INHIBITION OF CELL DIVISION BY INTERFERON: CHANGES IN CELL CYCLE CHARACTERISTICS AND IN MORPHOLOGY OF EHRlich ASCITES TUMOUR CELLS IN CULTURE

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SUMMARY
The rate of division of Ehrlich ascites tumour cells in culture is inhibited by mouse interferon. A 75% reduction in cell number is achieved after 3 days and this effect is species specific. The growth inhibition is associated with a marked increase in modal cell volume and with extensive changes in cellular morphology. The cells also become more resistant to detachment from the substrate by mild trypsin treatment.

Analysis of the duration of specific phases of the cell cycle indicates prolongation of mitosis (by 150%), G1 (by 44%) and S-phase (by 100%), but a slight shortening of G1, as a result of interferon treatment. There is also a dramatic rise in the proportion of cells in culture which contain 2 or more nuclei. As a result of these changes, together with the increase in cell size, the mean cellular contents of protein, RNA and DNA are considerably elevated.

There is a small but significant increase in 3'5' cyclic AMP (cAMP) content of Ehrlich cells after exposure to interferon, and exogenous cAMP or its analogues cause morphological changes similar to those elicited by interferon. However, many of the other effects of the latter are not mimicked by the cyclic nucleotide, suggesting that cAMP is only involved in part of the complex pattern of responses of cells to interferon which result in inhibition of growth and division.

INTRODUCTION
Interferons are glycoproteins synthesized and secreted by many vertebrate cell types in response to infection by viruses (Stewart, 1979). They are believed to bind to cell surface receptor(s) in a manner possibly analogous to that of the polypeptide hormones and to cause the induction of an antiviral state in cells with which they come into contact. It is now widely accepted that interferons also have several effects in addition to their induction of the antiviral state. Of particular interest is their ability to inhibit the proliferation of many cell types in culture and to slow the growth of certain tumours introduced experimentally into animals (Gresser & Tovey, 1978). Interferon that has been purified to homogeneity has now been shown to have these properties (Gresser, Demaeyer-Guignard, Tovey & Demaeyer, 1979).

In the work described in this paper we have analysed a number of aspects of the
interferon-mediated inhibition of the growth of Ehrlich ascites tumour cells in culture. The results we have obtained indicate that this inhibition is associated with changes in the duration and characteristics of specific phases of the cell division cycle. We have also observed morphological changes which may be related to variations in cellular levels of cAMP. These effects may be relevant to the actions of interferon against these and other tumour cells in vivo (Gresser & Bourali, 1970).

MATERIALS AND METHODS

Materials

Ehrlich ascites tumour cells were grown from a stock generously provided by Dr E. C. Henshaw (University of Rochester Medical Centre, Rochester, N.Y.); Daudi cells were a gift from Dr F. B. Bollwill (Imperial Cancer Research Fund, London). Eagle's Minimal Essential Media (MEM) and new-born calf serum were obtained from Flow Laboratories. Mouse interferon at a specific activity of 2 - 3 x 10^7 units/mg protein was purchased from Professor K. Pauker (Medical College of Pennsylvania, Philadelphia). Dibutyryl 3'5' cyclic AMP and theophylline were obtained from the Sigma Chemical Company and [3H]thymidine from the Radiochemical Centre, Amersham. K5 photographic emulsion was supplied by Ilford.

Cell culture

Ehrlich cells were routinely grown in suspension culture in MEM suspension medium supplemented with 10% (v/v) new-born calf serum, 100 units/ml kanamycin sulphate and 20 mM Mops (morpholinopropanesulphonic acid) buffer, pH 7.3. The concentration of cells was maintained in the range 4 x 10^4 - 5 x 10^5 cells/ml. Monolayer cultures were established in plastic Petri dishes by seeding with cells which had been taken from suspension culture and resuspended in MEM monolayer medium.

Daudi cells were grown as stationary suspensions in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum and 100 units/ml kanamycin sulphate.

Interferon treatment

Interferon was added to resuspended cells before seeding at the concentrations indicated in the figure legends. Cells were then grown for up to 3 days without renewal of medium or interferon. Four international reference units of interferon were equivalent to one effective unit in giving a 50% reduction in virus yield from EMC virus-infected Ehrlich cells, as measured by a haemagglutination assay (Martin, Malec, Sved & Work, 1961). All interferon concentrations quoted in this paper are in international reference units.

