Effect of Interferon on Mouse Cells
Chronically Infected with Murine Leukaemia Virus: Kinetic Studies on Virus Production and Virus RNA Synthesis

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SUMMARY

NIH/3T3 cells chronically infected with the Moloney strain of murine leukaemia virus were incubated with interferon (IF). There was no effect on virus production during the first 4 h, but thereafter an antiviral state gradually developed, reaching a maximum at about 12 h. When IF was removed, the antiviral state (expressed in terms of inhibition of release of virus) remained constant for 10 h, after which there was an abrupt return to the normal rate of virus release. Analysis of IF-treated cells showed that there was a three to fourfold increase in the amount of virus RNA in the nucleus at 48 h after IF addition, and still a slight increase at 72 h. There were no increases in the amounts of virus RNA in the cytoplasm during 72 h after the addition of IF. These results agree with the postulate that IF inhibits a late stage in the maturation of virus in chronically infected cells.

INTRODUCTION

Interferon (IF) is known to arrest the replication of a wide range of viruses. The precise mechanism of its action is not yet completely understood, but most data support the view that either virus RNA transcription or its translation is blocked in IF-treated cells (for a review see Metz, 1975).

In most cases the effect of IF is fully expressed only if virus infection occurs sufficiently long after IF addition, because the antiviral state requires several hours to reach its optimal level (Sonnabend & Friedman, 1973). Nevertheless, RNA tumour viruses are exceptional in this respect. It has been reported that IF can inhibit the release of these viruses from chronically infected cells, even if these were infected many generations before IF treatment (Billiau et al. 1973, 1974, 1975, 1976; Friedman & Ramseur, 1974; Liberman et al. 1974; Friedman et al. 1975, 1976, 1977; Aboud et al. 1976; Allen et al. 1976; Pitha et al. 1976). Studies of the mode of IF action on these viruses have indicated that IF does not inhibit the synthesis of virus RNA (Billiau et al. 1974) or virus-coded proteins (Friedman & Ramseur, 1974; Liberman et al. 1974; Friedman et al. 1975, 1976, 1977; Shapiro et al. 1977), but appears to block the final release of the virion from the cell surface. However, other data suggest that IF interferes, to a certain extent, with assembly of virions (Pitha et al. 1976) and that the major result is the formation of incomplete virions (Van Griensven et al. 1971; Pitha et al. 1976; Chang et al. 1977).

In the present investigation we studied the kinetics of the development of the effect of IF on virus release by cells chronically infected with murine leukaemia virus (MLV), the persistence of this effect after IF removal, and the effect of IF on the amount of virus RNA...
METHODS

Cell lines. NIH/3T3 mouse fibroblasts chronically producing Moloney murine leukaemia virus [NIH/3T3 (M-MLV)] were used throughout this study. Uninfected mouse NIH/3T3 fibroblasts and rat XC cells (Rowe et al. 1970) were used to assay infectious virus particles. Unless otherwise indicated, the cells were grown in 50 mm dishes (NUNC, Denmark) in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated calf serum, and maintained in this medium with 2.5% serum for IF studies.

Reverse transcriptase assay. Reverse transcriptase activity was measured in 50 μl samples of fluid as described by Aboud et al. (1976). In Fig. 4 and 5 and Table 1, the reverse transcriptase activities shown represent the incorporation of 3H-TTP into material binding to DEAE-cellulose during a reaction time of 45 min; in Fig. 1, the reaction time was 4 h.

Assay of infectious virus particles. This assay was performed essentially according to the standard XC-test (Rowe et al. 1970) with slight modifications. About 5 × 10^4 NIH/3T3 cells were plated per dish in 5 ml medium containing 8 μg/ml polybrene (Sigma Chemical Co., St Louis, Mo. U.S.A.). After 16 to 18 h the medium was removed and the cells were overlaid with 0.3 ml of tenfold serial dilutions of the virus sample under test, made in medium containing 8 μg polybrene per ml. After 1 h at 37 °C the cells were washed with PBS and covered with 5 ml of fresh medium. The cells were incubated for 4 to 5 days and then irradiated by a Philips 15 W TUV lamp for 30 s at a distance of 270 mm. The cultures were then covered with about 10^6 XC cells per dish in 5 ml medium. After additional incubation for 2 to 3 days the cultures were fixed with methanol for 20 min, stained with Giemsa for 30 min, rinsed with tap water and examined under the microscope. The number of syncytia was determined.

