Effects of Leukocyte and Fibroblast Interferon on Events in the Fibroblast Cell Cycle

By E. LUNDBRED, I. LARSSON, H. MIÖRNER AND Ö. STRANNHEGARD

Institute of Pathology and Department of Virology,
University of Umeå, Sweden

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SUMMARY

Serum-depleted human foetal skin fibroblasts were stimulated by addition of 10% foetal calf serum to proliferate synchronously for at least one cell cycle. This proliferation was suppressed by leukocyte or fibroblast interferon (IF), which prolonged the GI phase and diminished the rate of DNA synthesis during the S phase in a dose-dependent manner. When used in identical concentration, as judged in terms of units of antiviral activity, fibroblast IF had more pronounced effects on cell cycle events than leukocyte IF. Interferon exerted its effect in early GI, before the cells were irreversibly committed to DNA synthesis.

INTRODUCTION

Interferons (IF) have been shown to inhibit the growth of proliferating cells, the so-called anticellular activity (Paucker et al. 1962; Gresser et al. 1970a). This activity of IF has mostly been studied in transformed cell lines (Gresser et al. 1970a, b; Adams et al. 1975) but has also been found with normal cell lines and even with primary cultures (Lindahl, 1974; Dahl & Degré, 1976). Such non-neoplastic cell lines are subject to a strict growth control, and some important details of the mechanisms involved have been worked out (Smith & Martin, 1973; Pardee, 1974; Lindgren et al. 1975). In attempting to define the mechanism of the anticellular action of IF, we have therefore chosen to use proliferating normal cells. In the present paper we report that IF prolongs the GI phase and reduces the height of the S peak by reducing the number of cells in the S phase. The point of attack of IF seems to be some early events during GI.

METHODS

Interferon. Partially purified human leukocyte IF (PIF) with a sp. act. of $2.5 \times 10^5$ units/mg protein was kindly supplied by the Finnish Red Cross Blood Service. Fibroblast IF was produced by inoculating human embryonic fibroblast cell cultures with 600 haemagglutination units per ml of Sendai virus. The medium from inoculated cultures was harvested after 24 h and then dialysed, first against 0.1 M-glycine-HCl buffer, pH 2.0, for 24 h and then against phosphate-buffered saline, pH 7.3, for 48 h. The preparation was concentrated by precipitation with 30% polyethylene glycol 4000 to achieve a sp. act. of $2.5 \times 10^4$ units/mg protein.

Interferons were assayed on amnion U cells in microtrays with vesicular stomatitis virus as described by Havell & Vilček (1972). An internal laboratory reference IF standard, calibrated against the research standard 69/19 (National Institute for Medical Research, London, England), was included in each assay.
Cell and culture conditions. The fibroblast cell lines were derived from the skin of human foetuses from abortions induced during the 16th to 24th weeks of pregnancy. The cells were used between the 5th and 20th passage. Cell lines from five different foetuses were used, but no differences between them were found in the parameters measured.

Cells were grown in 90 mm plastic dishes (Nunc, Roskilde, Denmark) with Eagle's minimum essential medium (MEM) supplemented with antibiotics and 10% foetal calf serum (FCS), unless otherwise stated. The cultures were incubated in humidified air with 5% CO₂ at 37°C. The cells were split in a 1:2 ratio every 3rd or 4th day.

Assay of cell proliferation

Thymidine incorporation. The fibroblast cells were suspended in MEM containing 10% FCS and seeded at a density of 5 × 10⁴ cells/cm² growth surface in microtrays (Falcon Plastics Co., Oxnard, California). Three days later, when the cell number was about 1.5 × 10⁵ cells/cm², the medium was changed to MEM with 0.5% FCS and the cultures were further incubated for 24 h (the cells at this stage are termed 'serum-deprived' cells in this paper). The experiments started with a change to medium with 10% FCS (serum stimulation) or as described in the Results. At the appropriate time later the cells in the microtrays were pulsed for 60 min with 1 μCi/ml tritiated thymidine (³H-dThd; sp. act. 61 mCi/mM) in MEM without serum. The cultures were then trypsinized, washed and precipitated by TCA to a glass fibre filter by a semi-automatic multiple sample processor (Hartzman et al. 1972).