Cell counting and determination of size distribution

Cells were removed from Petri dishes by treatment with trypsin (0.25% w/v) at 37 °C and were counted with a Coulter counter (Model ZBI). The size distributions were obtained using a P128 size distribution analyser attached to the Coulter counter.

Protein, RNA and DNA estimations

The protein content of thoroughly washed cells was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) using a bovine serum albumin standard. RNA was measured by a modification of the method of Fleck & Munro (1962). DNA was estimated fluorimetrically using ethidium bromide and a calf thymus DNA standard (Blackburn, Andrews & Watts, 1973).
Cell cycle analysis

Mitotic index. The mitotic index (the fraction of a cell population in mitosis) was estimated by microscopic examination of a total of 500 cells per culture.

Continuous labelling with \(^{3}\text{H}\)thymidine. After 2 days' growth of cells with or without interferon, \(^{3}\text{H}\)thymidine together with unlabelled thymidine was added directly to cultures to give final concentrations of 0.3 μCi/ml and 40 μM, respectively. This method avoids perturbation of the cells caused by addition of any fresh medium. Incubation at 37 °C was continued and cultures were sampled at appropriate intervals by swelling the cells in 17 mM sodium citrate, fixing with methanol:acetic acid (3:1 v/v) and drying in air. The cells were then stained with methyl green, dried, overlayed with K5 photographic emulsion and exposed for 14 days. Cultures were scored for percentages of labelled metaphase and of labelled interphase nuclei (Stanners & Till, 1960).

Quantitation of multinucleate cells. Accurate counts of multinucleate cells were obtained by first swelling the cells in distilled water for 5 min and then fixing them by gently adding 2 ml of 3:1 (v/v) methanol:acetic acid mixture. The percentages of multinucleate cells were scored immediately by microscopic examination before deterioration of whole cells occurred.

Calculation of cell cycle parameters. The average lengths of time for each phase of the cell cycle were estimated from the results of the procedures described above. The length of mitosis, \(t_m\), was calculated from the mitotic index using the formula

\[
   t_m = \frac{1}{k} \ln \left( \frac{M}{N} + 1 \right),
\]

where \(k\) is the population growth rate constant and \(M/N\) the mitotic index (Nachtwey & Cameron, 1968). The length of the \(G_1\) phase, \(t_{G_1}\), was obtained from the kinetics of appearance of labelled mitoses in cells incubated continuously with \(^{3}\text{H}\)thymidine (Stanners & Till, 1960). The time taken to label 100% of the interphase nuclei, which is the sum of \(t_{G_1} + t_m + t_{G_2}\), was measured to give the length of the \(G_2\) phase, \(t_{G_1}\). By subtraction of \(t_{G_1} + t_m + t_{G_2}\) from the cell doubling time during exponential growth the time spent in \(S\)-phase, \(t_s\), was also estimated.

The proportion of cells in each phase of the cycle was calculated from the above parameters using the formulae described by Stanners & Till (1960).

3'5' cAMP determinations

2 × 10^6 Ehrlich cells were seeded on to 10 cm Petri dishes in the presence or absence of interferon. After 3 days' growth the medium was removed and the cells rapidly lysed with 2 ml cold 0.5 M perchloric acid. Debris was scraped off and added to the lysate and the mixtures sonicated at 4 °C for 10 s at 100 W. The lysates were centrifuged at 10000 g for 10 min, the supernatants neutralized with 1 M KOH and the potassium perchlorate precipitates removed by centrifuging at 5000 g for 10 min. The supernatants were freeze-dried and reconstituted in 1 ml of 10 mM Tris buffer, pH 7.4. The 3'5' cAMP content was estimated using a protein-binding assay (Gilman, 1970) provided in the form of a kit by the Radiochemical Centre, Amersham.

RESULTS

Interferon-mediated inhibition of cell growth

The effect of interferon on the growth of Ehrlich cells in monolayer culture is shown in Fig. 1A. After an initial lag both control and interferon-treated cultures grow exponentially for 3 days with doubling times of 13–15 and 20–26 h, respectively. The inset to Fig. 1A shows that a 75% reduction in cell number is observed in interferon-treated cultures relative to control cultures after 3 days. The dependence of the growth inhibition on interferon concentration is illustrated in Fig. 2; 50% inhibition is
The effect of mouse interferon on the proliferation of mouse Ehrlich ascites tumour cells and human Daudi cells. A. Ehrlich cells were seeded at $4 \times 10^4$ per 3-cm Petri dish in the presence (■) or absence (□) of 1000 U./ml of interferon. Cell numbers in duplicate dishes were counted daily using a Coulter Counter. Percentage inhibition of cell number by interferon on successive days is shown in the inset.

