

EFFECT OF INTERFERON ON GROWTH AND DIVISION CYCLE OF FRIEND ERYTHROLEUKEMIC MURINE CELLS IN VITRO

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ABSTRACT

The administration of appropriate doses of interferon to cultures of Friend leukemia cells causes a pronounced inhibition of cell growth. Several lines of evidence indicate that this effect is due to interferon itself, rather than to unknown contaminants of interferon preparations.

Autoradiograph analysis of growth parameters of Friend leukemia cells during treatment with interferon demonstrates that the rate of entry into the S phase, the percent decline of unlabeled mitoses, and the mitotic indexes are significantly lower in interferon-treated cell cultures than in control untreated cultures when tritiated thymidine was added 12 h after the administration of interferon. These data indicate that fractions of interferon-treated cell population are delayed in both G₁ and in G₂ phases of the cell cycle. This was confirmed by exact measurements of the length of the various phases of the cycle.

The interferon-induced inhibition of growth of Friend leukemia cells is reversible after removal of the compound. Autoradiograph data obtained from control cultures and from cultures previously treated with interferon that had been washed free of interferon and reseeded in interferon-free medium, demonstrate that during the first 12 h after removal of interferon, a large majority of the cells previously treated with interferon had a deranged flow into the S phase, a high number of unlabeled mitoses, and a low mitotic index. These data provide further evidence for the above-mentioned prolongations of G₁ and G₂ phases of the cell cycle. All growth parameters tested reverted to normal values within 12 h after washing out interferon.

KEY WORDS interferon · cell growth · cell cycle · Friend cells

Interferon preparations exhibit an inhibitory effect on the multiplication of both normal (12, 15) and neoplastic cells (8, 11, 17), without any significant effect on cell viability. Extensive studies on the effect of interferon on the division cycle of growth-inhibited L1210 cells in vitro pointed out that this phenomenon was seemingly due to a decrease in

the doubling potential of each interferon-treated cell (17).

We had previously shown that interferon also inhibits the multiplication of Friend virus (FLV)-induced erythroleukemic cells (4) in the spleens of irradiated and nonirradiated, histocompatible DBA/2 mice (22).

The data presented here show that the inhibitory effect of interferon on the growth of Friend leukemia cells in vitro is reversible. Autoradi-

ograph data of the effect of interferon on the cell cycle during both treatment with and after removal of interferon, provide evidence that interferon-treatment results in a delay of release of some interferon-treated cells from both G₁ and G₂ phases of the cell cycle.

MATERIALS AND METHODS

Cultures

Friend leukemia cells (clone 745A, obtained from Dr. C. Friend, Center for Experimental Cell Biology, Mount Sinai School of Medicine, New York) were grown in nonagitated suspension cultures in Dulbecco's modified Eagle's medium (Gibco [Grand Island Biological Co.], Grand Island, N.Y.), supplemented with 15% fetal calf serum and antibiotics, in Falcon plastic 60-mm petri dishes (BioQuest, BBL & Falcon Products, Becton Dickinson & Co., Cockeysville, Md.) kept in a humidified incubator in a 5% CO₂ atmosphere at 37°C. Cell counts were done daily in a hemocytometer, and viability was assessed by the trypan blue dye-exclusion test. In the figures, each point represents the average cell count of three to four cultures.

Several mouse interferon preparations were used in the present study. They were obtained from either Dr. I. Gresser (Institut des Recherches Scientifiques sur le Cancer, Villejuif, France), or from Dr. F. Dianzani (Istituto di Microbiologia, University of Torino, Torino, Italy). Procedures for interferon production, partial purification, and titration have been published (7, 14, 15). All interferon dosages employed here are based on the "mouse interferon unit" which equals four "mouse interferon reference standard units." Control (mock interferon) preparations consisted of medium from uninduced cell cultures. The various doses of interferon indicated in the text were added to the cultures at the time of seeding at the density of 10⁵ cells/ml. No further medium changes were made.