Measurement of incorporation of radioactive precursors into cellular macromolecules. NIH/3T3 (M-MLV) cells plated at 1.2 × 10^6 cells per dish with or without IF were incubated for 24 h, rinsed with PBS and re-fed with 2 ml of fresh medium containing 6 μCi/ml of either 3H-thymidine (20 Ci/mmol), 3H-uridine (11 Ci/mmol), 3H-L-proline (3 Ci/mmol), or 3H-L-alanine (42 Ci/mmol), all obtained from New England Nuclear, Boston, Mass., U.S.A. The cultures were incubated for 1 h and then rinsed three times with cold PBS. The cells were dissolved in 0.5% SDS, allowed to stand at room temperature for 15 min and samples were precipitated by 10% cold TCA on 3 MM filters (Whatman, Maidstone, Kent, England). The filters were dried and placed in vials containing toluene-based scintillation fluid; their radioactivity was determined in a scintillation counter.

Labelling of extracellular virions. NIH/3T3 (M-MLV) cells were plated at 8 × 10^5 cells per dish with or without IF, incubated for 24 h, rinsed with PBS, re-fed with 2 ml of medium containing 15 μCi/ml of either 3H-uridine or 3H-proline, and further incubated for 5 h. The medium was collected and centrifuged at 1000 g to remove cell debris and the labelled virus was sedimented in a Spinco SW 41 rotor at 105000 g for 45 min. The pellet was suspended in 0.4 ml of NTE buffer (0.1 M-NaCl; 0.01 M-tris-HCl, pH 7.4; 0.001 M-EDTA) and layered on a 15 to 60% (w/v) linear sucrose gradient made in NTE. The gradients were centrifuged in the SW 41 rotor at 105000 g for 60 min. Fractions were collected through a puncture made at the bottom of the tubes. Each fraction received 50 μg of bovine serum albumin (Sigma) as a carrier, and was precipitated with cold 10% TCA for counting, as described above.
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Interferon preparation and titration. Mouse IF was prepared and titrated by the reverse transcriptase reduction assay previously described (Aboud et al. 1976). The IF preparation used throughout this study contained $3.3 \times 10^3$ 50% reverse transcriptase reduction dose (RTRD$_{50}$) units per mg of protein. (One RTRD$_{50}$ is the IF dose reducing reverse transcriptase activity by 50% in our test. One RTRD$_{50}$ unit is equivalent to 20 research reference units of interferon in terms of the National Institutes of Health, Bethesda, Md., U.S.A., reference standard for mouse interferon, catalogue number G-002-904-511.)

RNA extraction from nuclear and cytoplasmic fractions. Cells were dispersed by trypsinization, washed three times with cold PBS and suspended in 2 ml of 10 mM-tris-HCl, pH 7.6, 10 mM-NaCl and 1.5 mM-MgCl$_2$. This suspension was brought to 1% Nonidet P40 (Fluka AG, Buchs, Switzerland), allowed to stand at room temperature for 10 min and then homogenized by 15 strokes of a loosely fitting pestle (B) of a glass Dounce homogenizer (Wheaton Scientific, Millville, N.J., U.S.A.). The nuclear fraction was pelleted by centrifuging at 1200 g for 10 min. The supernatant was removed, and the pellet was carefully suspended in 2 ml of the same buffer to which 0.5% deoxycholate and 1% Nonidet P40 were added. After 10 min at room temperature, the nuclei were pelleted and found by phase contrast microscope examination to be free of cytoplasmic residues. The two supernatants were combined, and total macromolecules were precipitated with 2.5 vol. of ethanol at $-20 \, ^\circ\mathrm{C}$ overnight. This precipitate, as well as the nuclear pellet, was suspended in 5 ml of a buffer containing 0.05 M-Na acetate, pH 5.1, and 0.1 M-EDTA. RNA was extracted from both, as detailed by Salzberg et al. (1977). No significant amount of ribosomal RNA was detected when nuclear RNA was analysed on a 15 to 30% linear sucrose gradient.

