviral actions of IFN- α and - β . A

Table II. Reduction in Viral Yields in Parental and Variant Clones in Response to IFNs

Type of IFN	J774.2		J7 γ R5		J7 γ R1		J774.2-7	
	VSV	EMCV	VSV	EMCV	VSV	EMCV	VSV	EMCV
IFN- γ	10 ⁴	10 ⁵	<10 ¹	10 ⁵	10 ⁴	10 ³	<10 ¹	10 ¹
IFN- α	10 ⁶	10 ⁶	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ⁶	10 ⁴

Viral yields were determined as described in Table I, in response to IFN- γ (1,000 IU/ml) and IFN- α (500 IU/ml).

Table III. Dissociation of the Antiviral and Antiproliferative Effects of IFN- γ

Clone	Phenotype	Growth inhibition	Reduction of VSV yields
		%	<i>pfu/ml</i>
J774.2	G ⁱ /V ^p	85	10 ⁴
J7 γ R5	G ⁱ /V ^s	0	<10 ¹
J7 γ R1	G ⁱ /V ^p	0	10 ⁴
J774.2-7	G ⁱ /V ^s	90	<10 ¹

Growth inhibitory effects of IFN- γ were derived from data in Figs. 1 and 2. Data for reduction of VSV yields were derived from Tables I and II.

P1/eIF-2 α protein kinase, in the presence of dsRNA, autophosphorylates itself and subsequently eukaryotic initiation factor-2, preventing the recycling of eIF-2 α which thereby inhibits polypeptide chain initiation. In contrast to the significant P1/eIF-2 α protein kinase activity induced by IFN- γ in the parental J774.2 line, the Gⁱ/V^s variant exhibited no demonstrable P1/eIF-2 α protein kinase activity, and the Gⁱ/V^s variant showed a diminished, but reproducible level of activity (Fig. 3). The Gⁱ/V^p (growth resistant, but antivirally protected by IFN- γ) cell line had similar P1/eIF-2 α kinase activity as the parental line, indicating that long-term growth of these cells in IFN- γ does not nonspecifically depress enzyme activity (Fig. 4). Two explanations for the lack, or decrease, in the Gⁱ/V^s and Gⁱ/V^s variants, respectively, of P1/eIF-2 α protein kinase activity, were considered. One possibility is that the mutant selected is a variant containing a defective P1/eIF-2 α protein kinase. Alternatively, it is possible that only the regulation of the kinase by IFN- γ is defective, the enzyme itself being fully functional. To distinguish between these two possibilities, we examined whether IFN- α could induce the antiviral state in these sets of variants. Since the same variants, summarized in Table II, were protected against VSV and EMCV infection by IFN- α , it was possible critically to test whether the Gⁱ/V^s and Gⁱ/V^s variants were capable of producing a functional dsRNA-dependent P1/eIF-2 α protein kinase in response to IFN- α . The results indicated that the Gⁱ/V^s and Gⁱ/V^s variants had an equal or greater amount of P1/eIF-2 α protein kinase activity after IFN- α treatment as compared to the parental clone (Figs. 5 and 6), indicating that the lack of antiviral activity in response to IFN- γ is most likely due to a defect in its regulation by IFN- γ .

The second biochemical mechanism associated with antiviral activity is the induction of the dsRNA-dependent 2',5'-(A)_n synthetase activity by the IFNs. This enzyme, in the presence of dsRNA, converts ATP into a 2',5'-(A)_n that activates a latent endonuclease capable of degrading viral RNA. The spectrum of results in our variants followed a pattern very similar to that obtained in P1/eIF-2 α protein kinase activity. While the parental line showed a 22-fold induction of 2',5'-(A)_n synthetase activity in response to IFN- γ treatment, the Gⁱ/V^s variant lacked any detectable 2',5'-(A)_n synthetase activity above background levels, while the Gⁱ/V^s variant had an approximately twofold higher basal level of 2',5'-(A)_n synthetase activity and a twofold increase above this level after IFN- γ treatment (Table IV). The Gⁱ/V^p variant had a similar increase in 2',5'-(A)_n synthetase activity in response to IFN- γ as the parental line. Interestingly, this variant had a fourfold higher basal level of 2',5'-(A)_n synthe-

