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Establishment of the Antiviral State in α,β -Interferon-resistant Friend Cells Treated with γ -Interferon

INDUCTION OF 67-KILODALTON PROTEIN KINASE ACTIVITY IN ABSENCE OF DETECTABLE 2-5A SYNTHETASE*

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Treatment with murine γ -interferon (IFN) preparations of variant sublines of Friend leukemia cells resistant to the α,β IFN-induced antiviral state (Affabris, E., Jemma, C., and Rossi, G. B. (1982) *Virology* 120, 441-452; Affabris, E., Romeo, G., Belardelli, F., Jemma, C., Mechti, N., Gresser, I., and Rossi, G. B. (1983) *Virology* 125, 508-512) results in the establishment of a bona fide antiviral state. In fact, γ IFN preparations are able to induce a dose-dependent reduction of endogenous virus release and of vesicular stomatitis or encephalomyocarditis viruses yields (up to 1.5 log).

Under these experimental conditions, no inducible 2-5A synthetase activity is detectable in cell extracts. The 67-kDa protein kinase, uninducible by treatment with α,β IFN (up to 13,000 units/ml), is instead induced upon treatment with γ IFN at a similar rate of activity as in wild-type Friend leukemia cells, both when assayed in solution and after immobilization on poly(rI)·poly(rC)-agarose.

Interferons (IFNs¹) which promote the establishment of an antiviral state in uninfected cells are also able to affect several metabolic activities such as, for example (a) inhibition of cell proliferation and cell motility (1, 2); (b) modulation of immunological functions (3), i.e. antibody response, expression of several surface antigen, macrophage activation, and stimulation of natural killer cells; and (c) stimulation (4, 5) or inhibition (6, 7) of globin synthesis depending upon the range of IFN doses used. All IFN types (α,β and γ), despite the diversity of cell origin, inducing agents, and antigenic and molecular properties (8-10), are able to affect these biological phenomena. In particular, the development of the antiviral

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¹ The abbreviations used are: IFN, interferon; FLC, Friend leukemia cells; VSV, vesicular stomatitis virus; EMCV, encephalomyocarditis virus; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; pfu, plaque-forming units; SDS, sodium dodecyl sulfate; FLV, Friend leukemia virus. 2-5A designates a series of oligonucleotides of the general formula pppA(2'p5'A)_n produced by the IFN-induced 2-5A synthetase.

response in IFN-treated cells has been shown to be often accompanied by the induction of at least two enzymatic pathways. The first one is a 2',5'-oligoadenylate synthetase capable of generating 2',5'-linked oligoadenylates from ATP, upon activation by double-stranded RNA (11, 12). These oligomers in turn activate RNase L, a latent endoribonuclease that cleaves single-stranded RNAs at preferred sites (13-20). The second enzymatic activity is a 67-kDa protein kinase that, if activated by double-stranded RNA, phosphorylates and thereby impairs the activity of the α peptide chain of eukaryotic initiation factor 2 (21-23). The activities of both protein kinase and 2-5A synthetase may account for the inhibition of overall protein synthesis that has been detected in lysates from IFN-treated cells.

In the past few years, we have been studying the effects of mouse IFN administration on Friend leukemia cells (FLC) and clones thereof which are resistant to the α,β IFN-induced antiviral state. FLC are mouse nucleated erythroid precursors able to differentiate *in vitro* when treated with various agents including dimethyl sulfoxide (24). α,β IFN is able to enhance or inhibit erythroid differentiation of wild-type FLC when administered at low or high doses, respectively (7, 26). The variant clones are resistant to the enhancement of erythroid differentiation caused by low doses of pure α,β IFN, but high doses thereof, which do not decrease Friend murine leukemia virus release and vesicular stomatitis virus (VSV) yields, inhibit erythroid differentiation of the resistant clones tested (27).

Both α,β IFN-resistant and -sensitive FLC clones show a specific saturable binding site for mouse α,β IFN with similar affinity constant. However, the variant clones appear to be unable to "induce" the 2-5A synthetase pathway even upon treatment with 10,000 units/ml α,β IFN (28).

In this report, we describe data showing that treatment with γ IFN of the variant clones results in the establishment of a bona fide antiviral state showing that γ - and α,β IFNs may have different interaction with the same cell system. Furthermore, the antiviral state induced by γ IFN takes place in the absence of any detectable 2-5A synthetase activity. As for the 67-kDa protein kinase activity, the variant FLC clones do induce it upon exposure to 20-500 units/ml γ IFN, whereas no such activity is detectable when the same cells are exposed to up to 13,000 units/ml α,β IFN.

EXPERIMENTAL PROCEDURES

Materials—Adenosine 5-[γ -³²P]triphosphate triethylammonium salt (3000 Ci/mmol) and [*methyl*-³H]thymidine 5'-triphosphate (60

Ci/mmol) were obtained from the Radiochemical Centre, Amersham, United Kingdom. Poly(rI)·poly(rC) and poly(rI)·poly(rC)-agarose were from P-L Biochemicals. Benzidine dihydrochloride was from Ciba. Poly(A) was from Sigma. Oligo(dT)₁₂₋₁₈ from Collaborative Research Inc., Waltham, MA. All other chemicals and solvents were of reagent grade.

