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Establishment of the Antiviral State in \(\alpha,\beta\)-Interferon-resistant Friend Cells Treated with \(\gamma\)-Interferon

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

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From the \$Departments of Virology, Istituto Superiore di Sanità, and of Cellular and Developmental Biology, University of Rome, Rome, Italy and the \$Laboratoire de Biochimie des Proteines, Equipe de Recherche Associée Centre Nationale de la Recherche Scientifique 482, University of Montpellier II, Montpellier, France

Treatment with murine \(\gamma\)-interferon (IFN) preparations of variant sublines of Friend leukemia cells resistant to the \(\alpha,\beta\)-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 135, 508–512) results in the establishment of a bona fide antiviral state. In fact, \(\gamma\)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, inducible by treatment with \(\alpha,\beta\)IFN (up to 13,000 units/ml), is instead induced upon treatment with \(\gamma\)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(riC)-agarose.

Interferons (IFNs) 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 (\(\alpha,\beta\) and \(\gamma\)), despite the diversity of cell origin, inducing agents, and antiganic and molecular properties (8–10), are able to affect these biological phenomena. In particular, the development of the antiviral 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 \(\alpha\) peptide chain of euakaryotic 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 \(\alpha,\beta\)IFN-induced antiviral state. FLC are mouse nucleated erythroid precursors able to differentiate in vitro when treated with various agents including dimethyl sulfoxide (24). \(\alpha,\beta\)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 \(\alpha,\beta\)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 \(\alpha,\beta\)IFN-resistant and -sensitive FLC clones show a specific saturable binding site for mouse \(\alpha,\beta\)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 \(\alpha,\beta\)IFN (28).

In this report, we describe data showing that treatment with \(\gamma\)IFN of the variant clones results in the establishment of a bona fide antiviral state showing that \(\gamma\)- and \(\alpha,\beta\)IFNs may have different interaction with the same cell system. Furthermore, the antiviral state induced by \(\gamma\)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 \(\gamma\)IFN, whereas no such activity is detectable when the same cells are exposed to up to 13,000 units/ml \(\alpha,\beta\)IFN.

EXPERIMENTAL PROCEDURES

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

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Ci/(mmol) were obtained from the Radiochemical Centre, Amersham,
United Kingdom. Poly(rI)-poly(rC) and poly(rI)-poly(rC)-agarase were
from P-L Biochemicals. Benzidine dihydrochloride was from
Ciba. Poly(A) was from Sigma. Oligo(T)$_{12-18}$ 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.

VSV and EMCV—(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. Titters ranged between $10^5$ and $10^6$ plaque-forming units/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. Titters ranged between 300 and 600 hemagglutinating
units/ml.

Reverse Transcriptase Assay—The assay was performed in a final
volume of 100 μl containing 20 mM Tris-HCl, pH 7.8, 60 mM NaCl,
10 mM MgOAc, 5 mM dithiothreitol (DTT), 2 μg/ml poly(A), 5 μg/ml oligo(T)$_{12-18}$, 2 μM TTP. Samples of clarified supernatants, ranging from 5 to 30 μl, with additional medium containing
5% fetal calf serum to a total volume of 30 μl, 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 pmol of poly(rI).poly(rC)-agarase-bound enzyme was prepared by mixing cell extracts (30 μl) with poly(rI).poly(rC)-agarase (20 μl) previously equilibrated with 10 mM Hepes buffer, pH 7.6, 90 mM KCl, 100 mM MgOAc, 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)-agarase-bound enzyme fraction (20 μl) was added to portions of the crude extracts, which were assayed as described previously.
Effects of γIFN in α,βIFN-resistant Friend Cells

Table I

| Cell clones | Friend virus release Reduction of VSV yield | α,βIFN | γIFN | α,βIFN | γIFN |
|-------------|---------------------------------------------|--------|------|--------|------|
|             | % RTA ± S.E. | Δlog(pfu/5 × 10⁶ cells) ± 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 |

The partial reduction of VSV yield and reverse transcriptase activity observed in cultures of the α,βIFN-resistant FLC clones could be ascribed to cell toxicity exerted by the crude preparations of γIFN used. Cells of wild-type and two variant FLC clones, treated for 20 h with 500 units/ml murine γIFN, were washed out of γIFN and then plated in soft agar medium. Data in Table II (left) show that the plating efficiency of α,βIFN-sensitive and resistant FLC do not significantly differ as a consequence of γIFN 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 reovirus), virus yields were titrated in aliquots of supernatants from cell cultures infected with 1 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 IFN-induced reduction of virus replication in α,βIFN-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 γIFN of α,βIFN-resistant FLC variants was the result of the induction of the 2-5A synthetase pathway (uninduced by α,βIFN treatment) (28), 2-5A synthetase activity was analyzed in sensitive and resistant FLC treated with γIFN. 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,βIFN, analysis of 2-5A synthetase activity reveals that treatment of 10⁶ cells/ml with 500 units/ml γIFN 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,βIFN-resistant clones. The 2-5A synthetase-specific activity at different doses of γIFN is presented in Fig. 3 for wild-type cells and for MRI 745 3C18 clone. The results obtained for the other α,βIFN-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,βIFN-resistant FLC clones given any IFN type, it was of obvious interest to check whether the other major IFN-induced en-
Effects of γIFN in α,βIFN-resistant Friend Cells