Autoradiography. Cells were grown on coverslips placed in Linbro tissue culture trays (FB-24TC, Flow Laboratories, Irvine, Scotland) in MEM with 10% FCS, with or without IF, and containing 1 μCi/ml of ³H-dThd. After 24 h the cultures were fixed in methanol-acetic acid for 1 h, washed in 70% ethanol, air dried and dipped in Ilford G5 emulsion (Ilford Ltd, Ilford, England). After an exposure time of 2 to 3 weeks the autoradiograms were developed, stained in May-Grünwald-Giemsa and counted in duplicate by light microscopy using an oil-immersion objective. Only cells with more than 10 to 20 grains over the nuclei were counted as S phase cells.

Cell number estimation. Cells were seeded at a density of 1.5 x 10⁵ cells/cm² growth surface. After different time periods in the experimental media, the cells were trypsinized and counted in an electronic cell counter (Linson Instrument AB, Stockholm, Sweden).

RESULTS

Effects of serum on cell growth

When cells serum-deprived for 24 h were incubated for a further 24 h with fresh medium containing different amounts of serum, the rate of ³H-dThd incorporation increased progressively with the serum concentration and reached a maximum with 10% FCS. In a similar experiment, cell numbers were determined after 72 h of growth with different serum concentrations. A threefold increase in cell number was achieved with 10% FCS (Fig. 1). There was at least one additional round of cell division in all the cultures if they were then re-fed with 10% FCS, regardless of the previous serum concentration. Therefore, with 10% FCS the rate of DNA synthesis and the increase in cell number was optimal; also, the cells were not adversely affected by exposure to low serum concentration. Therefore, in the following experiments, growth arrest and growth stimulation was achieved by culturing the cells in 0.5% and 10% FCS, respectively.

Fig. 2 shows the kinetics of ³H-dThd incorporation after shift from 0.5% to 10% FCS.
Interferon and cell cycle events

Fig. 1. Effect of foetal calf serum (FCS) on cell number (calculated as the cell density in the Petri dish). Serum-deprived cells were re-fed with fresh medium containing FCS in the indicated concentrations. ○—○, Cell number after 72 h; ●—●, cell number after another 72 h period, during which the serum concentration in the medium in all the cultures was changed from that indicated to 10% FCS.

Fig. 2. Kinetics of ³H-dThd incorporation in serum-deprived cells cultured in 10% FCS. At the times indicated after change to 10% FCS the cultures were pulsed with ³H-dThd.

The pre-replicative G1 phase as measured from the start of serum stimulation to the 50% point of the ascending limb of the ³H-dThd incorporation curve amounted to 19.3 ± 0.4 h. The S phase, measured as the distance between the 50% points on the ascending and descending parts of the curve, lasted 14.8 ± 1.0 h. In many experiments a second wave of synchronous DNA-synthesis was easily discernible.

Effect of IF on cell growth

Fig. 3. shows that IF preparations had a marked effect on cell growth. When 1000 reference units/ml were used, leukocyte IF reduced the increase in cell number during 144 h by 34%. An equivalent amount (in terms of antiviral units, as measured on amnion U cells) of fibroblast IF had a bigger effect: the increase in cell number was reduced by 90%. These effects were abolished by treatment of the IF preparations with trypsin, but not by treatment with RNase and pH 2.

To analyse this effect on growth, the rate of ³H-dThd incorporation into TCA-precipitable material was studied during the cell cycle. Fig. 4 shows that when leukocyte IF was added to serum stimulated cultures, G1 was prolonged and the height of the S peak was depressed in a dose-dependent way. There was no obvious change in the duration of the S phase. Fibroblast IF had a similar dose-dependent effect on G1 and S phases (data not shown).