Cells—FLC were grown in RPMI 1640 medium supplemented with 5% fetal calf serum and antibiotics. L929 and C-243-3 mouse cells were grown in minimum essential medium supplemented with 5% fetal calf serum and newborn calf serum, respectively.

Viruses—VSV (Indiana strain) and encephalomyocarditis virus (EMCV) stocks, obtained by infecting L929 cell monolayers with low multiplicity of infection (0.1 pfu/cell), were titrated by plaque assay on the same cells. Titers ranged between $10^{8.5}$ and $10^{9.0}$ pfu/ml. Newcastle disease virus, strain F, stocks were obtained by infecting 10-11-day-old embryonated eggs in allantoic cavity. The virus was harvested after 3 days of incubation at 35 °C and titered by hemagglutination assay. Titers ranged between 300 and 600 hemagglutinating units/ml.

Reverse Transcriptase Assay—The assay was performed in a final volume of 100 μ l containing 20 mM Tris-HCl, pH 7.8, 60 mM NaCl, 1 mM MnCl₂, 5 mM dithiothreitol, 0.015% Nonidet P-40, 50 μ g/ml poly(A), 5 μ g/ml oligo(dT)₁₂₋₁₈, 2 μ M TTP. Samples of clarified supernatants, ranging from 5 to 30 μ l, with additional medium containing 5% fetal calf serum to a total volume of 30 μ l, were added to the reaction mixture. To rule out artifactual results due to the presence of inhibitors in the fluids, the enzymatic activity was derived from the linear portion of the curves. Values were expressed as picomoles of TTP incorporated into acid-insoluble materials/ 10^6 cells.

Interferons—Mouse α , β IFN was essentially prepared by infecting mouse C-243-3 cells with Newcastle disease virus as described (29). The specific activities of α , β IFN preparations averaged 10^5 units/mg of protein. Amounts of IFN are given throughout this paper in mouse units, i.e. the amount of IFN reducing by 50% plaque production by VSV. This unit equals 4 research reference units of the National Institutes of Health Search Standard preparation code G-002-904-511 whose titer was 12,000 international units/ml when reconstituted. Details about this standard preparation are reported in Research Reference Reagents Note 15 (56).

Mouse γ IFN preparations were generous gifts of different laboratories. γ IFN obtained from Drs. H. Heremans and A. Billiau (Rega Institut, Leuven, Belgium) was produced in cultures of mouse spleen cells stimulated with *Staphylococcus aureus* lysate and partially purified by silicic acid (30). The preparations obtained from Drs. F. Marcucci and H. Kirchner (Institut für Virusforschung, Heidelberg, West Germany) were produced in cultures of mouse spleen cells stimulated with concanavalin A and partially purified by (NH₄)₂SO₄ precipitation (31). The preparation from Dr. S. Landolfo (Istituto di Microbiologia, Università di Torino, Italy) was produced in cultures of a T-cell tumor clone (L12-R4) stimulated with phorbol myristate acetate and partially purified by (NH₄)₂SO₄ precipitation (32). Specific activities ranged between 10^4 and 10^5 units/mg of protein. In absence of an international standard, amounts of γ IFN are derived from the same assay described for α , β IFN, i.e. are given in laboratory units. Recombinant murine γ IFN from *Escherichia coli* was a generous gift of Böehringer Institut (specific activity 7.2×10^6 units/mg of protein).

Cell Cloning in Soft Agar Medium—Cells serially (10-fold) diluted in 5 ml of RPMI 1640 supplemented with 23% fetal calf serum, then added with melted Bacto-Agar (2%, 1 ml) to obtain a final 0.33% Bacto-Agar concentration, were plated in a 60-mm Petri dish. After medium solidification, cultures were incubated at 37 °C in a humidified 5% CO₂ atmosphere. Clones were counted 6-8 days later.

Cell Extracts—Extracts (postmitochondrial supernatant fractions) were prepared at 0-4 °C from packed cells lysed into 1.5 volumes of homogenization buffer (10 mM Tris-HCl, pH 7.5, 7 mM EtSH, 10 mM KCl, 1.5 mM MgOAc, 0.5% Nonidet P-40). The homogenate was brought to 3 mM Tris-HCl, pH 7.5, 90 mM KCl, 3.5 mM MgOAc, and 7 mM EtSH and centrifuged at $10,000 \times g$ for 10 min. The supernatant (S-10) was either assayed immediately or stored in aliquots at -80 °C.