B. Daudi cells were grown in stationary suspension culture in the presence (■) or absence (□) of 1500 U./ml of interferon. Cell numbers were counted daily using a hemocytometer.

Both the cell growth inhibitory activity and the antiviral activity of interferon were eliminated either by heating it to 60 °C for 1 h or by treating it with an anti-interferon antiserum (results not shown). Further evidence that the growth inhibitory activity is due to interferon itself is the demonstration of its species specificity. The mouse interferon used throughout this work had no effect on the growth in culture of the Daudi line of human lymphoblastoid cells (Fig. 1B) although these cells are highly sensitive to human leukocyte
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Fig. 2. Inhibition of cell proliferation as a function of interferon concentration. Ehrlich cells were seeded as described in Fig. 1A and grown for 2 days in the presence of the indicated concentrations of interferon. The numbers of cells per culture were counted and percentage growth inhibitions calculated as in Fig. 1A.

![Graph showing inhibition of growth as a function of interferon concentration.](image)

Fig. 3. The effect of interferon on Ehrlich cell size distribution. Cells were grown on 3-cm Petri dishes with or without interferon (1000 U./ml) for 3 days and their size distributions measured electronically with a Coulter Counter. The ordinate shows the fraction of cells of a given volume relative to the peak number of cells. Control cells (---); interferon-treated cells (— — —).

![Graph showing size distribution of Ehrlich cells.](image)

interferon (Hilfenhaus, Damm, Karges & Mantley, 1976, and unpublished observations of D. R. Gewert and M. J. Clemens). The growth inhibition of the Ehrlich cells was completely reversible when medium containing interferon was replaced with fresh medium lacking this agent. The absence of significant cytotoxic action of interferon was indicated by the fact that there was greater than 95% trypan blue exclusion in both control and experimental cultures (results not shown).
The effect of interferon on cell size and morphology

We have observed that the inhibition of cellular proliferation by interferon is accompanied by changes in cell size and shape. The cell size distributions of control and interferon-treated Ehrlich cells after 3 days of growth in monolayer are shown in Fig. 3. There is a 33% increase in modal cell size caused by interferon and a skew towards much larger sizes. This increase has also been observed directly using a phase contrast microscope fitted with a graticule lens (results not shown).

Table 1. Effects of interferon treatment on protein, RNA and DNA content of Ehrlich cells

<table>
<thead>
<tr>
<th>Measure</th>
<th>Protein</th>
<th>RNA</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Interferon-treated</td>
<td>Control</td>
</tr>
<tr>
<td>μg per culture</td>
<td>51</td>
<td>31</td>
<td>60</td>
</tr>
<tr>
<td>μg per 10⁶ cells</td>
<td>90</td>
<td>150</td>
<td>14</td>
</tr>
</tbody>
</table>

Cells were grown on Petri dishes of 3 (protein and DNA estimations) or 6 cm (RNA estimations) diameter for 3 days, with or without interferon (1000 U./ml). Cells were counted and their protein, RNA and DNA contents estimated as described in Materials and methods.

The interferon-induced increase in cell size is reflected by increases in the content per cell of protein, RNA and DNA (Table 1), with slightly greater elevation of the former 2 values than of that for DNA. Although the cellular content of these macromolecules increases there is an overall decrease per culture due to the lower cell numbers after exposure to interferon.

The inhibition of cell proliferation is also accompanied by morphological changes of the Ehrlich cells. When these cells are grown on plastic tissue culture dishes in the absence of interferon, only part of the population attaches to the substratum. Those cells which do attach appear to be spread to very variable extents even after several days' growth. However, exposure to interferon induces a marked morphological change and alters the interaction between cells and substratum. Typical microscopic fields of view after 3 days of growth are presented in Fig. 4. The time-dependent change in morphology of interferon-treated cultures compared to controls is characterized by a greater proportion of fibroblastic-like cells. This effect has been quantitated in two ways. Table 2 shows the percentage of non-rounded cells in control and interferon-treated populations after different lengths of time in culture (column A). Also shown are percentages of completely flattened cells in which the nuclear outlines become easily distinguishable by phase-contrast microscopy (column B). The interferon-induced increase in flattened fibroblastic-like cells is accompanied by an increase in the proportion of cells which are attached to the substratum (data not shown). There is also increased resistance of the cell population to detachment by trypsin (Fig. 5).

