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Interferon as an Inhibitor of Cell Growth: Studies with Mouse Melanoma Cells (40760)

LEE RATNER,* JAMES J. NORDLUND,† AND PETER LENGYEL*

*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520 and †Department of Dermatology, West Haven VA Hospital, West Haven, Connecticut 06516

Introduction. Interferons are glycoproteins which are synthesized in various animal cells following viral infection. They are excreted, interact with other cells, and inhibit in them the replication of a broad range of viruses (1). Many other effects of interferon have been described including suppression of the multiplication of nonviral organisms such as chlamydiae (2, 3), rickettsiae (4, 5), protozoa (6, 7), and bacteria (8), inhibition of cell growth in tissue culture and in animals (9–11), sensitization of cells to the effects of various toxic agents such as double-stranded RNA (12, 13) and to amino acid starvation (14), and alterations of the immune system (15). We have chosen to study the effect of interferons on the growth of Cloudman mouse melanoma cells in tissue culture and in mice.

Materials and methods. Chemicals. Minimal essential media F10, F14, F15, fetal calf serum, and penicillin and streptomycin were obtained from Grand Island Biological Company, horse serum from Flow Labs, and controlled pore glass (CPG-10-240 A, 120–200 mesh) from Accurate Chemical and Scientific Corporation.

Interferons. A partially purified interferon preparation (specific activity 2.0×10^7 units/mg protein) was obtained by infecting Ehrlich ascites tumor (EAT) cells with Newcastle disease virus (Slattery *et al.*, unpublished data), and purifying the interferon produced by precipitation with ammonium sulfate and chromatography on carboxymethyl–Sephadex (16). This interferon preparation was further purified by chromatography on phosphocellulose as described by Kawakita *et al.* (16). The active fractions were pooled and diluted 10-fold with water, dialyzed against 10 m*M* potassium phosphate, pH 7.5, 146 m*M* NaCl, 2.7 m*M* KCl, and 0.5% (v/v) glycerol (buffer A) and applied to a column of controlled pore glass which had been preequilibrated with buffer A. The active fraction was eluted with 0.4 *M* glycine·HCl, pH 3.5, 0.5% (v/v) glycerol; the specific activity of this interferon preparation was 9.0×10^8 units/mg protein. For tissue culture experiments, interferon samples were diluted in medium, and sterilized by filtration through 0.22 μm Nalgene filters without loss of activity. For experiments in mice, interferon was sterilized by filtration through 0.22 μm Nalgene filters, diluted in 140 m*M* NaCl, 0.5 m*M* sodium phosphate, pH 7.4 (buffer B), and aliquots were stored at −70°. All interferon units are NIH mouse reference standard units. Interferon of specific activity 2.0×10^7 units/mg protein was used for all experiments except where designated differently.

Cells and viruses. Cloudman melanoma 91, clone PCla cells were obtained from J. Pawelek (17) and were grown in tissue culture at 38° in F10 medium supplemented with 2% (v/v) fetal calf serum, 10% (v/v) horse serum, 0.1 mg/ml penicillin, and 0.1 mg/ml streptomycin (supplemented F10 medium), in the presence of 5% CO_2. Cell viability as measured by trypan blue staining was generally 99%.

Melanoma cells were also maintained in syngeneic DBA/2 mice. Tumors were produced by a subcutaneous injection into the back of a mouse of 1.5×10^6 cells from tissue culture or 8.0×10^5 cells from a suspension made from a tumor resected from a donor mouse. Tumors approximately 1 cm in diameter were excised from an animal killed by cervical dislocation. The tumor was minced and gently pushed through a fine screen. Large clumps of debris were removed by centrifugation at 4° and 700 rpm for 5 min. The preparations contained a large amount of cellular debris and 30–50% dead cells (J. J. Nordlund, manuscript submitted).