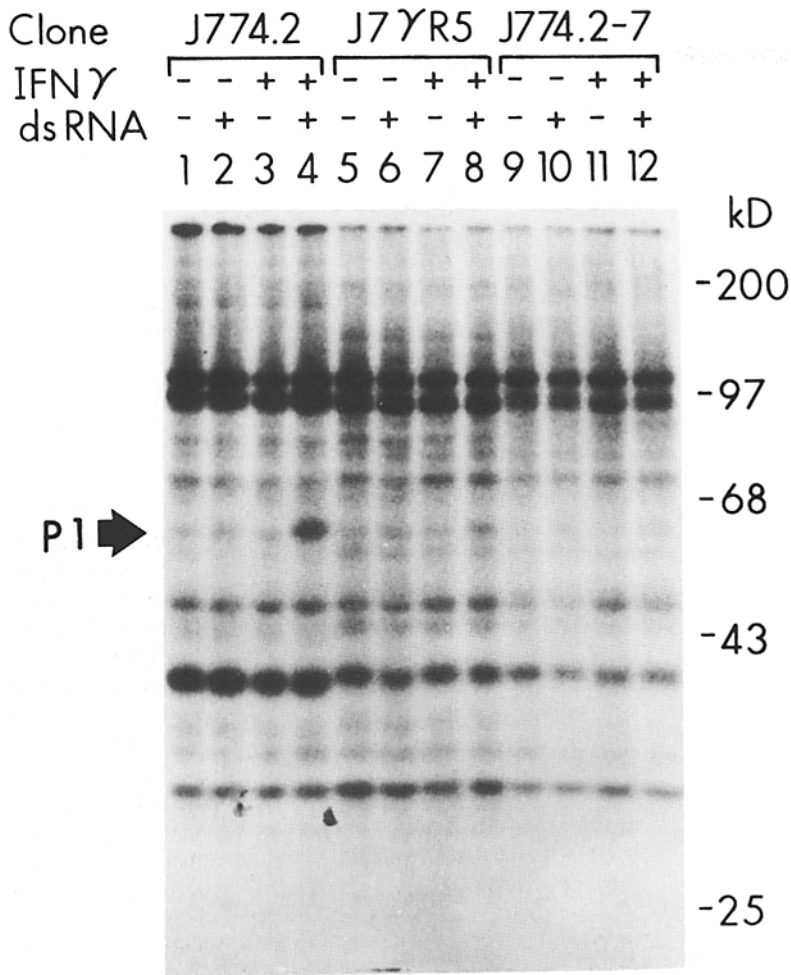


Figure 3. Assay of the P1/eIF-2 α protein kinase system in cell extracts of parental (J774.2, Gⁱ/V^s) and variant clones (J77R5, Gⁱ/V^s; J774.2-7, Gⁱ/V^s). Cells were incubated for 21 h in the absence or presence of IFN- γ (1,000 IU/ml) as indicated. Postmitochondrial cell extracts were prepared and incubated with [γ -³²P]ATP in the presence (even lanes) or absence (odd lanes) of 2.0 μ g/ml poly(I):poly(C) (dsRNA). The labeled proteins were analyzed by 10% SDS-PAGE. The positions and the molecular masses (kD) of the protein markers are indicated. The arrow points to the position of the protein P1 autophosphorylation. An autoradiogram of the dried gel is shown.

tase activity as compared with the parental line. Again, to differentiate whether the Gⁱ/V^s and Gⁱ/V^s variants contained a structural defect in the 2',5'-(A)_n synthetase or was defective in their regulation by IFN- γ , the induction of 2',5'-(A)_n synthetase activity after IFN- α treatment was measured. The 2',5'-(A)_n synthetase activity induced by IFN- α in these variants was fully comparable to that of the parental cell line, indicating again that there is a defect in the regulation of this enzyme by IFN- γ (Table IV). The results derived from the Gⁱ/V^s variant implicate for the first time a common regulatory pathway inducing both the 2',5'-(A)_n synthetase and P1/eIF-2 α protein kinase activities by IFN- γ , which must be distinct from both the pathway involved in regulating the antiproliferative effects mediated by IFN- γ , as well as the pathway involved in the antiviral response mediated by IFN- α . These results are consistent with the possibility that IFN- γ and IFN- α may use the same effector mechanisms to induce the antiviral state in J774.2 cells, and that only the regulation of these enzymes by IFNs differs.