Assay of 2',5'-Oligoadenylate Synthetase Activity—30 μ l of cell extract were incubated with 20 μ l of poly(rI)·poly(rC)-agarose for 1 h at room temperature. The enzyme bound to the poly(rI)·poly(rC) was washed three times with 200 μ l of 20 mM Hepes, pH 7.6, 8.5 mM KCl, 50 mM MgOAc, 50 mM KCl, 7 mM EtSH, 20% (v/v) glycerol and spun at $10,000 \times g$ for 20 s. The enzyme was then incubated with

20 μ l of the same buffer containing 3 mM ATP (neutralized with NaOH) overnight at 30 °C with gentle shaking. The yield of 2-5A was assayed by high pressure liquid chromatography analysis. The high pressure liquid chromatography analysis was carried out on a Lichrosorb RP-18 column (Merck) run in 50 mM ammonium phosphate, pH 7.0, at 1 ml/min with a 25-min linear gradient of 50:50 methanol:water to reach a final value of 25% (33).

Assay of Protein Kinase Activity (in Solution)—5 μ l of cell extract were incubated for 15 min at 37 °C in 10 mM Hepes buffer, pH 7.6, 90 mM KCl, 10 mM Mg(OAc)₂, 1 mM dithiothreitol, 100 μ M ATP, 20% (v/v) glycerol, 2 μ Ci of [γ -³²P]ATP, with or without poly(rI)·poly(rC) (1 μ g/ml), in a final volume of 25 μ l. After incubation, 2 volumes of electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 100 mM EtSH, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 0.005% (w/v) bromophenol blue) were added to the kinase assay mixture which was then heated at 95 °C for 15 min and analyzed by electrophoresis on 9% polyacrylamide slab gels containing 0.5% (w/v) SDS (34). The phosphorylated proteins were detected by autoradiography.

Assay of Protein Kinase Activity (in Solid Phase)—Cell extracts were first incubated for 15 min at 4 °C in the presence of heparin (100 units/ml) before addition of poly(rI)·poly(rC)-agarose. The poly(rI)·poly(rC)-agarose-bound enzyme was prepared by mixing cell extracts (30 μ l) with poly(rI)·poly(rC)-agarose (20 μ l) previously equilibrated with 10 mM Hepes buffer, pH 7.6, 90 mM KCl, 10 mM Mg(OAc)₂, 1 mM dithiothreitol, 20% (w/v) glycerol, incubating for 15 min at 37 °C, and then washing extensively with the 90 mM KCl buffer. The poly(rI)·poly(rC)-agarose-bound enzyme fraction (20 μ l) was incubated for 60 min at 37 °C with 20 μ l of the Hepes buffer in the presence of 2 μ Ci of [γ -³²P]ATP. After incubation, 2 volumes of electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 100 mM 2-mercaptoethanol, 10% (v/v) glycerol, 2% (w/v) SDS, 0.005% (w/v) bromophenol blue) were added to the kinase assay which was then heated at 95 °C for 15 min and centrifuged, and the supernatant solution was analyzed by electrophoresis on 9% polyacrylamide slab gels containing 0.5% (w/v) SDS (34). The phosphorylated proteins were detected by autoradiography.

RESULTS

Induction of the Antiviral State in α , β IFN-resistant FLC Clones Treated with Murine γ IFN—As shown in the dose-response curves of Fig. 1, a 20-h treatment of wild-type FLC (745A clone) with a crude murine γ IFN preparation results in a 3-log reduction of VSV yield (Fig. 1, lower panel). The same treatment is able to reduce VSV yield in two α , β IFN-resistant FLC clones, but at a lesser extent than in wild-type cells. Release of FLV is also affected by the same treatment (Fig. 1, upper panel). Table I shows the extent of reduction of VSV yield and reverse transcriptase activity in wild-type and five α , β IFN-resistant FLC clones treated for 20 h with 200 units/ml murine γ -IFN preparations.

Specificity of the Antiviral State Induced by γ IFN in FLC Variants—The results described above were obtained with crude murine γ IFN preparations. Control experiments have obviously been performed to demonstrate that the observed reduction of virus yield is indeed the consequence of the induction of a bona fide antiviral state. Steps taken were as follows.

1) Data obtained with the murine γ IFN preparations from Drs. Marcucci and Kirchner (and presented in Fig. 1) were perfectly superimposable (data not shown) with those obtained when batches of murine γ IFN prepared by various procedures in different cell lines (see "Experimental Procedures") were also used.

2) VSV yields and reverse transcriptase activity were reduced in culture supernatants also following a 20-h treatment of sensitive and resistant cells with a preparation of recombinant mouse γ IFN (7.2×10^6 units/mg of protein) (Fig. 2).