Virus purification and cDNA preparation. NIH/3T3 (M-MLV) cells were seeded in Roux bottles at a density of $4 \times 10^6$ cells/bottle in 100 ml of medium. The medium was changed every 12 h for 2 to 3 days. Virus was collected from the medium and purified, as described by Salzberg et al. (1973). The purified virus was used to prepare the virus complementary DNA strand (cDNA) by the endogenous reverse transcriptase reaction in a final vol. of 5 ml containing 50 mM-tris-HCl, pH 8.2, 5 mM-dithiothreitol, 100 mM-NaCl, 0.5 mM-MnCl$_2$, 0.1 mmol of dATP, dGTP and dCTP, 0.01 mm-3H-TTP (50 Ci/mmol), 0.03% Nonidet P40, 75 $\mu$g/ml of actinomycin D and an amount of purified virus equivalent to 350 $\mu$g protein/ml. This mixture was incubated at 37 $\, ^\circ\mathrm{C}$. Samples were taken at 1 h intervals to follow the reaction course. When DNA synthesis reached a plateau (usually after 3 to 4 h) the reaction was stopped by adding 0.5% SDS and 0.01 M-EDTA. After an additional 10 min at 37 $\, ^\circ\mathrm{C}$, nucleic acids were twice extracted by phenol (saturated with 100 mM-NaCl, 10 mM-tris-HCl, pH 7.4, and 1 mM-EDTA) and a mixture of chloroform-isooamyl alcohol (24:1). Escherichia coli tRNA (50 $\mu$g/ml, Sigma) was added as a carrier to the aqueous phase and the nucleic acids were precipitated with 2.5 vol. ethanol at $-20 \, ^\circ\mathrm{C}$ overnight. The precipitate was dissolved in 1 ml of ten times diluted SSC (SSC = 0.15 M-NaCl and 0.015 M-Na-citrate, pH 5.5), and the RNA was hydrolysed by treatment with 0.2 M-NaOH for 60 min at 37 $\, ^\circ\mathrm{C}$. After neutralization with HCl, 50 $\mu$g of E. coli DNA was added as carrier and the DNA was precipitated with ethanol. The specific activity of the cDNA thus obtained was $2 \times 10^7$ ct/min/µg. It was 95% sensitive to St nuclease digestion and 88% resistant after hybridization to an excess of virus RNA.

Quantification of virus RNA. Virus RNA in cellular fractions was estimated by hybridizing RNA samples to about 600 ct/min of cDNA for 40 h at 68 $\, ^\circ\mathrm{C}$, as described by Salzberg et al. (1977). The amount of virus-specific RNA in each sample was computed from a standard curve which was established by determining the percent of input cDNA hybridized as a function of the amount of 70S virus RNA added to the annealing reaction. The curve was
linear up to 50% hybridization, corresponding to 5 ng of 70S virus RNA. Accordingly, in calculating the cellular virus-specific RNA, only those C_t (mol/l of nucleotides × time of the annealing reaction in seconds) values of total RNA not exceeding 50% hybridization were considered. These values, usually approx. 1.5 × 10^2, were identical for both IF-treated and control samples at each time interval (Fig. 5). Since during RNA extraction we used no internal standard for estimating the extraction efficiency, we express our results as ng of virus RNA per μg of total RNA. Hence, no estimates of the absolute amount of virus RNA either in the nucleus or cytoplasm are given in this study.

RESULTS

Reliability of reverse transcriptase assay for studies of IF effect on virus release

Reverse transcriptase provides an easy method for quantification of oncornaviruses (Aboud et al. 1976). However, if this method is to be used in studies with IF and these viruses, it is important to verify that any observed effect of IF on this enzyme indeed reflects an effect on virus release.