To further delineate the nature of the defects in the Gⁱ/V^s and Gⁱ/V^s variants, induction of 2',5'-(A)_n synthetase mRNA levels in response to the IFNs were measured. Northern blot analysis after induction with IFN- γ for 8 h revealed that the 2',5'-(A)_n synthetase mRNA levels in the Gⁱ/V^s variant was comparable to the parental clone (Fig. 7). Interestingly, IFN- γ did not induce 2',5'-(A)_n synthetase mRNA levels in the

Gⁱ/V^s variant. Thus, the nature of the defects in the Gⁱ/V^s and Gⁱ/V^s variants are different, involving possibly transcriptional and posttranscriptional processes, respectively.

Mechanisms of Antiproliferative Effects of IFN- γ and IFN- α

If IFN- γ and IFN- α mediate their antiproliferative effects via different pathways, one might expect to find significant differences in their kinetics of action and dose-response curves. Therefore, we compared the sensitivity of J774.2 cells to the antiproliferative and antiviral effects of both IFN- γ and IFN- α . The antiproliferative and antiviral effects of IFN- α were dose dependent, but 100–1,000-fold greater amounts were required to inhibit cell growth than to engender the antiviral state (Fig. 1). In contrast, the concentration of IFN- γ required maximally to induce cells for the antiviral state was sufficient completely to arrest growth of the cells. Further, a kinetic difference exists between the two IFNs, in that IFN- γ -mediated effects appear only after 18–20 h, allowing the cells to divide at least once, whereas IFN- α can prevent the cells from dividing. These data are most consistent with the interpretation that the antiproliferative effects of IFN- α and IFN- γ are mediated by separate pathways.

A more rigorous genetic approach was undertaken to explore this question. We have previously reported that the an-

