Effects of Interferon on the Human Clonal Cell Line, RSa: Inhibition of Macromolecular Synthesis

By A. Fuse and T. Kuwata

Department of Microbiology, School of Medicine, Chiba University, Chiba 280, Japan

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SUMMARY

Multiplication of the human clonal cell line, RSa, is completely inhibited by human leukocyte interferon preparations. Synthesis of DNA and protein is markedly reduced in these cells in proportion to the concentration of interferon applied. Interferon treatment leads to accumulation of cells of an epithelial morphology which do not enter the division cycle. It is suggested that the growth inhibitory effects of interferon on RSa cells may result from these effects.

INTRODUCTION

The relationship between interferon and cell growth has been a subject of investigation for many years. Many workers have found that the multiplication of transformed cells was inhibited by interferon preparations, whereas normal fibroblasts were not affected or were less affected (see review by Gresser, 1972). Macieira-Coelho et al. (1971) suggested that the inhibition of cell multiplication was caused by a delay in the entry of interferon-treated cells into the division cycle. Inhibitory effects of interferon on the synthesis of macromolecules such as protein (Johnson, Lerner & Lancz, 1968) and DNA (Macieira-Coelho et al. 1971; O'Shaughnessy, Lee & Rozee, 1972; O'Shaughnessy et al. 1974; Tovey, Brouty-Boyé & Gresser, 1975), and on polynucleosome formation (Brouty-Boyé et al. 1973), have also been reported.

On the other hand, it has been shown that in interferon-treated cells, virus multiplication in vivo and in vitro is inhibited at the level of translation or transcription (see review by Oxman, 1973). Revel et al. (1975) reported that translation of both virus and cellular RNAs was decreased in a cell-free protein-synthesizing system obtained from interferon-treated cells.

We have previously described some highly interferon-sensitive human clonal cell lines (Kuwata et al. 1976a; Kuwata, Fuse & Morinaga, 1976b, previous paper). In this paper we report that there is inhibition of protein and DNA synthesis and of cell division in one of these lines, RSa, when treated with human leukocyte interferon.

METHODS

Cells and interferon. RSa cells and the interferon-resistant IFr cells were described in the accompanying paper (Kuwata et al. 1976b), as were the human leukocyte interferon and mock interferon preparations. Cell monolayer cultures were prepared by inoculating 60 mm plastic plates with approx. 2 to 3 x 10⁶ cells in 5 ml of Eagle's Minimal Essential Medium.
(MEM) supplemented with 10% bovine serum and antibiotics (100 μg/ml streptomycin and 100 units/ml penicillin G). Cultures were used for experiments one or two days after seeding.

**Measurement of DNA, RNA and protein synthesis.** RSA and IF<sup>T</sup> cells in 60 mm plastic dishes were re-fed with MEM containing various concentrations of interferon and incubated in a 5% CO<sub>2</sub> atmosphere at 37 °C. The medium was changed every day.

The incorporation of <sup>3</sup>H-thymidine, <sup>3</sup>H-uridine and <sup>14</sup>C-amino acids (Daiichi Pure Chemicals Co., Tokyo) into acid-precipitable and soluble fractions was determined as follows. After the appropriate incubation with or without interferon, the medium was decanted and the monolayers were rinsed with MEM. The cells were then incubated simultaneously with 3 μCi of <sup>3</sup>H-uridine (27.4 Ci/mmol) and 3 μCi of <sup>14</sup>C-protein hydrolysate (40.3 Ci/mmol) in 3 ml of MEM (without amino acids) for 20 min at 37 °C in 5% CO<sub>2</sub>. Three μCi of <sup>3</sup>H-thymidine (10.7 Ci/mmol) in 3 ml of MEM were added to another set of plates which were incubated for 40 min under the same conditions as above. After incubation, the medium was decanted and the monolayers were washed three times with 5 ml of ice-cold phosphate buffered saline (PBS), at pH 7.4. The cells were removed from the plastic plates with 0.25% (w/v) trypsin in PBS (without Ca<sup>2+</sup> and Mg<sup>2+</sup>) and then centrifuged, resuspended in ice cold PBS, diluted appropriately in PBS and counted with a haemocytometer.

The cells were disrupted by mild sonic oscillation (20 kHz, 15 W, 5 s) and the extracts precipitated by adding equal volumes of 20% (w/v) trichloroacetic acid (TCA). Acid-precipitable materials and soluble fractions were separated by filtration through glass fibre papers (Whatman GF/C). Acid-precipitable materials on filters were washed three times with 5 ml of 5% TCA and dried. The filter papers were placed in vials containing 8 ml of toluene scintillant, and radioactivities were counted in a Beckman LS-235 liquid scintillation counter. Samples of acid-soluble fractions were transferred to GF/C glass fibre papers. The papers were dried and their radioactivity was similarly measured.

**Cell protein content.** Cells were disrupted by sonic oscillation and their protein content was measured as described by Lowry *et al.* (1951). Crystalline bovine plasma albumin was used as the standard.