EAT and L929 cells were grown in sus-

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pension in F14 medium supplemented with 6.7% (v/v) fetal calf serum, 0.1% (w/v) glucose, 0.1 mg/ml penicillin, and 0.1 mg/ml streptomycin, or in monolayer in F15 medium supplemented with 6.7% (v/v) fetal calf serum, 0.1 mg/ml penicillin, and 0.1 mg/ml streptomycin (supplemented F15 medium) in the presence of 5% CO_2. The Indiana strain of vesicular stomatitis virus (VSV) was propagated on L929 cells as described by Stampfer *et al.* (18).

Yield reduction assays. The 1.0×10^5 melanoma cells were added to each 25-cm^2 Falcon flask and incubated with supplemented F10 medium at 38° for 24 hr. The medium was then replaced with fresh-supplemented F10 medium containing 0–10 units/ml interferon and incubated at 38° for an additional 24 hr. The medium was removed and seven plaque-forming units (pfu) per cell of VSV in 0.5 ml supplemented F15 medium was added to each flask. Incubation was continued at 38° for 90 min, with occasional swirling. The medium was removed, cells were washed with supplemented F15 medium, and 5 ml supplemented F15 medium was added. The cells were incubated at 38° for an additional 10 hr. The supernatant fractions were removed and stored at −70°. Virus concentrations in the supernatant fractions were determined by plaque assays on L929 cells as described by Vassef *et al.* (19).

Growth measurements. (*a*) *Tissue culture experiments.* Melanoma cells were released from monolayers in the presence of 5 m*M* EDTA in buffer C (0.5 m*M* sodium phosphate, pH 7.4, 140 m*M* NaCl, 2.7 m*M* KCl, 40 m*M* glucose), sedimented by centrifugation at 500*g*, and resuspended in supplemented F10 medium at $2–8 \times 10^4$ cells/ml. The 3 ml of the cell suspension was added to each 25-cm^2 Falcon flask. After incubation at 38° for 24 hr, the medium was removed and 3 ml of supplemented F10 medium containing 0–4000 units/ml interferon was added. Medium was removed from the cultures every 3 days, and fresh medium containing the same concentration of interferon was added. Cell counting was done with a Coulter counter Model B after releasing cells with 5 m*M* EDTA in buffer C and addition of 10 ml ice-cold buffer C. Three 0.5-ml portions of each cell culture were counted, and measurements on triplicate cultures averaged. The standard error was less than 10%. (The following settings on the Coulter counter were used: 1/amplification, 2; 1/aperture current, 1/2; lower threshold, 5; upper threshold, 100.)

(*b*) *Experiments in mice.* Melanoma cells were prepared from tissue culture or animal tumors. The cells were sedimented by centrifugation at 700*g*, washed twice in F10 medium (not supplemented), and injected into a subcutaneous site over the shaved back. The tumor was visible immediately after injection as a small black dot under the thin skin of the host. Its growth was quantified by measurement with a caliper of the longest axis and the perpendicular axis (both in the plane of the surface of the animal) ((20) and J. J. Nordlund, manuscript submitted). Interferon was administered by intraperitoneal injection (0.5 ml). Groups of mice receiving interferon consisted of five animals, mice in the control group, consisting of eight animals, were injected with 0.5 ml of buffer B.

Scanning microspectrophotometry. The 3 ml melanoma cells (1.5×10^5 cells) were added to each 25-cm^2 Falcon flask and incubated in the presence or absence of 3900 units/ml interferon for 5 days as described above. The control cells doubled every 40 hr, while the number of interferon-treated cells did not change. (Over 90% of the interferon-treated cells were viable after the treatment as measured by trypan blue staining.) At this time, the medium was removed and the cells were fixed and stained by the procedure of Feulgen and Rossenbeck (21). The absorbance of 100 cell nuclei from each sample was determined on a Zeiss Type 05 microscope photometer with a Wang 700 calculator (22).