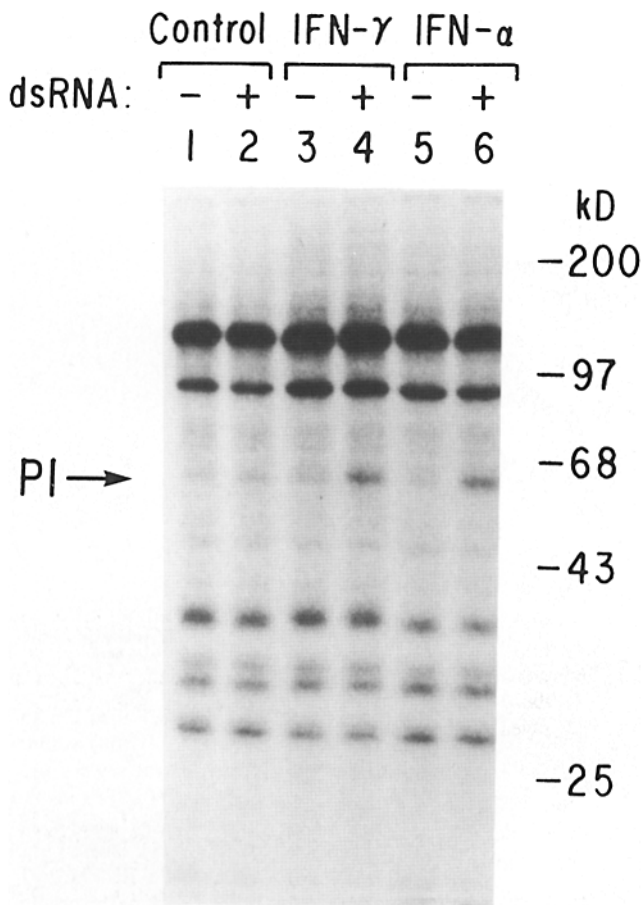


Figure 4. Assay of the PI/eIF-2 α protein kinase system in cell extracts of the Gⁱ/V^p (J7 γ R1) variant. Cells were incubated for 21 h in the absence or presence of IFN- γ (1,000 IU/ml), or IFN- α , as indicated. Postmitochondrial cell extracts were prepared and incubated with [γ -³²P]ATP in the presence (*even lanes*) or absence (*odd lanes*) of 2.0 μ g/ml poly(I):poly(C). The labeled proteins were analyzed by 10% SDS-PAGE. The positions and the molecular masses (*kD*) of the protein markers are indicated. The arrow points to the position of the protein PI autophosphorylation. An autoradiogram of the dried gel is shown.

tiproliferative effects of IFN- α on the J774.2 cell line was cAMP dependent (32, 39). To test whether the same was true for the IFN- γ antiproliferative effects, cholera toxin-resistant clones were tested for their sensitivity to IFN- γ -mediated growth inhibition. Cholera toxin-resistant clones have been previously shown to be defective in adenylate cyclase, and were found here to be fully sensitive to the antiproliferative effects of IFN- γ (Fig. 8 B). Furthermore, all the IFN- γ growth-resistant variants remain fully susceptible to growth inhibition by cholera toxin (Fig. 8 A). These results provide formal genetic evidence that the antiproliferative response of J774.2 induced by IFN- γ is cAMP independent. Finally, we examined whether the IFN- γ growth-resistant variants were sensitive or resistant to IFN- α -mediated antiproliferative effects. All of the IFN- γ growth-resistant mutants were fully sensitive to the antiproliferative effects of IFN- α (Fig. 2). These results provide genetic evidence that IFN- γ and IFN- α elicit antiproliferative responses in the J774.2 cell line through different signaling pathways.

Discussion

Three different classes of variants in the J774.2 macrophage cell line defective in their responses to IFN- γ have been derived from these studies (summarized in Table V). From a careful analysis of their phenotypes, we believe that several inferences regarding the regulatory mechanisms by which interferons act on these cells can be drawn. (a) IFN- α and IFN- γ mediate their antiproliferative activity via different signaling mechanisms. (b) The antiproliferative effect of IFN- γ , in contrast to IFN- α , is mediated by a cAMP independent pathway in these cells. (c) The mechanisms of the antiproliferative and antiviral activities of IFN- γ can be genetically dissociated. (d) dsRNA-dependent PI/eIF-2 α protein kinase and 2',5'-(A)_n synthetase, induced by both IFN- α and IFN- γ , may be involved in the antiviral effects against at least some RNA viruses, but the regulation of these enzymes by the different IFNs is mediated by different pathways. (e) The antiviral mechanisms induced by IFN- γ against VSV and EMCV have been genetically dissociated, and must be distinct at some level.

By way of commentary, it is not surprising that the seemingly similar antiproliferative response to IFN- α and IFN- γ in the same cloned cell line could be mediated via different regulatory signals. A wide variety of growth factors, hormones, lymphokines, and differentiation-inducing factors are known to act on the same structural gene by regulatory pathways involving different second messengers and *trans*-acting factors. For example, metallothionein genes are regulated by such diverse agents as the glucocorticoids, zinc and cadmium ions, cAMP, TPA, and IFN- α , through distinct