In the experiment shown in Fig. 1, NIH/3T3 (M-MLV) cells were plated with or without 4 RTRD_{50} units of IF per ml. After 24 h the cultures were rinsed three times with PBS and provided with fresh medium free of IF. After an additional 4 h the culture fluids were...
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Fig. 2. Effect of IF on the production of labelled virions. Cells were plated with (○--○) or without IF (■--■). After 24 h the cultures were rinsed with PBS and overlaid with 2 ml of medium containing (a) $^3$H-proline (15 μc/ml) or (b) $^3$H-uridine (15 μc/ml). After 5 h at 37 °C, the fluid from each culture was centrifuged and the virus pellet was overlaid on a 15 to 60 % sucrose gradient. Fractions were collected from the bottom of the tube and acid-insoluble radioactivity was determined for each fraction.

assayed for reverse transcriptase activity. As can be seen, the enzymic reaction proceeded at a linear rate for at least 60 min whether or not the culture had been treated with IF, but the enzyme activity in fluid from the treated culture was much lower. This could be because some inhibitor for this enzyme appears in the medium. Such a possibility was ruled out by the finding that a 1:1 mixture of the two fluids had an activity which was equal to the average value of the two separate activities (Fig. 1). The reduction in reverse transcriptase activity was comparable to the reduction in infectious virions released into the medium (Table 1). In a total of four similar experiments, the same ratio between IF-treated and untreated cells in both assays was obtained. However, it could still be argued that perhaps
Table 1. *Virus production and synthesis of cellular macromolecules in IF-treated and untreated cells*

| Interferon (RTRD₀₀ units/ml) | Reverse transcriptase (ct/min × 10⁻²) | Infectious virus units/ml (ct/min × 10⁻³) | Cell number | Incorporation (ct/min × 10⁻²) of
|-----------------------------|-------------------------------------|------------------------------------------|-------------|--------------------------------|
| 0                           | 49.5                                | 5.4 × 10⁸                                | 1.4 × 10⁶  | 42.4 90.7 2.1 11.0
| 3                           | 10.5                                | 1.0 × 10⁸                                | 1.3 × 10⁶  | 40.5 83.7 2.5 10.3

* Cells were plated with or without IF. After 24 h the cultures were rinsed with PBS, provided with fresh medium containing the appropriate radioactive label, further incubated for 1 h and analysed for incorporation of radioactive label. Other cultures received no label and were incubated for additional 4 h before measurement of reverse transcriptase activity, infectious virus and cell number.

IF had no effect on the overall formation of the virus, but rather introduced some defect in the reverse transcriptase, thus reducing the infectivity which is highly dependent on this enzyme (Hanafusa & Hanafusa, 1971; Hanafusa et al. 1972). To clarify this point, NIH/3T3 (M-MLV) cells were plated with or without 4 RTRD₀₀ units of IF per ml. After 24 h the cultures were washed with PBS, overlaid with medium containing ³H-uridine or ³H-proline and further incubated for 5 h to allow the release of virions labelled either in their RNA or protein. Fig. 2 shows that in both cases the reduction in the particle-associated radioactivity was comparable to that previously observed in reverse transcriptase and infectivity. The IF
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Development of the antiviral state in NIH/3T3 (M-MLV) cells

We have previously shown that when NIH/3T3 (M-MLV) cells have been treated with IF, virus is released into the medium after IF removal at a rate which is inversely proportional to the log of the IF dose (Aboud et al. 1976). The lowered rates of virus release represent, therefore, the level of the IF-induced antiviral state. This offers an easy way to follow the kinetics of the development of the antiviral state. For this purpose two groups of cultures were treated with, respectively, 1.5 and 3 RTRD₅₀ units of IF per ml. After different times of incubation one culture from each group was rinsed three times with PBS and provided with IF-free medium. Reverse transcriptase was assayed in the medium of each culture 2 h after IF removal and compared to that of a control culture which received no IF. Virus release was unaffected by IF up to 4 h after its addition (Fig. 3). With both concentrations of IF, inhibition was about half maximum at 6 h, and maximum at about 12 h. Only the level of the antiviral state was affected by the IF dose, but not the kinetics of its development.
Fig. 5. Effect of IF (4 RTRD$_{50}$/ml) on virus release and on virus-specific RNA in the nucleus and cytoplasm of NIH/3T3 (M-MLV) cells. (a) Nuclear and (b) cytoplasmic fractions from cultures treated with IF for 24, 48 or 72 h were assayed for virus-specific RNA. One culture at each time was rinsed with PBS, supplied with fresh IF-free medium, incubated for 4 h and tested for virus released by the reverse transcriptase assay (c). Empty bars: untreated control cells. Filled bars: IF-treated cells.