3) Recently, Tying *et al.* (35) have shown that even 30-fold purified mouse γ IFN preparations have direct cytolytic activity against a number of tumor and normal cells. Thus,

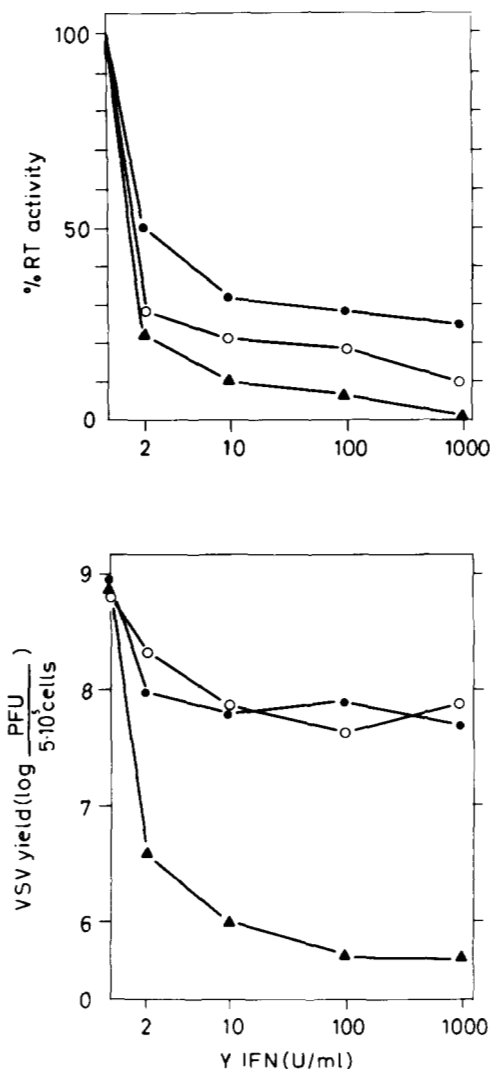


FIG. 1. Dose-response curves of VSV yield (lower panel) and FLV release (upper panel) in α,β IFN-sensitive and -resistant FLC treated with murine γ IFN. Cells seeded at 5×10^5 ml in the presence of graded doses of a murine γ IFN preparation obtained from Drs. F. Marcucci and H. Kirchner (see "Experimental Procedures"), were harvested 20 h later and gently centrifuged. Culture supernatants were analyzed for reverse transcriptase (RT) activity (see "Experimental Procedures"), whereas cell pellets were infected with 1 pfu/cell VSV (1 h, 37 °C in water bath), and then washed three times with culture medium and reseeded at 5×10^5 cells/ml. Each culture was harvested 8 h after infection. Virus yields in supernatants were determined by plaque assay on confluent L929 cell monolayers. \blacktriangle , 745A; \circ , MRI 745 3C18; \bullet , MRI 745 1C11. Control values (100%) for reverse transcriptase activity (picomoles of TTP/ 10^6 cells) were as follows: 745A = 40, MRI 745 3C18 = 46, and MRI 745 1C11 = 50.

the partial reduction of VSV yield and reverse transcriptase activity observed in cultures of the α,β IFN-resistant FLC clones could be ascribed to cell toxicity exerted by the crude preparations of γ IFN used. Cells of wild-type and two variant FLC clones, treated for 20 h with 500 units/ml murine γ IFN, were washed out of γ IFN and then plated in soft agar medium. Data in Table II (left) show that the plating efficiency of α,β IFN-sensitive and -resistant FLC do not significantly differ as a consequence of γ IFN treatment.

4) Since it has been reported (36) that several cell lines exhibit a range of sensitivity to IFN when infected with different RNA lytic viruses (*i.e.* VSV, EMCV, Mengo, or

TABLE I

Antiviral activity induced by 20-h treatment with 200 units/ml murine α,β - or γ IFN

Cells were seeded at 5×10^5 /ml with or without 200 units/ml murine α,β - or γ IFN. 20 h later, reverse transcriptase activity was evaluated in culture supernatants (see "Experimental Procedures"), and VSV yield was determined by infecting cell pellets as described in the legend to Fig. 1. Control values \pm S.E. for VSV yields (*i.e.* log pfu/ 5×10^5 cells) and for reverse transcriptase (RTA) activity (*i.e.* picomoles of TTP/ 10^6 cells) were as follows: 745A, 8.65 ± 0.12 (42 ± 11); MRI 745 1C11, 8.8 ± 0.1 (46.5 ± 6); MRI 745 3C18, 8.56 ± 0.14 (4 ± 7); MRI 745 7, 8.87 ± 0.14 (45 ± 13); MRI 745 8, 8.86 ± 0.1 (50 ± 13); and MRI 745 9, 8.85 ± 0.1 (42 ± 10). Values are means of three different experiments performed with the three different crude murine γ IFN preparations described under "Experimental Procedures."