Results. Interferon inhibits the growth of melanoma cells in tissue culture. The mouse melanoma cells used are good hosts for VSV replication, yielding 1280 pfu/cell. Furthermore, this cell line is quite sensitive to interferon; it shows 50% reduction in the single-cycle VSV yield at 1.7 units/ml interferon at a cell density of 7×10^4 cells/cm^2, i.e., confluence (data not shown), and

at about 0.1 unit/ml interferon at a cell density of 4×10^3 cells/cm^2 (Fig. 1, upper part). With the latter initial cell density, interferon markedly inhibited the growth of melanoma cells in tissue culture; a 50% reduction in cell number after 11 days of growth was achieved with 0.48 units/ml of a highly purified interferon preparation (specific activity 9.0×10^8 units/mg protein) or 0.92 units/ml of a less purified interferon preparation (specific activity 2.0×10^7 units/mg protein) (Fig. 1, lower part). This represents an average increase in the cell generation time (compared to that of the control cells), from 40 to 45 hr. (It should be noted that by 11 days even the cells grown in the absence of interferon did not reach confluency, whereas after a longer time even the cells grown in the presence of interferon did reach confluency.)

The difference in the concentration of the two interferon preparations required to achieve 50% inhibition of VSV replication (Fig. 1) is not significant since it is within the standard error of the interferon assay (E. Slattery, unpublished data).

Interferon did not affect cell viability; over 90% of the cells were viable after 11 days of treatment at all interferon concentrations. The effects of interferon treatment were reversible. Treating cells with 3900 units/ml interferon for 9 days decreased cell accumulation by greater than 90%. The cells recovered a normal growth rate within 7 days after replacement of the interferon-containing medium with interferon-free medium. The growth inhibitory effect of interferon was (a) independent of the total concentration of serum (horse serum and fetal calf serum in a ratio of 5:1 (v/v) in the range of 2 to 48% (v/v), and (b) the same whether the medium was changed every day or every 3 days (data not shown).

To establish if interferon delays the passage of cells through a specific phase of the cell cycle we examined the distribution of cells in various phases of the cycle by measuring the absorbance of individual Feulgen-stained nuclei by scanning microspectrophotometry. Figure 2A shows that most control cells exhibit a nuclear absorbance in the range of 35–45 which would be expected for G1 phase cells. Only about

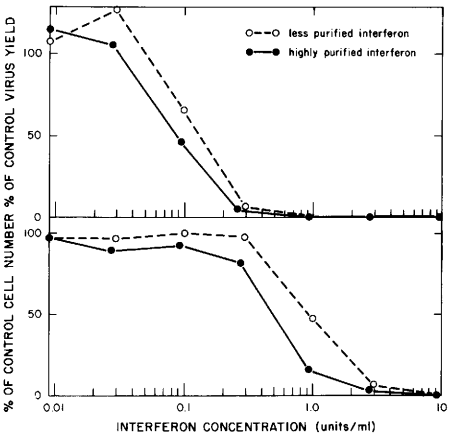

FIG. 1. Interferon of high specific activity inhibits the growth of melanoma cells. The 1.0×10^5 melanoma cells were added to each 25-cm^2 Falcon flask as described under Materials and Methods. After one day, the medium was replaced with fresh-supplemented Fl0 medium containing 0–10 units/ml of either an interferon preparation of specific activity 2.0×10^7 units/mg protein, "less purified" interferon" (○) or of an interferon preparation of specific activity 9.0×10^8 units/mg protein, "highly purified interferon" (●). The cells were grown as described under Materials and Methods. After 1 day of growth in the interferon-containing media or in the absence of interferon, one flask from each group was infected with VSV and the reduction of virus yield was determined (upper part of the figure) as described under Materials and Methods. The rest of the cultures were grown for a total of 11 days in interferon-containing media or medium free of interferon, with media changes every 3 days. Cells were then released from the monolayers and counted with a Coulter counter (lower part of figure). Each data point represents the average of three counts for each of three cultures; the standard error in this experiment was less than 5%. At this time, the interferon titers of an aliquot of the less purified and of the highly purified interferons were determined (16); the interferon titers showed no significant change from those determined before the experiment.