Conditions under which the cellular growth rate is slowed by other means also
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result in similar (but not identical) changes in morphology. We have observed that 3'5' cAMP, dibutyryl 3'5' cAMP and theophylline produce such effects within 2–4 h (Fig. 6); Kaminskas, Field & Henshaw (1976) have reported a very similar phenomenon, with an accompanying growth inhibition. Table 3 shows that interferon treatment of Ehrlich cells does in fact result in raised levels of endogenous 3'5' cAMP. These increases are similar to changes which have been observed in other cell types (Weber & Stewart, 1975; Fuse and Kuwata, 1978; Tovey, Rochette-Egly & Castagna, 1979). These results are consistent with the possibility that 3'5' cAMP plays a role in the growth inhibition and morphology changes brought about by interferon. However, it has been observed (Panniers, 1980) that the growth inhibition produced by exogenous 3'5' cAMP does not cause any increase in cell size, and does not lead to the formation of multinucleate cells as occurs when Ehrlich cells are treated with interferon (see below).

The effect of interferon on the cell division cycle

We have investigated the question of whether the size distribution of cells as a result of interferon treatment may be explained by an alteration of the proportions of cells in the various phases of the cell cycle. The effects of interferon on the length of time that asynchronous populations of Ehrlich cells spend in each phase of the cell cycle have been measured after 2 days of growth. The proportions of cells in mitosis and the times spent in mitosis, as measured by mitotic indices, are shown in Table 4. It can be seen that interferon prolongs the length of this phase 2-5-fold. The other cell cycle parameters have been measured by using a continuous [3H]thymidine labelling technique (Stanners & Till, 1960). Fig. 7 shows the kinetics of appearance of labelled metaphases in control and interferon-treated cell populations. The results indicate that the G2 phase in Ehrlich cells is increased from 4-5 to 6-5 h as a result of exposure to interferon. Similar results have also been obtained from pulse-chase experiments with [3H]thymidine (Panniers, 1980). The time taken to label 100% of interphase nuclei, which is an estimate of the combined lengths of G2 + M + G1, is shown in Fig. 8. From these data the lengths of time spent in all phases of the cell cycle can be calculated, using the cell doubling time during exponential growth as a measure of the generation time. The values obtained for these parameters are summarized in Table 4. It can be seen that the lengths of the phases are all extended by interferon treatment, except that of G1 which is slightly shortened. The greatest increases are in S-phase and mitosis. The increase in the duration of S-phase following interferon treatment was also established by measurement of the cellular labelling index after incubation with [3H]thymidine (not shown).

The proportions of the cell populations in each phase of the division cycle can be calculated from the measured parameters (Table 4). The increases in proportions of cells in later stages of the cycle, relative to the proportion in G1, are consistent with the interferon-induced shift in modal-size observed in the Coulter counter size distribution analysis (Fig. 3). It should be noted that the slower rate of DNA synthesis (reflected in the longer S-phase) is accompanied by a higher proportion of cells in the asynchronous population which is carrying out this process. Thus there is only a
Fig. 4. Changes in Ehrlich cell morphology due to interferon treatment. Cells were grown in 3 cm Petri dishes with (A) or without (B) interferon (1000 U./ml) for 3 days. They were photographed under a Leitz inverted phase-contrast microscope with a 35 mm Leitz SLR camera using Ilford Pan F (ASA 50) black and white film. × 150.
Fig. 4B, For legend see opposite.
small change in the rate of incorporation of [3H]thymidine per 10⁶ cells (Panniers, 1980).