Cell clones	Friend virus release		Reduction of VSV yield	
	α,β IFN	γ IFN	α,β IFN	γ IFN
	% RTA \pm S.E.		Δ log(pfu/ 5×10^5 cells) \pm S.E.	
745A	12.5 ± 1.5	16 ± 2.6	3.44 ± 0.12	2.99 ± 0.25
MRI 745 1C11	91 ± 11	36 ± 6	0.07 ± 0.02	1.02 ± 0.34
MRI 745 3C18	81 ± 9	35 ± 7	0.14 ± 0.02	1.22 ± 0.19
MRI 745 7	110 ± 13	47 ± 12	0.08 ± 0.02	0.84 ± 0.17
MRI 745 8	96 ± 6	44 ± 6	-0.17 ± 0.01	1.47 ± 0.74
MRI 745 9	92 ± 13	40 ± 12	-0.02 ± 0.01	0.92 ± 0.13

reovirus), virus yields were titrated in aliquots of supernatants from cell cultures infected with 1 pfu/cell either VSV or EMCV. In fact, these viruses are characterized by distinct replication cycles, as VSV is an enveloped, RNA(-) virus and EMCV is a naked, RNA(+) virus. Data in Table II (right) show that both VSV and EMCV yields were markedly reduced in cells treated with γ IFN.

5) A similar reduction of VSV yield was also observed when (a) cells were infected with 10 pfu/cell or (b) besides testing culture supernatants, virus production was also evaluated in freeze-thawed aliquots of the same culture conditions (data not shown). The latter experiment rules out the possibility that the partial reduction of VSV yield observed in the resistant cell clones was due to accumulation of infectious virus particles inside the cells.

These data are taken to show that the observed γ IFN-induced reduction of virus replication in α,β IFN-resistant FLC is due to the establishment of a bona fide antiviral state.

IFN-induced 2-5A Synthetase Activity—To investigate whether the sensitivity to γ IFN of α,β IFN-resistant FLC variants was the result of the induction of the 2-5A synthetase pathway (uninduced by α,β IFN treatment) (28), 2-5A synthetase activity was analyzed in sensitive and resistant FLC treated with γ IFN. The enzyme was assayed in presence of poly(rI)·poly(rC)-agarose (see "Experimental Procedures"). The agarose-bound enzyme is stable and free of any phosphatase activity which may be present in cell extracts (37), thus allowing synthesis and accumulation of 2-5A. Similarly to the data previously shown for α,β IFN, analysis of 2-5A synthetase activity reveals that treatment of 10^6 cells/ml with 500 units/ml γ IFN for 20 h induces a 5-fold increase of oligomer concentration in the reaction mixture from FLC. No inducibility of this enzymatic activity is detectable in the α,β IFN-resistant clones. The 2-5A synthetase-specific activity at different doses of γ IFN is presented in Fig. 3 for wild-type cells and for MRI 745 3C18 clone. The results obtained for the other α,β IFN-resistant FLC variants are closely superimposable (data not shown).

IFN-induced 67-kDa Protein Kinase Activity—Since the 2-5A synthetase pathway appears to be blocked in α,β IFN-resistant FLC clones given any IFN type, it was of obvious interest to check whether the other major IFN-induced en-

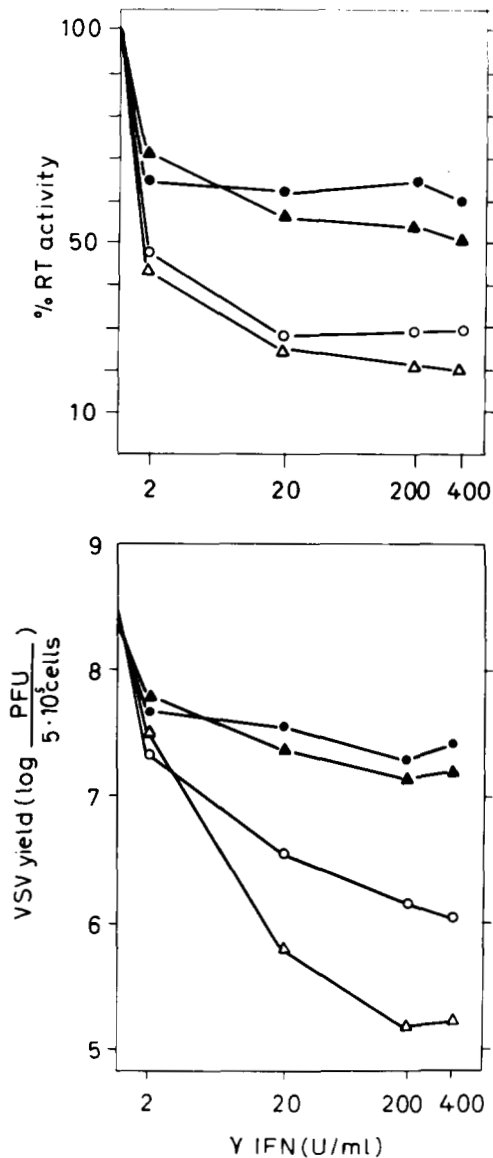


FIG. 2. Dose-response curves of VSV yield (lower panel) and FLV release (upper panel) in α, β IFN-sensitive and -resistant FLC treated with recombinant mouse γ IFN. Cells seeded at 10^6 /ml in the presence of graded doses of either crude murine γ IFN (triangles) or recombinant mouse γ IFN (circles) were harvested and gently centrifuged. 20 h later, reverse transcriptase (RT) activity was evaluated in culture supernatants (see "Experimental Procedures") and VSV yield was determined by infecting cell pellets as described in the legend to Fig. 1. \circ, Δ , 745A; \bullet, \blacktriangle , MRI 745 3C18. Control values (100%) for reverse transcriptase activity (picomoles of TTP/ 10^6 cells) were: 745A = 80 and MRI 745 3C18 = 55.