Autoradiography

DNA synthesis was measured as follows: CONTINUOUS LABELING: Duplicate cultures per each group were cultivated and labeled with tritiated thymidine ([³H]TdR) (0.5 μCi/ml; sp act: 2 Ci/mmol) either at various time intervals after cell seeding and exposure to interferon, or after cell reseeding after removal of interferon. At various time intervals, as indicated in the experiments, duplicate samples were washed twice with medium, pelleted, and fixed with acetic acid:methanol (1:3) for a total of 15 min, with three changes of fixatives, according to Moorehead et al. (18). Each sample was then spread on duplicate slides. After air drying, slides were dipped in nuclear track emulsion (NTB-2 Kodak) and developed after 7-9 days exposure at 4°C. Under the conditions described, background radioactivity never exceeded two grains per field at a magnification of 1,000,

and was considered negligible. The percentage of labeled interphases was obtained from analysis of 2,000 cells (500 cells/slide).

PULSE LABELING: This technique was used to measure the generation time. [³H]TdR was added (1 μCi/ml) to duplicate cultures in each group at day 1 after seeding and exposure to interferon. After 15-min labeling, the cultures were washed twice and resuspended in Eagle's medium with and (or) without interferon. Under these conditions, the labeling index of interphase nuclei remained constant. Cells were harvested and fixed at different times thereafter. The percentage of labeled mitoses per 2,000 cells was determined by counting 500 cells/slide. The duration of the phases of the cell cycle was determined by the labeled mitoses curve method.

KARYOTYPING: Cells from control untreated and interferon (1,000 U/ml)-treated cultures, as well as from cultures previously treated with interferon that had been washed and reseeded in interferon-free medium, were incubated for 6 h at 37°C in 10⁻⁷ M Colcemid (Ciba Pharm. Co., Summit, N.J.), washed, exposed for 5 min to 0.075 M KCl hypotonic solution, fixed as previously described for preparation of slides for autoradiography, and stained with aceto-orcein. 100 metaphases were scored for each sample.

RESULTS

Effect of Interferon on Cell Cycle of Friend Leukemia Cells

Fig. 1 illustrates cell growth curves of interferon-treated Friend leukemia cell cultures, as well as control untreated and mock interferon-

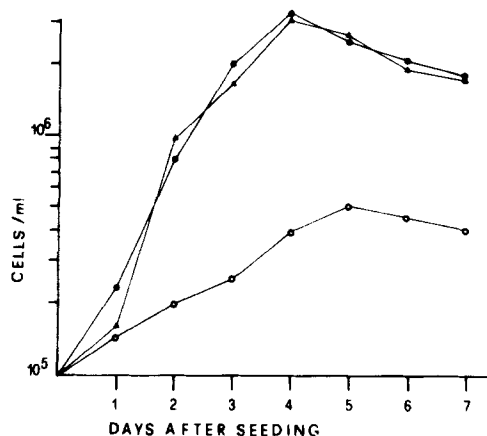


FIGURE 1 Growth curves of control (●), mock interferon (▲), and 1,000 U interferon-treated cultures (○). Interferon was added at the time of cell seeding. Percent viability values for interferon-treated cells were as follows from day 1 to day 7: 99.5, 98.7, 95.5, 95.0, 93.0, 91.2, 85.0.

treated cultures. The rate of growth of interferon-treated cultures was markedly lower than that of control cultures. The highest cell concentration was reached at a lower (> 6 -fold) cell density and 1 day later than in control and mock-treated cultures. Dead cell counts were always very low.

Fig. 2 compares the growth curves of control cultures and of cultures treated with various doses of interferon. The slopes of the curves and the maximal cell concentration are dose-dependent. Cultures given 250 U/ml per 10^5 cells of interferon reached a 10^6 cells/ml density, although still below control values (over 2×10^6 cells/ml). All other cultures were more or less growth-inhibited according to the employed concentration of interferon.

Because data obtained from mock interferon-treated cultures were consistently superimposable on those obtained from controls, they were omitted from all the following presentations of data for the sake of clarity.