An additional feature of the growth of Ehrlich cells in the presence of interferon is the formation of relatively large numbers of cells with more than one nucleus (Fig. 9). The nuclei cannot be easily visualized under normal conditions but by swelling the Table 2. The distribution of different morphology types in Ehrlich ascites cell cultures

<table>
<thead>
<tr>
<th>Material</th>
<th>2 days</th>
<th>3 days</th>
<th>4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Control</td>
<td>31 ± 4</td>
<td>4 ± 1</td>
<td>41 ± 4</td>
</tr>
<tr>
<td>Interferon-treated</td>
<td>43 ± 12</td>
<td>11 ± 3</td>
<td>60 ± 5</td>
</tr>
</tbody>
</table>

Cells were grown in the presence or absence of interferon (1000 U./ml) for the indicated time and then subjected to quantitative microscopic examination. Column A shows the percentages of non-rounded cells of all types. Column B shows the percentages of completely flattened cells. Each percentage (± s.d.) was calculated from observation of a total of 800 cells in 4 separate microscopic fields.

Fig. 5. The effect of interferon on cell detachment by trypsin. Ehrlich cells were grown as in Fig. 4, with (■) or without (●) addition of interferon (1000 U./ml) for the last 2 days. The medium was then removed and the cell monolayers washed twice with 1 ml phosphate-buffered saline. 1 ml of trypsin solution (0.02 % w/v in PBS) was overlayed and incubation at 37 °C was continued. Detached and attached cells were counted at the indicated times. Detached cells are defined as those cells removed by the trypsin solution and 2 subsequent gentle washes of 1 ml PBS.

cells in hypotonic medium and then fixing them, each nucleus can be clearly distinguished, thus allowing quantitation. Using this technique it was observed that control cultures contain very few multinucleate cells, usually constituting less than 1% of the population. In contrast, multinucleate cells appearing in response to interferon treatment amount to as much as 9–11% of the cell population after 2 days of growth (Fig. 10). These cells are mainly binucleate (Fig. 9); however, during longer periods of
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treatment with interferon increasing numbers of cells with 3, 4, 5, 6, 8 and 16 nuclei are seen (L. R. V. P., unpublished data). The formation of these cells is apparently due to the occurrence of multiple mitoses with incomplete cytokinesis, rather than to cell fusion events. This was established by mixing cells that had [3H]thymidine-labelled nuclei with unlabelled cells and allowing the mixed population to grow in monolayer in the presence of interferon. After 24 h of growth binucleate cells either contained 2 labelled or 2 unlabelled nuclei but never one of each (L. R. V. P., unpublished data). This phenomenon of incomplete cytokinesis may be a further explanation of the alteration in size distribution of the cells and, in particular, can account for the greater skew towards very large sizes seen in Coulter counter analyses (Fig. 3).

DISCUSSION

The ability of partially purified interferon preparations to inhibit the growth of cells in culture has been known for a long time (Paucker, Cantell & Henle, 1962; Macieira-Coelho, Brouty-Boyé, Thomas & Gresser, 1971). However, doubts had existed about whether such an effect was really due to the interferon molecules in these preparations. We have observed that although the mouse interferon we have used is only about 1% pure, the growth inhibition (and its accompanying features) show many properties which are characteristic of the action of interferon. These include species specificity, heat lability, neutralization by anti-interferon antiserum, lack of cytotoxicity, and complete reversibility of the effects. It has also now been shown that pure preparations of interferon are active cell growth inhibitory agents (Gresser et al., 1979). Nevertheless very little has been established concerning the mechanism by which interferon acts to produce this effect. Part of the problem lies in the apparent variations between cell lines in their sensitivities to growth inhibition by interferon. These differences may not necessarily reflect their relative sensitivities to the anti-viral activity of this agent (Borecky et al., 1977).

There has been a number of recent studies conducted on aspects of cell cycle control by interferon. Prolongation of the intermitotic time in a mouse mammary tumour cell line was reported (Collyn D’Hooghe, Brouty-Boyé, Malaise & Gresser, 1977) and Killander et al. (1976) and Balkwill, Watling & Taylor-Papadimitriou (1978) observed all phases of the cell cycle to be lengthened in asynchronously proliferating mouse leukaemia and human breast tumour cell cultures, respectively. In quiescent fibroblasts stimulated to divide by serum addition both G1 and S+G2 phases were prolonged after interferon treatment (Balkwill & Taylor-Papadimitriou, 1978). In contrast, in other synchronized cell populations the G1 phase has been reported to be the major site of interferon action (Sokawa, Watnabe, Watnabe & Kawada, 1977; Fuse & Kuwata, 1977). Matarese & Rossi (1977) noted a marked interferon-induced extension of G2 in DMSO-stimulated Friend leukaemia cells. These results, and the findings reported in this paper, together suggest that no single mechanism is responsible for the effects of interferon on division of cells in culture.
Fig. 6. Changes in Ehrlich cell morphology due to dibutyryl 3',5' cAMP and theophylline. Cells were grown on 3 cm Petri dishes and incubated for 4 h in the presence (A) or absence (B) of 1 mM dibutyryl cAMP and 1 mM theophylline. They were photographed as described in Fig. 4. × 350.
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Fig. 6b. For legend see opposite.
Table 3. The effect of interferon on the endogenous level of 3'5' cAMP in Ehrlich cells