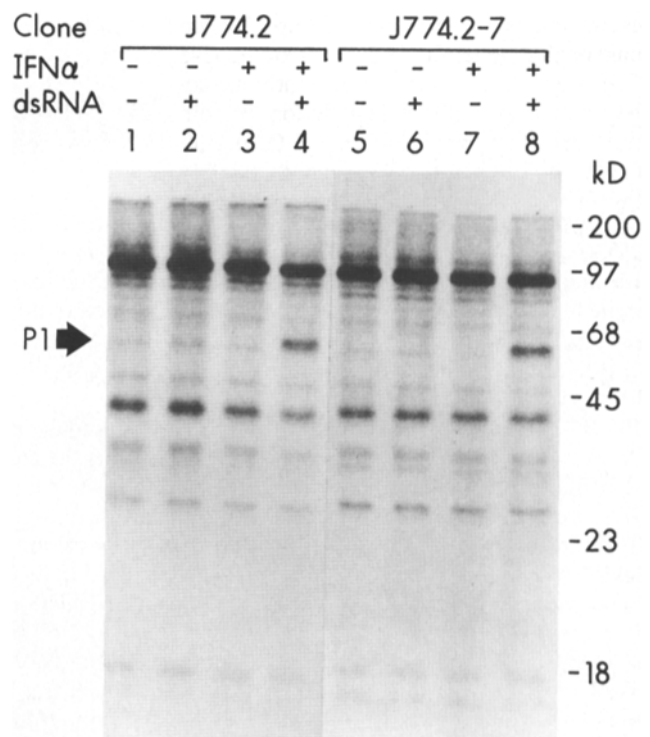


Figure 5. Assay of PI/eIF-2 α protein kinase activity in cell extracts of parental (J774.2, Gⁱ/V^p) and variant clone (J774.2-7, Gⁱ/V^s). Cells were incubated for 20 h in the presence or absence of IFN- α (200 IU/ml) and assayed as in Fig. 3.

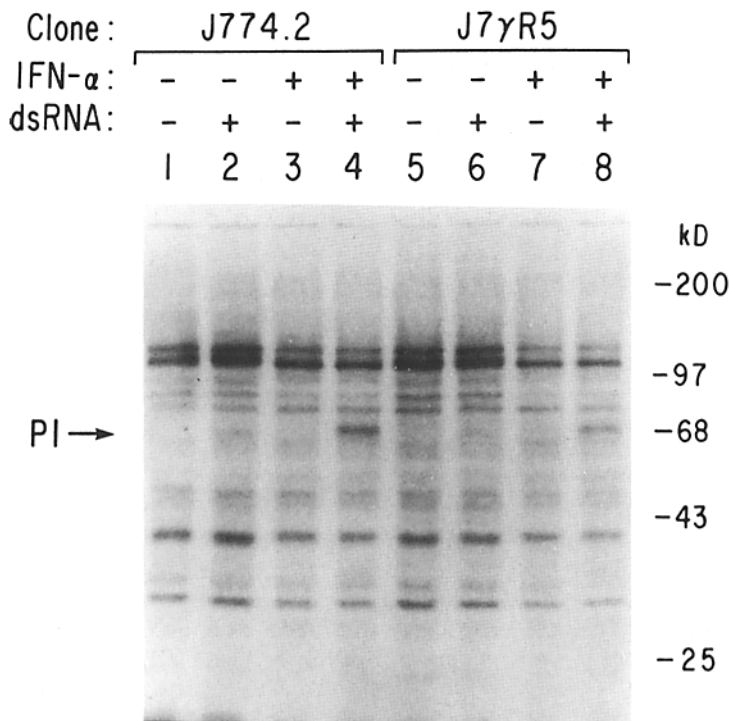


Figure 6. Assay of PI/eIF-2 α protein kinase activity in cell extracts of parental (J774.2, Gⁱ/V^p) and variant clone (J7 γ R5, Gⁱ/V^s). Cells were incubated for 20 h in the presence or absence of IFN- α (500 IU/ml) and assayed as in Fig. 3.

regulatory sequences in the 5' region of the gene (reviewed in 21). The proenkephalin gene has been shown to be transcriptionally activated by both the adenylate cyclase pathway via cAMP, as well as the phosphatidylinositol pathway via protein kinase C (11). Interestingly, the DNA sequences required for the regulatory effects for both cAMP and phorbol esters mapped to the same 37-bp 5' region of the human enkephalin gene. In bone marrow-derived macrophages, CSF-1 has been reported transiently to induce *c-fos* mRNA levels via phospholipid degradation, protein kinase C activation, and Ca²⁺ mobilization (6). Of particular interest, because of our previous finding that IFN- α exerts its antiproliferative effects in J774.2 macrophage cell lines via intracellular cAMP, it was similarly shown that dibutyryl cAMP or cholera toxin could lead to the stable induction of *c-fos* transcription in these same bone marrow macrophages. In this regard, we have preliminary genetic evidence indicating that the antiproliferative response of mouse macrophages to IFN- γ is mediated through a protein kinase C-dependent

pathway (Goldberg, M., L. S. Belkowski, and B. R. Bloom, manuscript submitted for publication). It will be fundamentally important to establish whether IFN- γ and IFN- α are using different signals to exert regulatory effects on the same effector genes that mediate the antiproliferative effects, e.g., transcriptionally inactivating the same proto-oncogenes, or whether IFN- α and IFN- γ affect the expression of distinct genes that lead to a common growth inhibitory effect.