Table 1 shows that even when serum and IF had been removed, IF still affected uptake of ³H-dThd into the cells during a 60 min pulsing period. At a concentration of 1000 units/ml both interferons reduced the TCA-soluble ³H-dThd pool by 75%. However, the pool size was the same after 10 h in 10% FCS as after 24 h, whether or not the cells displayed an S peak during the different treatments. Thus, transport of thymidine into the cells did not seem to be the rate limiting step. Similar results were obtained after 13 and 17 h (data not shown).
Fig. 3. Effect of interferon on serum-stimulated cell growth. Serum-deprived cells were re-fed at the start of the experiment with fresh medium containing 10% FCS and 1000 units/ml leukocyte IF (■—■), or 1000 units/ml fibroblast IF (▲—▲), or no IF (○—○). Cell numbers are calculated as the cell density in the Petri dish.

Fig. 4. Effect of leukocyte IF on cell cycle kinetics. Experimental conditions as in Fig. 3. Cells were cultured in absence of IF (○—○), or in presence of 50 units/ml (▲—▲), 250 units/ml (■—■), 500 units/ml (△—△), or 1000 units/ml (▲—▲) of IF.

| Table I. Uptake of $^3$H-thymidine by control and interferon-treated cells* |
|---------------------------------|------------------|------------------|
|                                 | TCA-precipitable | TCA-soluble      |
|                                 | fraction (ct/min) | fraction (ct/min) |
| Treatment                       | Duration (h)     |                  |                  |
| Control                         | 10               | 465 ± 40         | 1069 ± 76        |
|                                 | 24               | 2845 ± 234       | 977 ± 109        |
| Leukocyte IF                    | 10               | 323 ± 15         | 281 ± 16         |
|                                 | 24               | 2112 ± 152       | 307 ± 36         |
| Fibroblast IF                   | 10               | 185 ± 10         | 244 ± 11         |
|                                 | 24               | 125 ± 10         | 282 ± 36         |

* Fibroblast cells were grown in 10% FCS in the presence of the indicated interferon at 1000 units/ml for 10 h or 24 h and were then pulsed with $^3$H-dThd for 60 min in medium containing no serum or interferon. The mean ct/min (± s.e.) from four replicate cultures are shown.
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Fig. 5. Effect of IF on the entry of cells into S. Serum-deprived cells were stimulated with medium containing 10% FCS in the presence of ³H-dThd (1 μCi/ml). At the times shown the cells were fixed and autoradiograms were prepared. O—O, control; ■—■, 1000 units/ml leukocyte IF; ▲—▲, 10000 units/ml fibroblast IF.

Fig. 6. Comparison between the effects of serum depletion (O—O) and interferon addition (●—●) on cellular DNA synthesis. At the indicated times cultures were changed to serum-free medium, or leukocyte interferon (2500 units/ml) was added. Incorporation of ³H-dThd was determined 24 h after time 0 in all cultures by pulsing for 1 h.

Effects of IF on rate of entry into S phase

Autoradiography experiments were performed with cells labelled with ³H-dThd for 24 h after stimulation with 10% FCS in the presence or absence of 1000 units/ml of leukocyte or fibroblast IF. The results are represented in Fig. 5 according to the suggestions of Smith & Martin (1973) in terms of the fraction of unlabelled cells after different time periods, i.e. the fraction of cells remaining in G1 or the A state.

During the first 16 h there was a small decrease in the proportion of cells in G1 (unlabelled nuclei). Then the slope changed, indicating that the cells started to enter S at a constant rate, calculated as 0.045 h⁻¹. With cells treated with 1000 units/ml leukocyte IF, there was a delay before the cells changed their rate of entrance into S (constant estimated as 0.016 h⁻¹).

In contrast, during the observation period of 30 h, all the cells treated with 1000 units/ml fibroblast IF remained in G1. At comparable time periods, there was no obvious difference in the number of grains per nucleus, which indicates that IF did not exert its effect mainly by decreasing phosphorylation of thymidine. Therefore, our method of estimating ³H-dThd in TCA-precipitable material seems to be a reasonable way to estimate the rate of DNA synthesis.

