Antiviral, Anticellular and Enzyme-inducing Activities of Interferons in RD-114 Cells

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(Accepted 13 June 1983)

SUMMARY
RD-114 is a human sarcoma-derived cell line which is chronically infected with the RD-114 retrovirus. In a previous study, we found that treatment of these cells with human interferon-α or human interferon-γ causes a marked inhibition of RD-114 virus production, but that the replication of exogenous vesicular stomatitis or encephalomyocarditis virus is not impaired. In the present study, we report that neither type of interferon has strong inhibitory effects on DNA synthesis or on multiplication of the cells. We also failed to detect a double-stranded RNA-dependent protein kinase activity in extracts of both interferon-treated and untreated cells. However, a low level of 2',5'-oligoadenylate [2,5(A)] synthetase activity was detectable in extracts of interferon-treated cells. 2,5(A)-dependent endonuclease L activity was detectable in extracts of both untreated and interferon-treated cells. This was probably responsible for the inhibition of protein synthesis observed upon introduction of 2,5(A) to RD-114 cells. In many cells, interferon has been found to induce synthesis of several proteins demonstrable by autoradiographic analysis of slab gels on which extracts of interferon-treated and radiolabelled cells are separated. Using a similar method, no such induced protein synthesis was detectable in interferon-treated RD-114 cells. Our results indicate that RD-114 cells are resistant to most known actions of interferons except for the antiretroviral action to which they are as sensitive as any other cell line.

INTRODUCTION
Interferons not only inhibit the replication of exogenously infecting cytopathic viruses, but also inhibit continuous production of retroviruses by cells that are chronically infected with these viruses (Friedman, 1977; Sen, 1982). It is well-established that the conventional antiviral action and the antiretroviral actions of interferons are exerted at different stages of the virus replication process. Whereas protein synthesis of exogenously infecting viruses is inhibited in interferon (IFN)-treated cells, this step is unaffected for retroviruses in a chronically infected cell. On the other hand, retrovirus assembly and release from the plasma membrane are impaired in IFN-treated cells (Friedman et al., 1975; Pitha et al., 1976; Billiau et al., 1976; Sen & Sarkar, 1980).

A double-stranded (ds)RNA-dependent protein kinase pathway and a dsRNA-dependent 2',5'-oligoadenylate [2,5(A)] synthetase–endonuclease pathway are thought to be instrumental in inhibiting protein synthesis in IFN-treated virus-infected cells. In addition to the protein kinase and 2,5(A) synthetase, synthesis of several other proteins is induced by IFN (Lengyel, 1982; Sen, 1982). The roles of these proteins in various actions of interferon are yet to be elucidated.

IFN treatment of many cell lines decreases their rates of multiplication (Taylor-Papadimitriou, 1980). Moreover, IFN-γ has cytolytic activity against many transformed lines (Rubin & Gupta, 1980; Rubin et al., 1983). The 2,5(A) system may play a role in the anticellular action of IFN as well (Williams & Kerr, 1978; Hovanessian et al., 1979; Kimchi et al., 1979; Panet et al., 1981).
In the recent past, several murine lines have been described in which the antiretroviral activity of IFN is fully or partially dissociated from the conventional antiviral activity of IFN against vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) (Allen et al., 1976; Czarniecki et al., 1981; Epstein et al., 1981; Sen & Herz, 1983). Recently, we reported that retrovirus production by the human line RD-114 is extremely sensitive to both IFN-α and IFN-γ, but replication of neither VSV nor EMCV is inhibited by IFN treatment of these cells (Sen et al., 1982; Herz et al., 1983). We wanted to understand the biochemical basis for the lack of classical antiviral actions of IFN in these cells. Moreover, we were interested to know whether these cells would respond to the anticellular actions of IFN. In this report, we present the results of these investigations.

METHODS

Cells. RD-114 is a human sarcoma-derived cell line which is chronically infected with the RD-114 retrovirus (McAllister et al., 1972). The procedures for culturing these cells have been described previously (Herz et al., 1983).

Interferons. Human IFN-α was produced by virus infection of human leukocytes and was partially purified to a specific activity of $10^6$ units/mg protein. Human IFN-γ was produced by stimulation of leukocytes with staphylococcal enterotoxin A and partially purified to a specific activity of $10^6$ units/mg protein as assayed on GM2767 cells, using VSV as the challenge virus and compared with an internal laboratory standard (Rubin et al., 1983).