10–15% of the cell population has a nuclear absorbance in the range of 70–90 as expected for G2 and M phase cells. This is consistent with a G2 plus M phase duration of 4–7 hr. The relatively long G1 phase duration in this cell line is a consequence of the long generation time (23). Cells with intermediate nuclear absorbances are S phase cells. Melanoma cells treated with 3900 units/ml interferon for 5 days showed essentially the same distribution of cells in

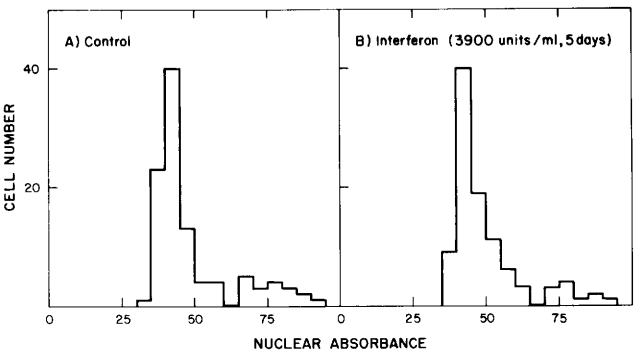

FIG. 2. Interferon treatment causes no alteration in the distribution of melanoma cells in different phases of the cell cycle. Cultures grown in the presence or absence of 3900 units/ml interferon for 5 days were stained by Feulgen reaction, and were used for scanning microspectrophotometry studies as described under Materials and Methods.

the cell cycle as untreated cells (Fig. 2B). This makes it unlikely that there is an interferon-induced block in the G2 or M phases of the cell cycle.

The cell morphology of melanoma cells was also examined by both light microscopy and scanning electron microscopy. No significant difference could be detected in the cell shape, cell attachment "fibers," or surface microstructure between control and interferon-treated cells.

Interferon inhibits melanoma cell growth in mice. Subcutaneous injection of 8×10^5 melanoma cells into syngeneic DBA/2 mice led to the formation of tumors in greater than 90% of the animals within 30 days. The growth of melanoma cells in mice is dependent on the source of the cells; cells maintained in animals give rise to larger tumors within the time course of the experiment than do cells derived from tissue culture (Fig. 3). Treatment of mice with interferon (10^6 units/day, 5 days/week) starting on the day of the injection of melanoma cells, retarded the growth of melanoma cells. Results were similar with larger cell inocula of $4-6 \times 10^7$ cells (Fig. 3) as with smaller doses of 8×10^5 cells (data not shown). In addition, inhibition of melanoma cell growth in mice was also demonstrated with 10^5 or 10^4 units/day interferon, although the effect was less marked (data not shown). In these experiments, no mice died and the average weight of interferon-treated mice was the same as that of control mice.

Discussion. Our studies reveal that the

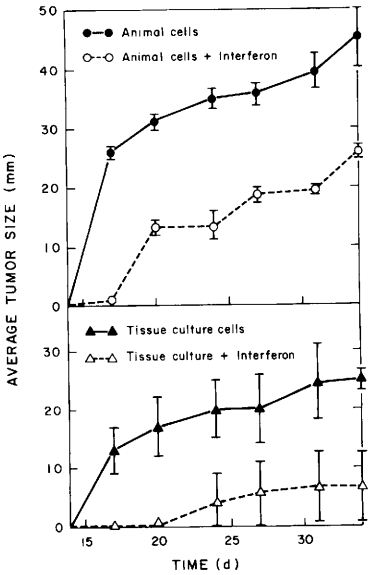

FIG. 3. Interferon inhibits the growth of melanoma cells in mice. The 4.0×10^7 viable melanoma cells in suspension prepared from tumors in mice or 6.0×10^7 melanoma cells from tissue culture were injected subcutaneously into DBA/2 mice as described under Materials and Methods. Mice were treated by intraperitoneal injection with 10^6 units of interferon or buffer B per day, 5 days per week. Tumor size was measured as described under Materials and Methods and is expressed as the sum of the lengths of the two axes measured. The bars in the graph represent standard errors. As revealed by plaque reduction assays with VSV on L929 cells the titer of the interferon preparation did not decrease significantly during the course of the experiment.