Fig. 3 shows autoradiograph data from cultures of Friend leukemia cells treated with interferon at zero time; [^3H]TdR was added at the same time as interferon (panels *a*, *b*, *c*), or at 12 h (panels *d*, *e*,

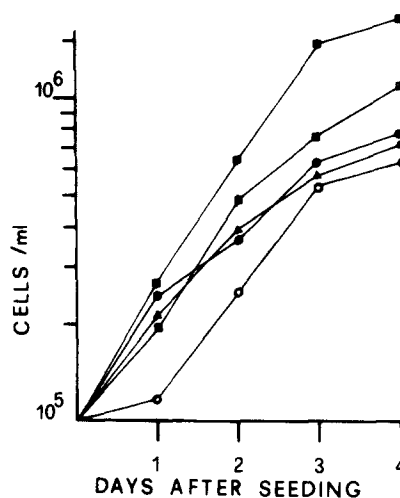


FIGURE 2 Growth curves of control (■) and experimental cultures treated with 2,000 U/ml (○), 1,000 U/ml (▲), 500 U/ml (●), and 250 U/ml (□) of interferon added on day zero.

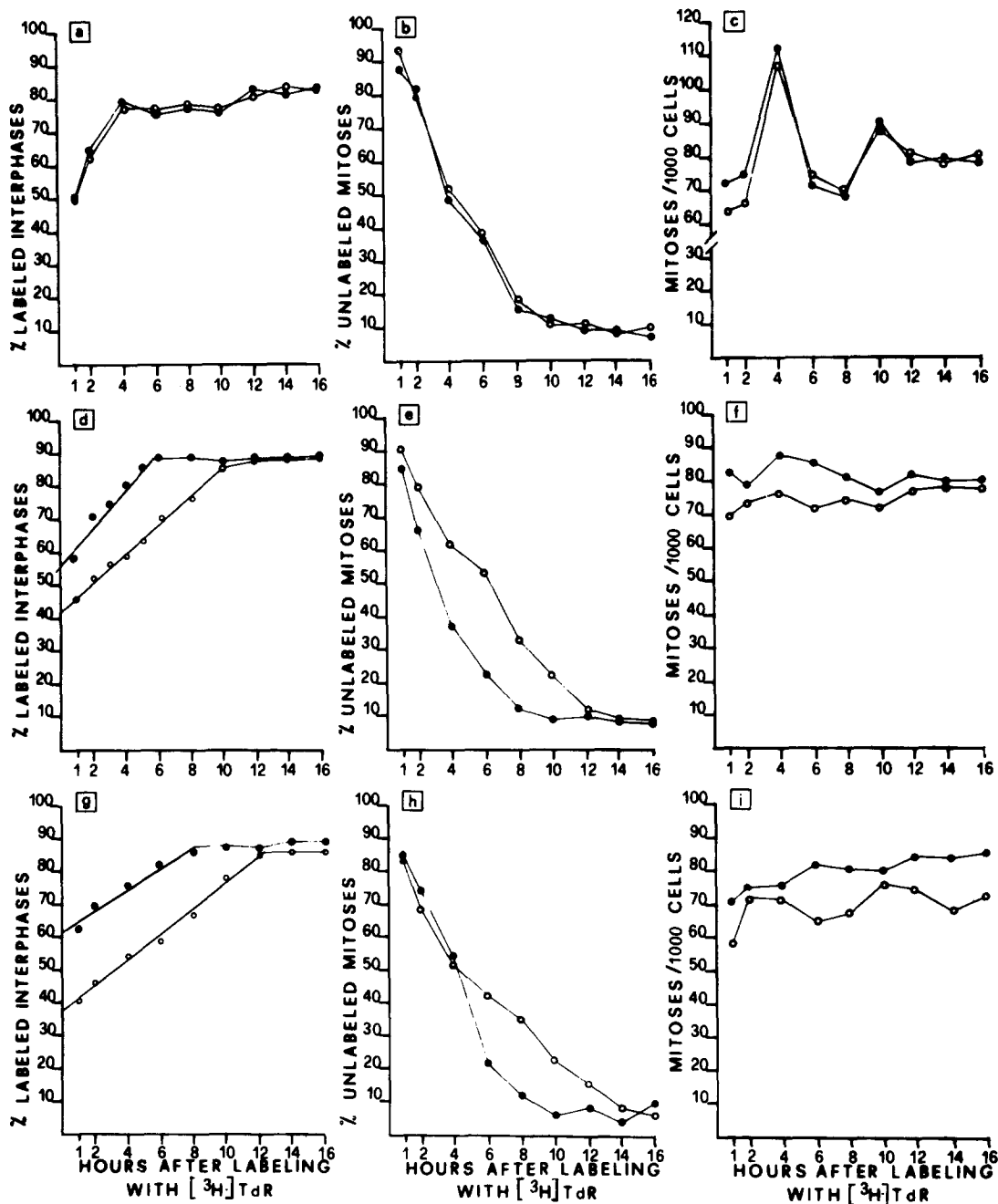
f), or 24 h after cell seeding (panels *g*, *h*, *i*). For each culture, the percentages of labeled interphases and of unlabeled mitoses, and the mitotic