zyme, the double-stranded RNA-dependent 67-kDa protein kinase, was induced in the variant cells exposed to γ IFN. To validate detection of kinase activity, this was determined both in solution (Fig. 4) and by immobilization on poly(rI)-poly(rC)-agarose (Fig. 5). When assayed in solution, the characteristic double-stranded RNA-dependent increase of 67-kDa band phosphorylation is clearly visualized in cell extracts of wild-type and variant FLC treated with 500 units/ml γ IFN. In the former cell extract, the kinase was activated also when the cells had been exposed to 200 units/ml α, β IFN. The kinase was also immobilized on poly(rI)-poly(rC)-agarose as described by Hovanessian and Kerr (37) and evaluated for its capacity to phosphorylate the 67-kDa endogenous substrate.

TABLE II

Plating efficiency in soft agar versus reduction of VSV and EMCV yields in α, β IFN-sensitive and -resistant Friend cells treated with 500 units/ml γ IFN

Cells were seeded at 5×10^6 cells/ml with or without 500 units/ml murine γ IFN. 20 h later, cells were infected with 1 pfu of VSV or EMCV as described in the legend to Fig. 1 to evaluate virus yields. Before virus infection, an aliquot of each culture condition was washed three times, and 1000, 100, and 10 cells were plated in triplicate in soft agar (0.33%) medium to evaluate plating efficiencies. Values are means of three different experiments performed with three different murine γ IFN preparations. Control values for VSV and EMCV yield expressed as pfu/ 5×10^5 cells were as follows: 745A, 8.79 ± 0.04 (7.41 ± 0.02); MRI 745 1C11, 8.57 ± 0.15 (7.35 ± 0.06); and MRI 745 3C18, 8.73 ± 0.03 (7.44 ± 0.08).

Cell clones	Plating efficiency		Reduction virus yield	
	-IFN	+IFN	VSV	EMCV
	% \pm S.E.		$\Delta \log(\text{pfu}/5 \times 10^5 \text{ cells}) \pm$ S.E.	
745A	82 ± 12	52 ± 19	2.65 ± 0.57	1.76 ± 0.7
MRI 745 1C11	56 ± 12	43 ± 16	1.22 ± 0.5	1.85 ± 0.54
MRI 745 3C18	75 ± 18	57 ± 26	1.41 ± 0.52	1.86 ± 0.06

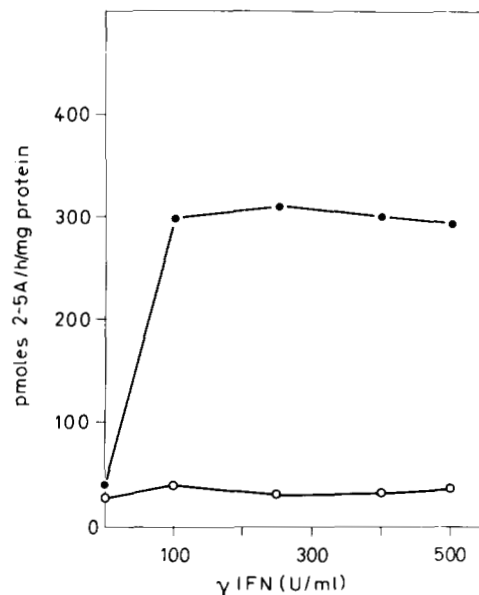


FIG. 3. Induction of 2-5A synthetase as a function of γ IFN dosage. 745A cells (\bullet) and MRI 745 3C18 (\circ) seeded at 10^6 /ml were treated with varying amounts of mouse γ IFN for 20 h. Cell extracts and 2-5A synthetase assay were performed as described under "Experimental Procedures." The yield of 2-5A was assayed by high pressure liquid chromatography analysis.

Here again, the results indicate that the enzyme activity is similarly induced in lysates from both wild-type and variant FLC treated with γ IFN, whereas no inducibility thereof is detectable in FLC variants treated with 13,000 units/ml α, β IFN. The pattern of phosphorylation (solid phase assay) of extracts from 745A, 3C18, 1C11, 7, 8, and 9 treated with α, β - or γ IFN is presented in Fig. 5.