**RESULTS**

**The effect of interferon on RSA cell growth**

RSA and IF<sup>T</sup> cells were re-fed with medium containing 500 units/ml of interferon. Growth curves for control and interferon-treated cells are shown in Fig. 1. When RSA cells were treated with 500 units/ml of interferon, their growth was completely suppressed after 2 days (Fig. 1a). In contrast, the effect of interferon at the same concentration was less marked in cells of the interferon-resistant IF<sup>T</sup> line (Fig. 1c). Mock interferon prepared in the same manner as the interferon had no apparent effect on the multiplication of RSA cells (Fig. 1b).

**Interferon treatment and DNA synthesis**

After 2 days treatment with interferon RSA cells were pulse-labelled with <sup>3</sup>H-thymidine. Fig. 2a shows that the greater the interferon concentration, the less was the incorporation of <sup>3</sup>H-thymidine into both acid-soluble and especially acid-insoluble materials in treated cultures. These effects on the incorporation of <sup>3</sup>H-thymidine were also dependent on the duration of interferon treatment (Fig. 3a), and maximum inhibitions of 31% in the acid-soluble fraction and 62% in the acid-insoluble fraction were observed when cells were treated with interferon for 3 days.
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Fig. 1. The effect of interferon on the multiplication of RSa and IF' cells. Cells were treated with interferon (500 units/ml) or mock interferon (equivalent to 5000 'interferon' units/ml) 24 h after seeding into 60 mm Petri dishes: (a and c) ••, no treatment; ◦◦, 500 units/ml interferon; (b) ••, no treatment; ◦◦, mock interferon.

Fig. 2. Dose-dependent effects of interferon on (a) DNA, (b) RNA and (c) protein synthesis in RSa cells. RSa cell cultures were treated with interferon at different concentrations for 2 days. Points show the radioactivity per interferon-treated cell as a percentage of that in control cells, and are the means from duplicate samples: ◦◦, acid-soluble; ••, acid-insoluble fractions.

Interferon-treated IF' cells were similarly pulse-labelled with 3H-thymidine. The incorporation of 3H-thymidine into both acid-soluble and insoluble fraction was inhibited slightly and at almost the same rate (Fig. 4a).

Mock interferon had no detectable effect on 3H-thymidine incorporation into the acid-insoluble fraction (Table 1). Thus, interferon preparations inhibit DNA synthesis of RSa cells preferentially in addition to having an effect on total 3H-thymidine uptake into cells.
Table 1. Effect of interferon (IF) at 500 units/ml and mock interferon preparations on DNA, RNA and protein synthesis in RSa cells

| Incorporation of radioactive precursors into macromolecules | $^{3}$H-thymidine ($\text{ct/min}^\star/\mu\text{g protein}$) | $^{3}$H-uridine ($\text{ct/min}^\star/\mu\text{g protein}$) | $^{14}$C-amino acids ($\text{ct/min}/\mu\text{g protein}$) |
|---|---|---|
| Control | 600 (100.0) | 145 (100.0) | 120 (100.0) |
| IF | 234 (39.0)† | 168 (115.8) | 95 (79.2) |
| Mock IF 1‡ | 616 (102.6) | 180 (124.1) | 139 (115.8) |
| 2§ | 615 (102.5) | 208 (148.9) | 141 (117.5) |

* Radioactivity of TCA-insoluble fraction after treatment for 2 days.
† % of control.
‡ Concentration equivalent to 1000 ‘interferon’ units/ml.
§ Concentration equivalent to 5000 ‘interferon’ units/ml.

Fig. 3. The effect of the duration of interferon treatment on (a) DNA, (b) RNA and (c) protein synthesis in RSa cells. Cells were treated with 500 units/ml of interferon for the number of days indicated: ○——○, acid-soluble; ●——●, acid-insoluble fractions.

Interferon treatment and RNA synthesis

Interferon treatment of RSa cells led to a slight increase in the rate of $^{3}$H-uridine uptake. There was an increase of specific radioactivity due to $^{3}$H-uridine in the intracellular pool (Fig. 2b and 3b), and the comparably increased rate of RNA synthesis presumably reflected this increase.

Mock interferon also stimulated the rate of $^{3}$H-uridine uptake by RSa cells (Table 1), so that the stimulation caused by interferon preparations was not caused by the interferon molecules present in them.

Interferon treatment and protein synthesis

The effect of interferon on $^{14}$C-amino acid uptake into the intracellular pool and on the synthesis of protein was also investigated. There was a significant decrease in protein
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Fig. 4. The effect of interferon on (a) DNA, (b) RNA and (c) protein synthesis in IF² cells. Cells were treated with 500 units/ml of interferon for the number of days indicated: ⋄—⋄, acid-soluble; ⌧—○, acid-insoluble fractions.

Fig. 5. The morphology of RSa cells: (a) control cells; (b) treated with 500 units/ml of interferon for 2 days.
Fig. 6. The effect of interferon on the protein content of RSa cells. The cellular protein content was
determined as described in the text. •—•, non-treated; ○—○, interferon treated (500
units/ml).

synthesis in interferon-treated RSa cells depending on the interferon concentration (Fig.
2c) and the duration of treatment (Fig. 3c). When RSa cells were treated with 500 units/ml
of interferon for 3 days, their protein synthesis decreased to 58% of that of non-treated cells
(Fig. 3c). In contrast to the changes in uptake in $^3$H-thymidine, uptake of $^{14}$C-amino acids
by the same cell cultures was inhibited only slightly more by increasing the duration of
treatment with interferon.