2,5(A) introduction into cells. Chromatographically purified trimer of 2,5(A) (Samanta et al., 1980) was used for these experiments. 2,5(A) was introduced into cells using the calcium phosphate co-precipitation technique described by Hovanessian & Wood (1980) with the modifications of Panet et al. (1981). Approx. 5 × 10^5 cells were plated in each well (10 cm² area) of a 6-well plate and allowed to grow overnight. The following day, regular medium was removed, cells were washed once with medium without serum and then incubated for 1 h with 0.5 ml of medium without serum. Dilutions of 2,5(A) were made in 0.5 ml of transfection buffer containing 20 mM-HEPES, 136 mM-NaCl, 5 mM-KCl, 0.7 mM-Na₂HPO₄ and 9 mM-glucose, pH 7.1; 2 M-CaCl₂ was added to these dilutions to a final concn. of 114 mM. The solutions were mixed well, incubated for 10 min at room temperature and added to the cell culture media. The cells were incubated at room temperature for 45 min and at 37 °C for 90 min. The transfection mixture was removed and 2 ml of regular medium containing serum was added. After 2 h incubation cells were labelled in 1 ml of medium containing 25 µCi of the usual concn. of methionine and 10 µCi [³⁵S]methionine for 2 h. Cells were washed once with cold phosphate-buffered saline (PBS) and then lysed in 0.5 ml cold PBS containing 1% SDS. A 0.2 ml sample of cell extract was used for determining hot trichloroacetic acid (TCA)-insoluble counts.

Analysis of IFN-inducible proteins. Confluent monolayers were incubated with or without IFN in a medium containing 25% of the usual concn. of methionine and 20 µCi/ml [³⁵S]methionine for 14 h. Cell extracts were made and centrifuged at 200,000 g; supernatants were prepared as described previously (Rubin & Gupta, 1980). Extracts containing equal amounts of labelled proteins were analysed by electrophoresis in a 15% polyacrylamide gel. Labelled molecular weight markers were electrophoresed in the same gel and the gels were subjected to fluorography.

Procedures for measuring reverse transcriptase activity, protein kinase activity, endonuclease L activity and 2,5(A) synthetase activity, and the methods for VSV and EMCV yield reduction assay, for [³H]thymidine incorporation assay and for measurement of the rates of cell multiplication have all been described previously (Sen & Herz, 1983).

RESULTS

Anticellular actions of IFN

We measured the effects of various doses of IFN-α and IFN-γ on thymidine incorporation into DNA in RD-114 cells (Fig. 1). At the highest dose tested (200 U/ml) there was 20 to 25% inhibition of thymidine incorporation. This degree of inhibition was much less than that reported for IFN-γ-treated FS-4 cells (Rubin & Gupta, 1980). Moreover, there was no remarkable difference between the potencies of IFN-α and IFN-γ in inhibiting thymidine incorporation. That this inhibition was due to differential thymidine transport is unlikely, since there were no differences in TCA-soluble counts in IFN-treated and untreated cells (data not shown).
Effects of interferons in RD-114 cells

Fig. 1. Effect of increasing doses of IFN-α and IFN-γ on the rate of [³H]thymidine incorporation into DNA of RD-114 cells. Cells were treated with various doses of IFN overnight and [³H]thymidine incorporation was measured as described in Methods. The amount of [³H]thymidine incorporated into DNA of untreated cells has been expressed as 100 arbitrary units. ●, IFN-α; ○, IFN-γ.

Fig. 2. Effect of IFN on the rate of multiplication of RD-114 cells. Approx. 4 × 10⁴ cells were plated out in 60 mm dishes and treated with no IFN (▲), 200 U/ml IFN-α (●) or 200 U/ml IFN-γ (○). Rates of cell multiplication were measured as described in Methods.