DISCUSSION

Recently, cell variants (38, 39) have been described which are partially or totally sensitive to γ IFN treatment, despite their α, β IFN-resistant phenotype. For example, α, β IFN-resistant murine leukemia L1210 cells, which lack high affinity binding receptor sites for α, β IFN (40), are sensitive to γ IFN. Human fibroblasts show different specific receptors for α - and β - versus γ IFNs (41). Competition binding experiments

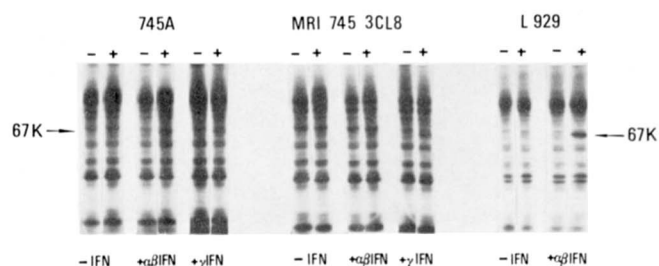


FIG. 4. Autoradiography of SDS-polyacrylamide gel showing phosphorylation reaction products (kinase assays in solution) of S-10 cell-free extracts of sensitive (745A) and resistant (MRI 745 3C18) FLC treated with α,β - or γ IFN. FLC were seeded at 5×10^6 /ml and grown in the presence of mouse α,β IFN (200 units/ml) or mouse γ IFN (200 units/ml) for 20 h. L929 cells were seeded at 3.5×10^4 /cm² 1 day before addition of mouse α,β IFN (200 units/ml) and treated for 20 h. Cell extracts, kinase assays in solution, and polyacrylamide gel electrophoresis were performed as described under "Experimental Procedures." The patterns of phosphorylation of the cell extracts are shown in the absence (–) or presence (+) of poly(rI)·poly(rC). The position of the M_r 67,000 polypeptide (arrow) is indicated on both sides of the gels. L929 cells are taken as a positive internal control. Bovine serum albumin (68,000), ovalbumin (45,000), and chymotrypsinogen (25,000) were used as molecular weight standards.

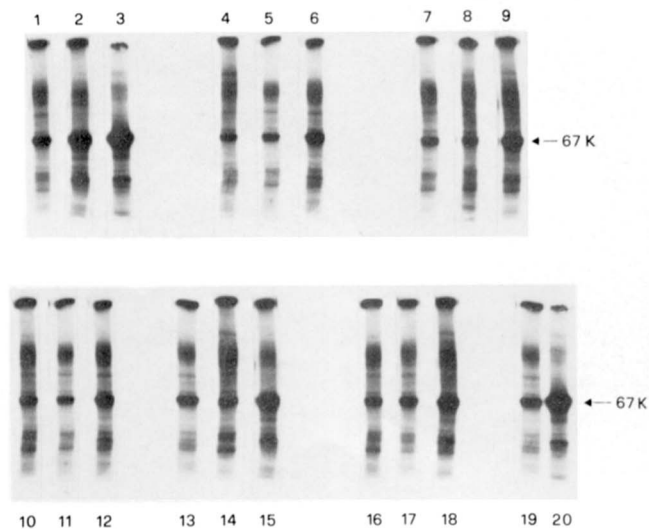


FIG. 5. Autoradiography of SDS-polyacrylamide gel showing phosphorylation reaction products (solid phase kinase assay) of S-10 cell-free extracts of sensitive and resistant FLC treated with α,β - or γ IFN. Cells were seeded at 10^6 /ml and grown in the presence of mouse α,β IFN (13,000 units/ml) or mouse γ IFN (500 units/ml) for 20 h. L929 cells were seeded at 3.5×10^4 /cm² 1 day before addition of mouse α,β IFN (200 units/ml) and treated for 20 h. Cell extracts, kinase assay, and polyacrylamide gel electrophoresis were performed as described under "Experimental Procedures." Assay mixtures applied to each lane were as follows: 1, 745A; 2, 745A + α,β IFN; 3, 745A + γ IFN; 4, MRI 745 1C11; 5, MRI 745 1C11 + α,β IFN; 6, MRI 745 1C11 + γ IFN; 7, MRI 745 3C18; 8, MRI 745 3C18 + α,β IFN; 9, MRI 745 3C18 + γ IFN; 10, MRI 745 7; 11, MRI 745 7 + α,β IFN; 12, MRI 745 7 + γ IFN; 13, MRI 745 8; 14, MRI 745 8 + α,β IFN; 15, MRI 745 8 + γ IFN; 16, MRI 745 9; 17, MRI 745 9 + α,β IFN; 18, MRI 745 9 + γ IFN; 19, L929; 20, L929 + α,β IFN. The position of the M_r 67,000 polypeptide (arrow) is indicated to the right of the gels. L929 cells are taken as a positive internal control. Molecular weight standards are as described in the legend to Fig. 4.

with radioiodinated purified human or mouse IFN (42, 43) confirmed the existence of distinct membrane receptors for distinct IFN types. In addition, some human lymphoblastoid cell lines (Daudi and PEHR-1) are sensitive to the antiviral

and anticellular effects of human α - and β IFNs, whereas they are resistant to the antiviral and anticellular actions of human γ IFN (44). Conversely, only recombinant γ IFN, but not cloned α_A IFN, inhibited the replication of reovirus in human amnion U and GM2767A cells (45). This indicates that each IFN type shows its own kind of interaction with target cells. Nonetheless, all IFN types are, in general, able to induce 2-5A synthetase and protein kinase (38, 46).