FIGURE 3 (*a*) Percentage of labeled interphases observed at different time intervals, in the continuous presence of [^3H]TdR, in control cells (●) and in cells exposed to 1,000 U/ml of interferon (○), added at the time of cell seeding and present in the medium throughout the experiment. (*b*) Percentage of unlabeled mitoses in the same experiment illustrated in Fig. 3*a*. (*c*) Mitotic indexes determined at different times after [^3H]TdR-labeling of cultures of the same experiment illustrated in Fig. 3*a*. All mitotic phases counted; observation with immersion oil to identify early prophase. Number of mitoses/1,000 cells include both labeled and unlabeled mitoses. (*d*) Percentage of labeled interphases observed at various time intervals in control cells (●) and in cells exposed to 1,000 U/ml of interferon (○) added at zero time and present in the culture throughout the experiment. [^3H]TdR was added 12 h after cell seeding. The straight lines were fitted by the least squares method, taking into consideration all values up to the first point on the plateaus and disregarding the following ones, i.e., for control cultures from the 1st to the 6th h and for interferon-treated cells from the 1st to the 10th h. The equation parameters (slope and intercept \pm standard errors) were as follows. Control: b , 5.802 ± 1.99 ; a , 56.673 ± 7.76 ; interferon-treated: b , 4.373 ± 0.40 ; a , 42.072 ± 2.45 . The analysis of the regressions gave the following F values: Control: 84.82 with a $P < 0.001$; interferon-treated: 1,178.28 with a $P < 0.001$. The significance of the difference between the slopes of the two lines was tested by the Student's t test for slopes. The t value was 2.932 with a probability value of $0.01 < P < 0.02$. (*e*) Percentage of unlabeled mitoses in the same experiment illustrated in Fig. 3*d*. (*f*) Mitotic indexes determined at different times after [^3H]TdR-labeling of cultures of the same experiment illustrated in Fig. 3*d*. For other conditions, see Fig. 3*c*. The mean values \pm standard errors of mitotic indexes of control (81.71 ± 1.52) and interferon-treated (73.00 ± 1.09) cultures are significantly different ($P < 0.001$) according to Student's t test. (*g*) Percentage of labeled interphases observed at various time intervals in control cells (●) and in cells exposed to 1,000 U/ml of interferon (○) added at zero time and present throughout the experiment. [^3H]TdR was added 24 h after cell seeding. (*h*) Percentage of unlabeled mitoses in the same experiment illustrated in Fig. 3*g*. (*i*) Mitotic indexes determined at different times after [^3H]TdR-labeling of cultures of the same experiment illustrated in Fig. 3*g*. For other conditions, see Fig. 3*c*. The mean values \pm standard errors of mitotic indexes of control (80.56 ± 1.66) and interferon-treated (70.78 ± 1.80) cultures are significantly different ($P < 0.002$) according to Student's t test.

index were determined per each time interval illustrated.

Panels *a*, *b*, and *c* demonstrate that treatment with interferon did not cause significant changes in the growth parameters of Friend leukemia cells for the first 12 h, because all curves obtained from treated cells were indistinguishable from those ob-

tained from control cultures. The unusual behavior of mitotic indexes shown in Fig. 3c needs some explanation. The values observed in both control and interferon-treated cultures show some fluctuations which can be ascribed to the conditions typical of freshly seeded cultures: seeding of cells at a low density, stimulation by fresh serum, changes



of temperature inherent in the technicalities of culture seeding, etc. If one compares the mitotic indexes of Fig. 3c with those shown in Figs. 3f and i, it is apparent that the values of the mitotic index tend to become rather stable only a few hours after seeding.