FIG. 2. Dose-response curves of VSV yield (lower panel) and FLV release (upper panel) in α,βIFN-sensitive and -resistant FLC treated with recombinant mouse γIFN. Cells seeded at 10^6/ml in the presence of graded doses of either crude murine γIFN (triangles) or recombinant mouse γIFN (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. O, △ 745A; ○, △ 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 γIFN. To validate detection of kinase activity, this was determined both in solution (Fig. 4) and by immobilization on poly(rC)-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 γIFN. In the former cell extract, the kinase was activated also when the cells had been exposed to 200 units/ml α,βIFN. The kinase was also immobilized on poly(rC)-poly(rC)-agarose as described by Hovanessian and Kerr (37) and evaluated for its capacity to phosphorylate the 67-kDa endogenous substrate.

FIG. 3. Induction of 2-5A synthetase as a function of γIFN dosage. 745A cells (○) and MRI 745 3C18 (○) seeded at 10^6/ml were treated with varying amounts of mouse γIFN 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 γIFN, whereas no inducibility thereof is detectable in FLC variants treated with 13,000 units/ml α,βIFN. The pattern of phosphorylation (solid phase assay) of extracts from 745A, 3C18, 1C11, 7, 8, and 9 treated with α,β- or γIFN is presented in Fig. 5.

DISCUSSION

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

TABLE II

| Cell clones       | Plating efficiency | Reduction virus yield |
|-------------------|--------------------|-----------------------|
|                   | ~IFN | γIFN   | VSV | EMCV |
|                   | % ± S.E. | pfu/10^6 cells | % ± S.E. | pfu/10^6 cells |
| 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 |

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

Cells were seeded at 5 x 10^6 cells/ml with or without 500 units/ml murine γIFN. 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 100, 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 γIFN preparations. Control values for VSV and EMCV yield expressed as pfu/5 x 10^6 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).
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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 γIFN. FLC were seeded at 5 x 10^6/ml and grown in the presence of mouse α,βIFN (200 units/ml) or mouse γIFN (200 units/ml) for 20 h. L929 cells were seeded at 3.5 x 10^4/cm^2 1 day before addition of mouse α,βIFN (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, 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 γIFN. Cells were seeded at 10^6/ml and grown in the presence of mouse α,βIFN (13,000 units/ml) or mouse γIFN (500 units/ml) for 20 h. L929 cells were seeded at 3.5 x 10^4/cm^2 1 day before addition of mouse α,βIFN (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,βIFN; 3, 745A + γIFN; 4, MRI 745 1C11; 5, MRI 745 1C11 + α,βIFN; 6, MRI 745 1C11 + γIFN; 7, MRI 745 3C18; 8, MRI 745 3C18 + α,βIFN; 9, MRI 745 3C18 + γIFN; 10, MRI 745 7; 11, MRI 745 7 + α,βIFN; 12, MRI 745 7 + γIFN; 13, MRI 745 8; 14, MRI 745 8 + α,βIFN; 15, MRI 745 8 + γIFN; 16, MRI 745 9; 17, MRI 745 9 + α,βIFN; 18, MRI 745 9 + γIFN; 19, L929; 20, L929 + α,βIFN. The position of the M, 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 radiiodinated 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 antineoplastic activities of human α- and βIFNs, whereas they are resistant to the antiviral and antineoplastic actions of human γIFN (44). Conversely, only recombinant γIFN, but not cloned α,βIFN, 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,βIFN as HeLa cells (52); and neither recombinant α,β- or recombinant γIFN 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,βIFN-resistant FLC variants with γIFN preparations induces a bona fide antiviral state. The extent of reduction of lytic viruses yield induced by γIFN in the α,βIFN-resistant FLC variants (1–2 logs) is lower than that induced in wild-type FLC (3–4 logs), but it is specific. Likewise, γIFN 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,βIFN 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,βIFN anti-murine leukemia virus activity but is poorly responsive to its anti-EMCV and -SVV activity (55). Little, if any, induction of 2-5A synthetase activity over the basal level was observed in these resistant cells treated with α,βIFN, 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,βIFN 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,βIFN. In contrast, treatment with graded doses of γIFN (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 γIFN 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,βIFN 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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