<table>
<thead>
<tr>
<th>Material</th>
<th>cAMP, pmol</th>
<th>Per culture</th>
<th>Per 10⁶ cells</th>
<th>Per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19 ± 1</td>
<td>5'1 ± 0'4</td>
<td>43 ± 3</td>
<td></td>
</tr>
<tr>
<td>Interferon-treated</td>
<td>17 ± 2</td>
<td>9'0 ± 0'3</td>
<td>57 ± 6</td>
<td></td>
</tr>
</tbody>
</table>

Significance of difference

- Control vs. Interferon-treated: Not significant
- Interferon-treated vs. Control: P<0.001
- Interferon-treated vs. Control: P=0.01

Cells were grown for 3 days in the presence or absence of interferon (1000 U./ml) and extracted for determination of cyclic AMP as described in Materials and methods. The results are means of quadruplicate measurements ± S.D.

Table 4. Compilation of the cell cycle parameters of control and interferon-treated Ehrlich cells

<table>
<thead>
<tr>
<th>Material</th>
<th>Generation time</th>
<th>Mitosis</th>
<th>G₁ phase</th>
<th>S-phase</th>
<th>G₂ phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>14'6 (75)</td>
<td>0'5 (2'5)</td>
<td>4'75 (38)</td>
<td>4'75 (38)</td>
<td>4'5 (25'5)</td>
</tr>
<tr>
<td>Interferon-treated</td>
<td>21'0 (4)</td>
<td>1'25 (4)</td>
<td>3'75 (25)</td>
<td>9'5 (48)</td>
<td>6'5 (25)</td>
</tr>
</tbody>
</table>

Cells were grown in the presence or absence of interferon (1000 U./ml) for 2 days and then subjected to cell cycle analysis as described in the text. Values for the lengths of time (h) spent in particular phases of the cell cycle were estimated from the mitotic index, the results of continuous [³H]thymidine labelling experiments (Figs. 7, 8) and the measurement of generation times. The percentages of cells in each phase (shown in parentheses) were calculated as described in Materials and methods.

Fig. 7. Kinetics of labelling of cells in metaphase with [³H]thymidine. Ehrlich cells were grown for 2 days in the presence (□) or absence (■) of interferon (1000 U./ml). [³H]thymidine and unlabelled thymidine were then added as described in Materials and methods and the percentages of labelled metaphases were determined as in Materials and methods. The duration of G₁ was obtained from the time taken to label 50% of metaphases.
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Fig. 8. Kinetics of labelling of interphase nuclei with [³H]thymidine. The experimental procedure was as described in Fig. 7 except that percentages of labelled interphase nuclei were measured. 1000 separate nuclei were examined. The combined duration of $G_s + M + G_1$ was obtained from the time taken to label 100% of the interphase nuclei. Control cells, ■; interferon-treated cells, □.

The possible effects of the appearance of multinucleate cells in the interferon-treated cultures on the measured cell cycle parameters should be taken into consideration. For example, it has been reported that cells of higher ploidy can show prolonged mitosis (Oftebro, 1968). However, in the case of the Ehrlich cells used here interferon caused prolongation of mitosis in mononucleate cells to the same extent as in the total cell population (Panniers, 1980). This observation, together with the fact that multinucleate cells constituted only 10% of the interferon-treated population, suggest that changes in cell cycle characteristics are not solely a consequence of the appearance of the multinucleate cells during inhibition of proliferation, although this remains a formal possibility.