The second question addressed in this work concerns whether different pathways were responsible for mediating the different biological responses to IFN- γ in the mouse macrophage cell line. We had previously derived variants that were resistant to the antiproliferative effects of IFN- α , and found them to be fully protected against lysis by VSV (33). In similar experiments, we have been able genetically to dissociate the antiviral and antiproliferative effects of IFN- γ by isolating variants specifically unresponsive to one, but not the other of these effects. Our results suggest for the first time the possibility that a common molecule regulates both the PI/eIF-2 α protein kinase and the 2',5'-(A)_n synthetase activities that must be distinct from molecules involved in regulating the antiproliferative responses to IFN- γ . The Gⁱ/V^s variant fails to show induction of both of these enzymatic activities after treatment with IFN- γ , even though the enzymes themselves are fully functional, as indicated from the induction of their activity by IFN- α . At what point does the bifurcation of the cellular responses to IFNs occur? The available evidence indicates the existence of a single biologically functional high affinity receptor for IFN- γ on many cell types including macrophages (1, 8), with which our preliminary Scatchard data on J774.2 are consistent. The receptor molecule itself could conceivably mediate different effects by binding to regulatory regions of different genes, as is the case for the glucocorticoid receptor (2); or, the different biologic effects could diverge subsequent to binding and triggering of

Table IV. Induction of 2',5'-Oligoadenylate Synthetase Activity after IFN Treatment

Clone	Phenotype	2',5' Oligoadenylate synthetase activity*		
		Control	IFN- γ 1,000 IU/ml	IFN- α 1,000 IU/ml
J774.2	G ⁱ /V ^p	3.5	101.5	186.7
J7 γ R5	G ⁱ /V ^s	8.0	18.6	149.9
J7 γ R1	G ⁱ /V ^p	14.3	102.5	336.7
J774.2-7	G ⁱ /V ^s	2.0	4.2	200.7

* Nanomoles ATP converted per milligram protein. Results are presented as the average 2',5'-(A)_n synthetase activity derived from two independent experiments except for the IFN- γ -treated J7 γ R5 clone and the IFN- α -treated J7 γ R1 clone in which the results represent a single experimental value.

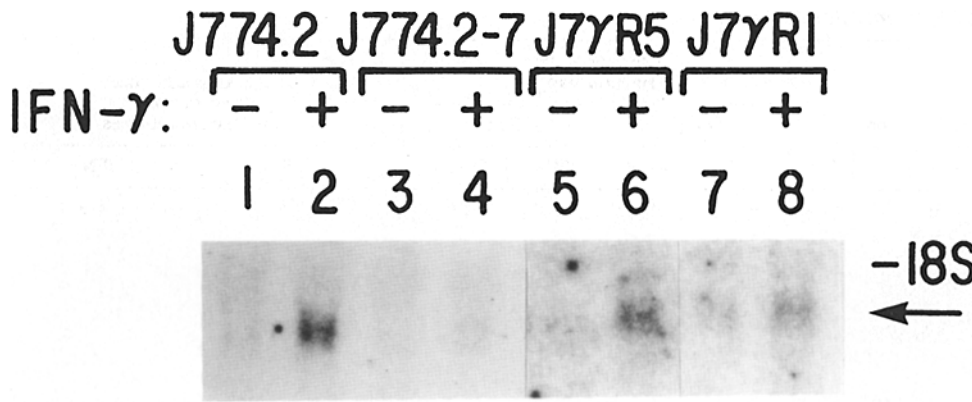


Figure 7. Induction of 2',5'-(A)_n synthetase mRNA in parental and variant clones. Cells were incubated in the absence or presence of 500 IU/ml of IFN- γ for 8.5 h. Cytoplasmic RNA was isolated and analyzed via Northern blot analysis. The Biodyne blot was hybridized with a ³²P-labeled riboprobe of the human 2',5'-(A)_n synthetase gene. An autoradiogram of the hybridized blot is shown. Lanes 1-4 and 5-8 are results from two different Northern blots, respectively,