growth of melanoma cells in tissue culture and in mice is inhibited by interferon. In tissue culture, a highly purified interferon preparation (specific activity 9.0×10^8 units/mg protein), thought to be at least 30% pure (16 and Cabrer *et al.*, unpublished data) is as efficient in inhibiting cell growth as a much less purified interferon preparation (specific activity 2.0×10^7 units/mg protein). This suggests that the cell growth inhibitory activity associated with the interferon preparation is due to interferon rather than to contaminants in the preparations. This conclusion is supported by the finding that the growth of mouse leukemia L1210 cells is also inhibited by essentially pure interferons (16, 24).

The continued viability of melanoma cells in the presence of interferon and the ability of the cells to recover a normal growth rate in interferon-free medium after experiencing a considerable interferon-induced growth depression seem to indicate that interferon does not damage the cells irreversibly. The decrease of the growth rate in the presence of interferon is probably not the consequence of an increased dependence on a nutrient (e.g., amino acid) which might become limiting (see however (14)). This is the case since an increase in the serum concentration or more frequent medium changes did not alleviate the growth depression. Furthermore an inhibition of the multiplication of L1210 cells by interferon when cultivated under steady-state conditions in a chemostat in which nutritional, pH, and gas modifications are neutralized was reported (25).

Our results concerning the cell cycle distribution of interferon-treated cells are consistent with other reports according to which interferon-treated cells are not arrested in any one phase of the cycle (26–28), However, Sokawa *et al.* report that interferon delays the entry into S phase of quiescent BALB/c3T3 cells after serum stimulation (29).

Some of the work described here has been duplicated with Ehrlich ascites tumor (EAT) cells in tissue culture. EAT cells treated with interferon manifest in addition to growth inhibition an increase in cell size: Cell volume increases 1.1-fold after a 1-day treatment with 130 units/ml interferon and 2.6-fold after a 1-day treatment with 3900 units/ml interferon. Furthermore, after treatment with 130 units/ml interferon for 4 days the cells become more flat, the number of attachment "fibers" increases, and the surface microstructure becomes less pronounced (data not shown).

The experiments with melanoma cells in DBA/2 mice also demonstrated an inhibition of cell growth upon interferon treatment. However, the smallest dose of mouse EAT interferon needed to obtain this effect was large compared to those required for growth inhibition in our tissue culture experiments (this paper and data not shown) and compared to doses of human leukocyte interferon used in antitumor studies in humans (11, 30, 31). It is possible that the mouse interferon used, which was produced by EAT cells in monolayer, may have properties analogous to those of human fibroblast interferon in humans. The latter produces serum levels 30-fold lower than a comparable dose of human leukocyte interferon (32), and is less effective than leukocyte interferon in several clinical situations (33).

The mechanism of the antitumor effects is unknown; it may involve (a) inhibition of virus replication required for tumor growth, (b) inhibition of cell proliferation directly, and/or (c) stimulation of the host immune system (9, 11).

Further work will seek methods to enhance the effects of interferon and to gain a more complete understanding of the criteria required for effective use of interferon as an antitumor agent.

Summary. Interferon treatment was found to inhibit the growth of Cloudman mouse melanoma cells in tissue culture. This was not associated with a block in the G2 or M phases of the cell cycle. A highly purified interferon preparation, approximately 30% pure, was as effective as a less purified interferon preparation, less than 1% pure, in inhibiting melanoma cell growth. This suggests that interferon rather than contaminants in the preparation is responsible for the effects seen. The growth of the melanoma cells in DBA/2 mice was also retarded upon interferon treatment.

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