The G1 phase of the cell cycle can be subdivided into two distinct stages. This is illustrated by the experiment in Fig. 6, in which 10% FCS medium was added to all cultures at the start. At the indicated times the serum-containing medium was changed to medium devoid of FCS. ³H-dThd incorporation was estimated by pulsing all cells after 24 h. After 11 h, but not after 7 h, of continuous serum stimulation, there was a significant increase in DNA synthesis. Calculated from the 50% point of the ascending limb, it appears that the cells were able to pass the G1/S transition point after 14.5 ± 0.6 h of continuous serum stimulat-
tion. As G\textsubscript{i} was 19.3 h, the serum-independent period, corresponding to the Glc period of Temin (1971), was 4.8 h. The same experiment was performed but with the addition of leukocyte IF at various times instead of a change to serum-free medium. As demonstrated in Fig. 6, addition of IF had the same effect on cell proliferation as serum depletion. Thus, IF suppressed DNA synthesis only if added before the start of the Glc phase.

DISCUSSION

Few and seemingly conflicting data are currently available concerning the effects of IF preparations on cell cycle events. From studies of the effect of IF on L1210 cells, Macieira-Coelho \textit{et al.} (1971) suggested that IF delays the entry of cells into the division cycle. Killander \textit{et al.} (1976), however, reported that L1210 cells were arrested in all stages of the cell cycle and not in a particular stage, and that the proportion of cells in the different stages were the same as in control cultures. However, these authors did not comment on their finding that it was more than 24 h before the cell number was decreased; this could mean that IF had to await specific cell cycle events to express its effect. Fuse & Kuwata (1976) claimed that virus-transformed human fibroblasts were blocked in the transition from G\textsubscript{i} to S if leukocyte IF was given late in G\textsubscript{i}. In L cells synchronized by double thymidine block, IF delayed the S phase by several hours if given when the block was released, i.e. at the G\textsubscript{i}/S transition (O'Shaugnessy \textit{et al.} 1972).

In these cited studies, only neoplastic cell lines were used and these may have defects in the control of the cell cycle. We have used serum-stimulated and non-neoplastic fibroblast cultures, which are subject to strict growth control, and our results are apparently different, for reasons which are not yet clear.

Several authors have pointed out the presence of two distinct restriction points in cell lines with strict growth control (Smith & Martin 1973; Pardee, 1974; Lindgren \textit{et al.}, 1975). One point is at the transition from the resting stage into the cell cycle (Go/G\textsubscript{i}), a step normally initiated by several hormone-like growth stimulating factors and serum. When the cells have passed the other point (transition from G\textsubscript{i} proper to Glc), they start DNA synthesis irreversibly and independent of the presence of these stimulatory factors.

Our results indicate that the primary point of attack of fibroblast and leukocyte IF in diploid, non-neoplastic fibroblasts is during the pre-replicative G\textsubscript{i} phase, prior to Glc. Once the cells have passed the G\textsubscript{i} proper/Glc transition, they do not seem to be sensitive to the anticyclical effect of IF.

IF exerted its effect during the growth factor dependent part of G\textsubscript{i}, but had no effect on DNA synthesis if added during Glc. It therefore seems certain that the prime target of attack is on the triggering system regulating cell proliferation and not on the process of DNA synthesis. Several interesting possibilities can be suggested to explain this: IF may compete with growth factors for receptors on the cell surface; it may diminish the number of receptors for growth factors; or it may alter the affinity of the receptors for growth factors. Our experimental data give no answers to these questions, but hint that interferon may be an important probe for the mechanism of growth control in normal and neoplastic tissue.

During the revision of this manuscript a report by Sokawa \textit{et al.} (1977) showed similar interferon effects on the cycle of 3T3 cells, but in their system the G\textsubscript{i} phase was not prolonged.
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