that were combined to form a single figure. Exposure times for the two blots were normalized to equal intensity of the IFN- γ -induced 2',5'-(A)_n synthetase mRNA transcript in the parental line. The arrow points to the 2',5'-(A)_n synthetase mRNA transcript.

the receptor, for example, by generating transducing factors that regulate different genes in different ways. Evidence on a human macrophage cell line, U937 cells, indicates clearly that transcriptional activation of IFN- γ -inducible genes can occur by at least two separate pathways, protein kinase C dependent and independent (14). Further, cycloheximide can block the transcriptional induction of only a subset of IFN- α and IFN- γ -induced genes implicating multiple pathways by which the IFNs exert their regulatory control (7, 13, 16, 29). From several studies on gene regulation by IFN- α and IFN- β , transcriptional regulatory regions of IFN-inducible genes appear to be necessary, but not fully sufficient for the regulatory effects induced by the IFNs. For example, IFN- α -inducible genes contain homologies in their 5' flanking regulatory regions (10, 15, 37, 40, 41), although the cytoplasmic mRNA levels for some of these genes is determined by posttranscriptional mechanisms (16, 25). It is not yet clear at what level IFN- γ regulates the 2',5'-(A)_n synthetase and PI/eIF-2 α protein kinase enzymatic activity in the J774.2 cells.

Our attempts to clarify the molecular mechanisms responsible for the antiviral effects of the two classes of IFN in these experiments presented an unexpected finding regarding the specificity of antiviral mechanisms for different RNA viruses. It has long been known that there are differences in both kinetics and mode of action in the induction of the antiviral state by IFN- γ and IFN- α . IFN- α generally requires a shorter time to induce the antiviral state than IFN- γ , the thought being that IFN- γ requires more than one round of protein synthesis to induce the antiviral state (3, 12). The Mx gene product involved in natural resistance to influenza infection in mice is specifically induced by IFN- α , but not IFN- γ (45). On the other hand, it appears that adenoviruses can be inhibited by IFN- γ , but not IFN- α (30). While we have no formal proof that the antiviral effects of IFN- γ are mediated through dsRNA-dependent PI/eIF- α protein kinase or 2',5'-(A)_n synthetase, our results are consistent with the interpretation that either or both of those pathways may be involved in resistance to VSV and EMC replication, although our data would suggest that IFN- α and IFN- γ must utilize different pathways to regulate these genes.

Throughout the interferon literature, correlations between the antiviral state and enzymatic activities of 2',5'-(A)_n synthetase and PI/eIF-2 α protein kinase induced by IFNs represent a major theme. For example, the role of the PI/eIF-2 α protein kinase system in the antiviral response has been strengthened from the analysis of adenoviruses and vaccinia viruses that have devised strategies to overcome the activity of the PI/eIF-2 α protein kinase system (22, 44, 51, 52). Strong support for a role of the 2',5'-(A)_n synthetase derives from transfection studies in Chinese hamster ovary (CHO) cells. High constitutive expression of the 2',5'-(A)_n synthetase correlated to resistance to infection by a picornavirus such as mengo, but not to the rhabdovirus, VSV (9). Consistent with this general view, the J774.2-7 variant, which is fully growth inhibited by IFN- γ , exhibited no demonstrable PI/eIF-2 α protein kinase activity or 2',5'-(A)_n synthetase activity after IFN- γ treatment, and was not protected against VSV or EMCV. Our genetic evidence is corroborated by biochemical data that shows IFN- γ completely inhibits VSV protein synthesis in the J774.2 cell line (data not shown). This finding contrasts to a report that implicates primarily a posttranslational step in the IFN- γ -induced antiviral activ-