In another experiment we tested the effects of IFN on the rate of multiplication of RD-114 cells (Fig. 2). Untreated cells multiplied exponentially over 5 days, after which the growth rate slowed down. When the cells were cultured continuously in the presence of either 200 U/ml IFN-α or 200 U/ml IFN-γ, the growth rates were somewhat slower. We consistently observed that after 2 to 3 days of treatment there were more cells in IFN-γ-treated culture than in IFN-α-treated culture. However, after this time the IFN-α-treated cells multiplied more rapidly, whereas multiplication of IFN-γ-treated cells slowed down. Seven days after the beginning of IFN treatment there were 55% fewer cells in the IFN-α-treated culture and 70% fewer cells in the IFN-γ-treated culture, as compared to the untreated culture. Although IFN-γ inhibited cell growth slightly more than IFN-α, there was no sign of cell death in the IFN-γ-treated culture. Moreover, the magnitude of growth inhibition of RD-114 cells by IFN-γ was far less than that reported for other transformed cells (Rubin & Gupta, 1980).

Double-stranded RNA-dependent protein kinase activity

Since neither IFN-α nor IFN-γ inhibits VSV or EMCV replication in RD-114 cells (Herz et al., 1983), and their effects on cell growth were also modest, we were interested to learn whether IFN treatment of these cells causes induction of the usually inducible enzyme activities such as the dsRNA-dependent protein kinase activity and 2,5(A) synthetase activity. We tested extracts of IFN-treated and untreated RD-114 cells for the presence of the protein kinase activity. As a positive control we included in this experiment an extract of IFN-treated mouse L929 cells, which are known to have this protein kinase activity. As shown in Fig. 3, presence of dsRNA in the incubation mixture specifically enhanced the phosphorylation of a 67000 mol. wt. protein in the L cell extract (lanes 1 and 2), indicating the presence of a dsRNA-dependent protein kinase; however, no dsRNA-dependent phosphorylation could be detected in extracts of either IFN-α- or IFN-γ-treated RD-114 cells (lanes 3 to 8). Similar results were obtained with several other extracts made from IFN-treated RD-114 cells. It appears, therefore, that IFN treatment does not induce this enzyme in RD-114 cells.
Fig. 3. Protein phosphorylation in extracts of L929 and RD-114 cells. Endogenous protein phosphorylation products of different cell extracts were analysed as described in Methods. Odd-numbered lanes represent incubations in the absence of dsRNA, and even-numbered lanes represent those in the presence of dsRNA. The arrow indicates the position of the major dsRNA-dependent phosphoprotein in L929 cell extract (Pt, mol. wt. about 67000). Lanes 1 and 2, extracts of mouse IFN (200 U/ml)-treated L929 cells; lanes 3 and 4, extracts of untreated RD-114 cells; lanes 5 and 6, extracts of RD-114 cells treated with 200 U/ml human IFN-α; lanes 7 and 8, extracts of RD-114 cells treated with 200 U/ml human IFN-γ.

Fig. 4. Inhibition of protein synthesis by introduction of increasing concentrations of 2,5(A) into RD-114 and L929 cells. The concentrations of 2,5(A) as indicated here were those of the dilutions before adding to the culture media. The control samples (100% protein synthesis) received similar treatments except that no 2,5(A) was added. ○, RD-114 cells; ▲, L929 cells.

Status of the endonuclease L pathway in RD-114 cells

Another dsRNA-dependent pathway which may be active in IFN-treated virus-infected cells is the RNase L pathway. We measured the levels of the two key enzymes of this pathway, 2,5(A) synthetase and RNase L, in IFN-treated and untreated RD-114 cells.

Extracts were made from untreated, IFN-α-treated and IFN-γ-treated RD-114 cells and the 2,5(A) synthetase levels in the extracts were measured. For the sake of comparison, the levels of 2,5(A) synthetase in comparable extracts of HeLa cells and L cells were also measured (Table 1). Untreated HeLa cells had a high level of 2,5(A) synthetase as noted by others (Minks et al., 1979). Treatment with 200 U/ml IFN-α or IFN-γ boosted this activity even further. Consistent with a published report (Baglioni & Maroney, 1980), IFN-α elevated the level higher than did IFN-γ. Untreated RD-114 cells had a 10-fold lower 2,5(A) synthetase level, as compared to HeLa cells. Both IFN-α and IFN-γ boosted the level of 2,5(A) synthetase in RD-114 cells. As in HeLa cells, IFN-γ boosted this activity less than did IFN-α. For two independent sets of extracts (Table 1, Expt. 1 and 2), slightly different values for the amounts of 2,5(A) synthetase activities were obtained but the relative degrees of stimulation by IFN were similar. It should be noted
Table 1. *Induction of 2,5(A) synthetase by IFN-α and IFN-γ*