In interferon-resistant IF$^r$ cells, interferon treatment did not affect uptake of $^{14}$C-amino
acids or their incorporation into proteins (Fig. 4c).

**Cellular protein content and morphology**

A striking morphological change became evident in RSa cells after interferon treatment
(Fig. 5). The number of fibroblastic type cells decreased and the percentage of epithelial-
type cells increased relatively after treatment for 24 h. Epithelial-type cells predominated at
48 h. The protein content of the cells in interferon-treated cultures was slightly lower than
that of untreated control cells at one day, but then increased during the next four days to
reach a level approximately twice that of control cells (Fig. 6). Since epithelial-type cells
accumulate in interferon-treated RSa cultures, the inhibitory effect of interferon is presumed
to result from these cells being prevented from entry into a particular phase of the cell cycle.
There were no such morphological changes in interferon-treated IF$^r$ cell cultures, or in
RSa cultures treated with mock interferon.
DISCUSSION

Since Paucker, Cantell & Henle (1962) first showed inhibitory effects of interferon on cell multiplication, there have been many reports of such effects, for example with human interferon in suspended cell cultures (Cantell, 1970; Adams, Strander & Cantell, 1975) and monolayer cultures (Lee, O'Shaughnessy & Rozee, 1972; Gaffney, Picciano & Grant, 1973). Like RSb cells (Kuwata et al. 1976b), RSA cells are very sensitive to human leukocyte interferon, which can completely suppress their growth, whereas with most of the cells previously described, interferon merely slowed their rate of growth. Therefore, RSA cells have advantages for a detailed analysis of the events which lead to inhibition of cell multiplication by interferon.

It has been suggested that the most probable explanation for interferon-mediated inhibition of cell multiplication is a delay in the entry of treated cells into the division cycle (Macieira-Coelho et al. 1971). Another report showed that the depression by interferon of growth of L-929 cells in synchronized cultures resulted from a delay in DNA synthesis both in terms of thymidine incorporation and of the appearance of the mitotic cells (O'Shaughnessy et al. 1974). We found inhibition of 3H-thymidine incorporation into both acid-soluble and especially acid-insoluble materials in human RSA cells treated with human leukocyte interferon preparations. However, further studies are needed to determine what causes this inhibition of 3H-thymidine incorporation into acid-insoluble materials (for instance, changes in the activity of DNA polymerase or in the size of the cellular nucleotide pool etc.). Tovey et al. (1975) reported inhibition of 3H-thymidine incorporation into acid-soluble and acid-insoluble extracts from mouse leukaemia L1210 cells cultivated in a chemostat in the presence of mouse interferon. Our results are consistent with theirs. On the other hand, they found no such effect in interferon resistant L1210 cells. As can be seen in Fig. 4a, the incorporation of 3H-thymidine into acid-soluble and acid-insoluble materials was reduced slightly and to the same extent in treated IFr cell cultures. The difference between the results of Tovey and colleagues (1975) and ours is due to the different sensitivity of the two cell lines to interferon (Kuwata et al. 1976b).

Mouse interferon inhibits the protein synthesis of L cells (Johnson et al. 1968). Our results show that human leukocyte interferon also inhibits the protein synthesis of human RSA cells.

Interferon preparations stimulated 3H-uridine uptake but this effect was also observed in RSA cell cultures treated with mock interferon preparations (Table 1), suggesting that it was caused not by interferon but by serum or other substances present in the interferon preparations.

The factor responsible for the inhibition of cell macromolecular synthesis is presumably interferon, as discussed in the previous paper (Kuwata et al. 1976b). However, more highly purified interferon preparations are needed help to solve this problem.

Gresser (1961) noticed morphological changes in interferon-treated human amnion cells. Microscopic studies showed that interferon treatment led to accumulation of RSA cells of an epithelial type. These were also found in non-treated cultures as one type of cell morphology during the cell cycle. These accumulated cells did not enter into division, and their cell volume estimated in terms of their protein content per cell increased on prolonged incubation with interferon. One possible explanation is that, as the result of a higher degree of inhibition of DNA synthesis than of protein synthesis, the cell volume increased even though the rate of protein synthesis was decreased. Thus we suggest that interferon may inhibit the multiplication of RSA cells because the cells are prevented from entering into a
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particular phase in the cell cycle. This could be the result of inhibition of DNA synthesis or of protein synthesis, or both. The consequence is that the cell eventually degenerates (Kuwata et al. 1976b).

To elucidate the precise action of interferon in the cell cycle, experiments using RSa cells in synchronized culture are currently in progress. Preliminary results show that DNA synthesis in the S phase is completely inhibited when interferon is added late in the G1 phase, and the cells remain in the G1/S boundary phase. These data give additional support to the hypothesis presented in this paper.

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REFERENCES


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