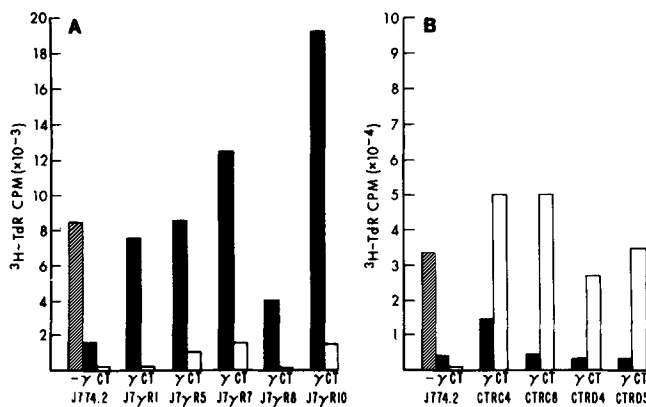


Figure 8. Growth of parental and variant clones in the absence (hatched bar) and presence of IFN- γ (1,000 U/ml, solid bar) and cholera toxin (250 ng/ml, open bar). Results are presented as the average number of counts per minute from triplicate wells. (A) IFN- γ -resistant clones were seeded with 2×10^3 cells/0.15 ml and pulsed with tritiated thymidine at 80 h. (B) Cholera toxin-resistant clones were seeded at 7.5×10^3 cells/0.15 ml and pulsed with tritiated thymidine at 65 h.

Table V. Summary of Parental and Variant Clone Phenotypes

Clone	Phenotype	Induction of the antiviral state							2',5' Oligoadenylate synthetase and eIF-2 kinase activities	
		Growth inhibition			IFN γ		IFN α		IFN γ	IFN α
		IFN γ	IFN α	C.T.	VSV	EMCV	VSV	EMCV		
J774.2	G ⁱ /V ^p	+	+	+	+	+	+	+	+++	++++
J774.2-7	G ⁱ /V ^s	+	+	+	-	-	+	+	-	++++
J7 γ R1	G ⁱ /V ^p	-	+	+	+	+	+	+	+++	++++
J7 γ R5	G ⁱ /V ^s	-	+	+	-	+	+	+	+	++++

ity in a human amniotic cell line (48, 49), raising the unhappy possibility that the antiviral effects of IFNs may be mediated by distinct molecular mechanisms in different cell types.

Another mutant defective in the IFN- γ -mediated antiviral response presents a unique phenotype. This variant, J7 γ R5, is resistant to the antiproliferative effects of IFN- γ and is protected against viral lysis caused by EMC virus, but not by VSV. This variant exhibits a small, but consistently detectable level of PI/eIF-2 α protein kinase activity and 2',5'-(A)_n synthetase activity induced by IFN- γ . A similar variant has been described in a human rhabdomyosarcoma cell line which is protected by human IFN- γ against EMCV, but not VSV (26). Constitutive expression of the cDNA encoding the 40-kD 2',5'-(A)_n synthetase recombinant protein in CHO cells resulted in reconstitution of resistance against a related picornavirus, mengo virus, but not VSV (9). The mechanism for this differential sensitivity of EMCV and mengo virus relative to VSV is unclear, although qualitative differences in mechanisms of replication of positive stranded viruses, such as EMC or mengo, relative to a negative-stranded virus, like VSV, may be important. Alternatively, the mutation that allowed this variant to grow in the presence of IFN- γ may also permit the transcriptional activation of the VSV genome, but not of the EMCV genome. Indeed, there is evidence that tubulin and microtubular-associated proteins can stimulate VSV transcription in vitro (19, 31). Curiously the J7 γ R5 variant, unlike the parental line, fails to undergo any morphologic changes in the presence of IFN- γ , and only poorly adheres to surfaces after treatment with IFN- γ .

Finally, we hope that the macrophage variants described, in addition to facilitating analysis of differential signalling pathways and possible effector mechanisms involved in cellular responses to IFNs, may provide unique possibilities for isolating genes encoding important effector proteins and *trans*-acting regulatory factors involved in interferon action.

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