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Cell extract</th>
<th>Interferon</th>
<th>2,5(A) synthesis (nmol AMP polymerized/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RD-114</td>
<td>None</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>RD-114</td>
<td>Human IFN-α</td>
<td>8.00</td>
</tr>
<tr>
<td></td>
<td>RD-114</td>
<td>Human IFN-γ</td>
<td>2.50</td>
</tr>
<tr>
<td>2</td>
<td>RD-114</td>
<td>None</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>RD-114</td>
<td>Human IFN-α</td>
<td>5.65</td>
</tr>
<tr>
<td></td>
<td>RD-114</td>
<td>Human IFN-γ</td>
<td>1.63</td>
</tr>
<tr>
<td>3</td>
<td>HeLa</td>
<td>None</td>
<td>7.00</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>Human IFN-α</td>
<td>50.00</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>Human IFN-γ</td>
<td>30.00</td>
</tr>
<tr>
<td>4</td>
<td>L929</td>
<td>Mouse IFN-α + β</td>
<td>52.20</td>
</tr>
</tbody>
</table>

* Cells were treated with 200 U/ml IFN for 18 h where indicated. Cell extracts were made by detergent disruption and 2,5(A) synthetase activities were measured as described in Methods. Amounts of 2,5(A) synthesized are presented as nmol AMP polymerized in extracts from 10^6 cells with the assumption that the products were all trimers.

Table 2. *2,5(A)-dependent endonuclease L activity in RD-114 cell extracts*

<table>
<thead>
<tr>
<th>Cell extract</th>
<th>IFN treatment</th>
<th>TCA-precipitable ct/min after incubation</th>
<th>RNase L activity (X – Y) x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>L929</td>
<td>Mouse IFN-α + β</td>
<td>629 – 295</td>
<td>53</td>
</tr>
<tr>
<td>RD-114</td>
<td>None</td>
<td>748 – 714</td>
<td>4.5</td>
</tr>
<tr>
<td>RD-114</td>
<td>Human IFN-α</td>
<td>750 – 656</td>
<td>12.5</td>
</tr>
<tr>
<td>RD-114</td>
<td>Human IFN-γ</td>
<td>765 – 708</td>
<td>7.5</td>
</tr>
</tbody>
</table>

* Incubation conditions have been described in Methods. Two different sets of RD-114 extracts and L cell extracts were tested and similar results were obtained with both sets. Input radioactivities in these reactions were 1200 ct/min.

that the level of activity present in extracts of IFN-α-treated RD-114 cells was similar to the level of activity in extracts of untreated HeLa cells. The observed lower 2,5(A) synthetase activity in extracts of IFN-treated RD-114 cells, as compared to HeLa or L cells, was probably not due to the presence of an inhibitor of this enzyme or to the presence of a potent 2,5(A)-degrading activity in these cells. The above conclusions were based on the observation that when equal amounts of extract from IFN-treated L cells and extract from IFN-treated RD-114 cells were mixed and tested for 2,5(A) synthetase activity, the total activity observed was a sum of the individual activities (data not shown).

2,5(A)-dependent endonuclease L activities in extracts from IFN-treated and untreated RD-114 cells were measured in another experiment (Table 2). 32P-labelled VSV mRNA was incubated with the extracts in the presence and absence of added 2,5(A), and the amount of residual TCA-precipitable radioactivity after the incubation was measured. The difference between the radioactivity in the reaction without 2,5(A) and the reaction with 2,5(A) was used as a measure of the 2,5(A)-dependent endonuclease activity. Low levels of 2,5(A)-dependent endonuclease activity were detectable in extracts of both IFN-treated and untreated RD-114 cells. Although the level was low, it was detected reproducibly in several sets of extracts.

Since we detected a low level of RNase L activity in extracts of RD-114 cells, we were interested to learn whether this activity is manifested in vivo. For this purpose, increasing amounts of 2,5(A) were introduced into the cells and the effect on protein synthesis was measured. L929 cells, in which 2,5(A)-mediated inhibition of protein synthesis has been demonstrated by others (Hovanessian et al., 1979; Kimchi et al., 1979; Williams & Kerr, 1978), were used as known positive controls. As shown in Fig. 4, introduction of 2,5(A) resulted in
inhibition of protein synthesis in both L929 and RD-114 cells. About 50% inhibition was obtained at $10^{-8}$ M-2,5(A) in RD-114 cells, whereas similar inhibition was obtained at $10^{-9}$ M in L929 cells. These results demonstrated that RNase L able to be activated is present in RD-114 cells and it can inhibit protein synthesis if 2,5(A) is made available to these cells.