Attempts to strictly correlate IFN-mediated induction of 2-5A synthetase and protein kinase with the antiviral activity observed *in vivo* have been performed. In spite of a widespread variability, data appear consistent with the assumption that the 2-5A synthetase-RNase L pathway is functioning *in vivo* and may mediate the antiviral activity of IFNs (for a review, see Refs. 47 and 48). On the other hand, the IFN-induced 67-kDa protein kinase activity may also be involved in the antiviral action of IFN. In fact, this is supported by (i) experiments of *in vivo* phosphorylation of the 67-kDa polypeptide measured in mouse L929 cells infected with reovirus (49) and (ii) the correlation between the kinetics of induction and decay of both these enzymatic activities and the antiviral state measured in L929 mouse cells treated with IFN (50, 51). In addition, cell lines vary widely in their sensitivity to IFNs and in the constitutive level or inducibility of these enzymes. In humans, both enzymes are induced in HeLa cells by IFN; neither is detectable in MRC5 cells, which are as sensitive to α,β IFN as HeLa cells (52); and neither recombinant α_A - or recombinant γ IFN induces the kinase activity in GM2767A fibroblasts, whereas only the former is able to induce the kinase in amnion U cells (45). In the murine system, both enzymes are induced in L929 cells, whereas only the protein kinase is induced in K/BALB and only the 2-5A synthetase in NIH/3T3 cells (53).

One way to approach this problem is to isolate and characterize cell variants lacking the enzymatic activities involved in the mechanism of IFN action. In the present study, we showed that treatment of the α,β IFN-resistant FLC variants with γ IFN preparations induces a bona fide antiviral state. The extent of reduction of lytic viruses yield induced by γ IFN in the α,β IFN-resistant FLC variants (1–2 logs) is lower than that induced in wild-type FLC (3–4 logs), but it is *specific*. Likewise, γ IFN treatment is also able to reduce the chronic release of FLV in sensitive and resistant FLC even if the extent of reduction is lower in resistant cells. It is noteworthy to keep in mind that the quantitative aspects of this effect in resistant *versus* susceptible FLC are hardly comparable, because the reverse transcriptase assay used to test FLV release is sensitive to detect only 1-log differences between IFN-treated and untreated cells, as the remaining tail of the curves tends asymptotically to zero.

Evidence has been provided that the α,β IFN antiviral effect may be effective on retroviruses but not on lytic viruses and vice versa (36, 54). As retroviruses establish a symbiotic relationship with the host cell, it is conceivable that IFN inhibition of murine leukemia virus release is mediated by pathways different from those involved in the case of lytic RNA viruses. In fact, Sen and Hertz (36) as well as Salzberg *et al.* (55) have described a subclone of murine leukemia virus-transformed NIH/3T3 cells which has retained susceptibility to α,β IFN anti-murine leukemia virus activity but is poorly responsive to its anti-EMCV and -VSV activity (55). Little, if any, induction of 2-5A synthetase activity over the basal level was observed in these resistant cells treated with α,β IFN, suggesting that the 2-5A synthetase pathway is not involved in IFN-induced inhibition of chronic retrovirus release. Sen and Hertz (36) suggest that both 2-5A synthetase and protein

kinase pathways do not play a direct role in the antiviral activity of IFN on retroviruses.

In the FLC system, treatment with doses of α , β IFN up to 13,000 units/ml is not able to induce any 67-kDa protein kinase activity in the α , β -resistant FLC clones. Thus, both these enzymatic pathways appear not to be activated in these variants treated with α , β IFN. In contrast, treatment with graded doses of γ IFN (up to 500 units/ml) is unable to induce 2-5A synthetase activity, but is still able to induce the 67-kDa protein kinase activity.

These data are compatible with the following: (a) the 67-kDa protein kinase activity may be responsible for the observed antiviral state; and (b) activation of the 2-5A synthetase is not *strictly* required for this effect. As the reduction of virus yield induced by γ IFN treatment is lower than that observed in wild-type FLC, the observed activation of the 67-kDa protein kinase may not be sufficient to completely inhibit virus replication. This is in agreement with the accepted hypothesis that the two different enzymatic pathways work independently and/or additively in the same direction. In addition, the uninducibility of 67-kDa protein kinase activity observed in the resistant FLC treated with α , β IFN suggests that the activation of this enzymatic pathway could be mediated by different intermediate steps subsequent to IFNs binding to their proper receptors.

Finally, the possibility that other IFN-induced proteins (25), in addition to those analyzed here, are involved in the mechanism(s) underlying the observed effects cannot be ruled out.

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