In terms of the transition probability model of the cell cycle (Smith & Martin, 1973; Shields & Smith, 1977; Shields et al. 1978; Brooks, Bennett & Smith, 1980), the probability of occurrence of one or more events necessary for cells to traverse the conventional cycle appears to be lowered by the action of interferon. It is possible that the raised 3'5' cAMP levels in interferon-treated cells may play a role in the regulation of several different stages within the cell cycle (Pardee, Dubrow, Hamlin & Kletzien, 1978).

Despite the observed changes in the cell cycle parameters we have found only a small inhibition by interferon of the rate of [³H]thymidine incorporation into DNA (per 10⁶ cells) and no effect at all on the absolute rate of protein synthesis (L. R. V. P. and M. J. C., manuscript in preparation). The latter is in marked contrast to the effects of interferon on virus-infected cells, in which both host and virus-specific polypeptide synthesis are often severely impaired (Metz & Esteban, 1972; Metz, 1975). It seems
Fig. 9. Appearance of binucleate cells after interferon treatment. Ehrlich cells were grown for 2 days in the presence (A) or absence (B) of interferon (1000 U./ml) and were then swollen and fixed as described in Materials and methods. The nuclei were photographed as described in Fig. 4. Some of the binucleate cells are indicated by arrowheads. × 350.
Fig. 9B. For legend see opposite.
unlikely that the same molecular mechanisms are responsible for the anticellular and antiviral activities of interferon.

Until recently there have been few descriptions of changes in cell size or morphology caused by interferon. Gresser (1961) showed that interferon altered the state of order of human amnion cells in monolayer culture; size increases and greater spreading of cells were noted by Fuse & Kuwata (1976). A detailed report on interferon-induced changes in these parameters, however, appeared while this manuscript was in preparation. Pfeffer, Wang & Tamm (1980) have described effects on human fibroblasts, after 3 days in the presence of 640 U./ml of human interferon, which are remarkably similar to our own observations. The changes seen included a 31% increase in mean cell volume, morphological alterations associated with the appearance of large actin-containing fibres, and a 5-fold increase in the fraction of binucleate cells in the population. The results of Pfeffer et al. and our own, obtained independently and with a different species of cell and of interferon, confirm each other in the conclusion that we are looking at real and important characteristics of the cell growth inhibition phenomenon. The expression of some cell surface proteins is enhanced by interferon treatment (Lindahl, Leary & Gresser, 1973, 1974) and this may also contribute to the morphological changes. Indeed, it now appears that the surface proteins affected include those actively involved in cell attachment to substrata, such as fibronectin (Pfeffer et al. 1980). The formation of multinucleate cells probably also contributes to the cell size increase. This phenomenon is a novel and specific effect of interferon action that we have not observed when cell growth was slowed by other methods. The effect very closely resembles that of cytochalasin B, which inhibits cytokinesis while allowing nuclear division to proceed (Carter, 1967). Interestingly, as seen in the overall population of interferon-treated cells in this study, binucleate cells produced in the
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presence of cytochalasin B have a shortened $G_1$ phase of the cell cycle (Fournier & Pardee, 1975).

A similar morphological change to that caused by interferon treatment is brought about by exposure of Ehrlich cells to dibutyryl $3'5'$ cAMP. This cyclic nucleotide has also been shown to produce marked morphological changes in other cell lines, associated with conversion of the cells from 'transformed' to 'normal' phenotypes (Johnson, Friedman & Pastan, 1971). Exogenous dibutyryl $3'5'$ cAMP also inhibits cell growth (including that of Ehrlich cells). However, it is difficult to distinguish between cause and effect in considering the relationship between morphological changes and inhibition of growth in the presence of interferon or the cyclic nucleotide. The inhibitions of growth of Ehrlich cells caused by interferon and by dibutyryl $3'5'$ cAMP do not have identical characteristics since the latter agent does not cause any increase in size or lead to the formation of multinucleate cells. However, as noted above, the observations of interferon-mediated increases in $3'5'$ cAMP levels in cells suggest that the nucleotide may play an important role in some (but not all) of the actions of interferon.

The relevance of the anticellular actions of interferon to the inhibition of tumour development in vivo remains to be fully established. It has been shown that interferon can also exert a variety of influences on the immune system which may play important roles in this respect (Johnson & Baron, 1977). However, direct inhibition of cell proliferation by interferon may well be at least partly responsible for reduced rates of tumour growth (Gresser, Maury & Brouty-Boyé, 1972) and the changes in cellular behaviour described in this paper can provide a basis for the further exploration of the mechanisms involved.

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