**IFN-inducible protein synthesis in RD-114 cells**

IFN treatment induces the synthesis of several specific proteins in both human and mouse cells (Lengyel, 1982; Sen, 1982). We investigated whether induction of any such protein in RD-114 cells could be detected using experimental procedures which are appropriate for detecting synthesis of these induced proteins in human fibroblasts. As shown in Fig. 5, treatment with IFN-γ enhanced the level of synthesis of several proteins in FS-4 cells; however, synthesis of none of these proteins was enhanced by treatment of RD-114 cells with IFN-α, -β or -γ. Although induction of these proteins has been studied mostly in human fibroblasts, their induction can also be detected in other human lines such as HeLa or Daudi (B. Y. Rubin et al., unpublished observation) using the same procedures as described here. It appears therefore that
none of these proteins is induced by IFN treatment of RD-114 cells. We cannot, however, rule out the possibility that they are synthesized in much lower quantities or that their presence is masked by other proteins co-migrating with them in the gel analysis. Moreover, other proteins in addition to the ones that are observed here may be induced by IFN both in FS-4 and in RD-114 cells. Detection of these putative IFN-induced proteins may need more powerful methods of analysis.

DISCUSSION

Our studies demonstrated that IFN-α and IFN-γ not only fail to inhibit the replication of VSV and EMCV in RD-114 cells, but also have only modest antiviral activities against these cells. In agreement with Tomita et al. (1982) we could not detect the dsRNA-dependent protein kinase activity in extracts of IFN-treated RD-114 cells. However, in contrast to their observations, we detected a low level of 2,5(A) synthetase activity in such extracts. This discrepancy could be due to a higher sensitivity of our assay method or due to a genuine difference between the properties of our cell line and those of the subclone of RD-114 line used by them. The 2,5(A) synthetase level in untreated RD-114 cells was very low, unlike the high constitutive level of 2,5(A) synthetase in HEC-1 cells, another human line in which VSV replication is insensitive to IFN (Verhaegen et al., 1980). Although introduction of 2,5(A) into RD-114 cells caused severe inhibition of protein synthesis, presumably through activation of the RNase L pathway, only low levels of RNase L activity could be detected in the cell extracts. It is possible that inclusion of protease inhibitors in the extracts would have resulted in stabilization of the nuclease activity (Nilsen et al., 1982).

The above information on the status of the two IFN-inducible dsRNA-dependent pathways in these cells allows some general conclusions to be drawn regarding the role of these pathways in inhibition of replication of different viruses. It appears that the RNase L activity or the dsRNA-dependent protein kinase activity is not needed for the antiretroviral action of IFN. A functional RNase L pathway seems to be necessary for inhibition of EMCV replication (Sen & Hertz, 1983; Epstein et al., 1981). In IFN-treated RD-114 cells, although RNase L is present, the low level of 2,5(A) synthetase may not be sufficient for activating this pathway. In contrast, in JLSV-11 cells IFN treatment induces a high level of 2,5(A) synthetase, but since the RNase L level is very low, EMCV replication is not inhibited (Sen & Herz, 1983). The protein kinase pathway probably does not play a role in the inhibition of either EMCV or VSV replication (Holmes & Gupta, 1982). Inhibition of VSV replication does not appear to be mediated through the RNase L pathway either, since VSV replication is inhibited by IFN in JLSV-11 cells and in NIH/MOL,C cells but not in NIH/MOL,B cells or RD-114 cells, although the RNase L pathway seems to be operative in all four cell lines. Impairment of virus mRNA methylation (Sen et al., 1977; De Ferra & Baglioni, 1981) may play a role in inhibition of VSV replication by IFN.

We thank B. K. Pal of California State Polytechnic University for the RD-114 cell line, P. Lengyel of Yale University for mouse interferon and 2,5(A), A. K. Banerjee of Roche Institute of Molecular Biology for VSV mRNA, and M. Krim of this Institute for IFN-α. We acknowledge the excellent technical assistance of D. Richardson and V. Davatelis. These studies were supported by National Institutes of Health grants CA-08748 and AI-17920, and by an American Cancer Society grant MV-146.

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*(Received 16 March 1983)*