Recovery from the antiviral state after IF removal

It was of interest to find out how long after IF removal the antiviral state was maintained in cells, and whether this period was affected by the IF dose. Cells were plated in the presence of 1, 2 or 4 RTRD$_{50}$ units of interferon/ml, or without IF (control cultures). After 24 h all cultures were rinsed three times with PBS and received fresh medium without IF. At various times thereafter, 50 µl samples of medium were assayed for reverse transcriptase. Fig. 4 shows that in all IF-treated cultures virus release proceeded linearly at a reduced rate for about 10 h after IF removal and then abruptly resumed approximately the normal rate of the control culture. The dose of IF affected the rate of virus accumulation, but not the time for which this lowered rate was maintained.
**Effect of IF on virus RNA accumulation**

In this study we investigated the effect of IF on virus production by NIH/3T3 (M-MLV) cells at the molecular level. Fig. 5 illustrates one representative out of five replicate experiments in which we compared the amounts of virus RNA in the cytoplasmic and nuclear fractions of cells treated for various periods with 4 RTRD₅₀ units/ml with those in fractions of parallel untreated cells. There were no significant differences in the nuclear virus-specific RNA of IF-treated and untreated cells at 24 h (Fig. 5a). However, at 48 h the amount of virus RNA in the nucleus of IF-treated cells was 3 to 4 times greater than in untreated cells, and a slight but reproducible difference was still observed after 72 h. In contrast, when the cytoplasmic fraction was analysed (Fig. 5b), there were virtually no differences between IF-treated and untreated cells at the three times, and virus release was inhibited to about the same extent (Fig. 5c).

**DISCUSSION**

IF was found to inhibit virus release by NIH/3T3 cells chronically infected with M-MLV. This was not a consequence of general cytotoxicity, since no effects of IF treatment on synthesis of cellular DNA, RNA or protein were detected. A similar effect of IF was observed when measured in terms of reverse transcriptase, infectivity or physical virus particles. This finding is consistent with that of Friedman & Ramseur (1974). However, Pitha et al. (1976) found IF had a much greater effect on the infectivity of the released virions than on their reverse transcriptase activity. They interpreted their observation as indicating that IF interferes with the assembly of the virions in a manner leading to the formation of non-infectious particles.

Virus release was inhibited to an extent dependent on the dose of IF used. In contrast, the rate at which the antiviral state developed was not affected by the IF dose; with each dose, the corresponding maximum level of the antiviral state was reached after 12 h incubation with IF. Similarly, the antiviral state lasted for 10 h after removal of IF, and was unaffected by the IF dose. These two findings suggest that the level of the antiviral state produced in each individual cell is about the same with all IF doses used, and that the parameter which varies according to the IF dose is the number of IF-affected cells in the culture. The apparent abrupt return of virus release to the normal rate can be explained by the concept that IF blocks a late stage in virus development.

Examination of the effect of IF on the amounts of virus RNA in nuclear and cytoplasmic fractions from treated cells revealed no significant difference from untreated cells at 24 h after the addition of IF. However, after 48 h there was a marked increase in virus-specific RNA in the nuclear fraction of IF-treated cells and a smaller though reproducible difference after 72 h. No such changes were detected in the cytoplasm of IF-treated cells. The reasons for this are not yet known. However, one can speculate that some control mechanism activated in IF-treated cells maintains normal levels of virus RNA in the cytoplasm by preventing further delivery of virus RNA from the nucleus. This leads to accumulation of more virus RNA in the nucleus than in untreated cells. When this accumulation exceeds a certain level, another mechanism is triggered which either halts the synthesis of virus RNA or leads to its degradation.

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