Panels d, e, and f, instead, show marked differences between control and interferon-treated cultures. The slopes of the straight lines, fitted with the least squares method, are significantly different (panel d) ($P < 0.02$), indicating that the interval including $G_2 + M + G_1$ phases is longer in interferon-treated cultures as compared to control cultures. Moreover, the analysis of the percentage of unlabeled mitoses (panel e) shows that in interferon-treated cultures the percentage curve did not decline as sharply as that of controls, but remained quite elevated until 10 h after labeling time, with a maximal differential of ~30 percentage points at the 6th h. The mitotic indexes (panel f) calculated at the various intervals indicated were also different, because every value obtained from interferon-treated cultures was smaller than the corresponding value obtained from controls, and their mean value was significantly smaller ($P < 0.001$) than that of controls. The lowering of the mitotic index of interferon-treated cells, when compared to that shown in Fig. 3c, indicates that 12 h after the addition of interferon, a significantly smaller number of cells entered mitosis.

Panels g, h, and i demonstrate that the interferon-induced changes of growth parameters observed at the earlier time interval (12 h) persisted at the 24-h interval, although the differences between control and treated cultures were less marked. The straight lines fitted through the percent-labeled interphases (panel g) reached the plateau with a 4-h delay for interferon-treated cells, but the slopes were not significantly different in this instance, whereas the kinetics of disappearance of unlabeled mitoses closely paralleled that observed at the earlier interval (panels e, h). The mitotic indexes (panel i) were again always lower in interferon-treated cultures than in control cultures, and their mean values differed significantly ($P < 0.002$).

Therefore, the data shown in Fig. 3 indicate that at 12 h after the addition of interferon, the treated cell population entered the S phase at a lower pace than control cells (panel d). They also suggest that the total length of the cell cycle of interferon-treated cells was prolonged ~4 h (panels d, g). Therefore, it was of interest to determine the

exact parameters of the generation time of control and interferon-treated cell populations. These data are shown in Fig. 4. The cells were pulse-labeled (15 min) 24 h after being exposed to interferon, which remained present throughout the experiment. The percentage of labeled mitoses was determined at 2-h intervals. The average generation times, the lengths of the G and S phases, were calculated according to the labeled mitoses curve method, and are given in the legend to Fig. 4. It is apparent that interferon-treated cells show a marked prolongation of T_{G1} (1.9 h as compared to 1.0 h of control cells) and of T_{G2} , which lasts twice as long. The remaining values were similar in both groups.

Reversibility of Interferon Effect

Cells cultivated for 96 h in the presence of interferon were centrifuged, washed three times with 20 ml of serum-free medium, and reseeded at the routine concentration of 10^5 cells/ml in the absence of interferon. Fig. 5 shows that the growth curves of control cells and of cells previously treated with interferon are indistinguishable, indicating that interferon-directed inhibition of

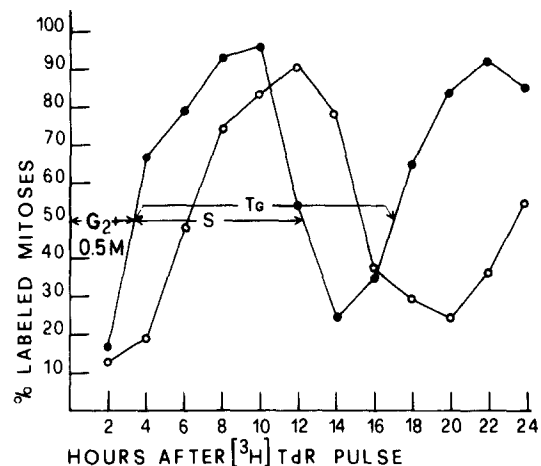


FIGURE 4 Percentage of labeled mitoses observed after pulse-labeling (15 min) of cultures during the 2nd day after seeding. Control (●) and interferon (1,000 U/ml)-treated (○) cultures. The length (h) of the G_1 , G_2 , and S phases of cell cycles was determined according to the labeled mitoses curve methods. The values of the duration of M phase were determined according to (mitotic index $\times T_G$ duration) formula. Control cells: T_G , 13.6; T_{G1} , 1.0; T_S , 9.0; T_{G2} , 2.9; T_M , 0.7. Interferon-treated cells: T_G , 17.6; T_{G1} , 1.9; T_S , 9.0; T_{G2} , 6.1; T_M , 0.7.

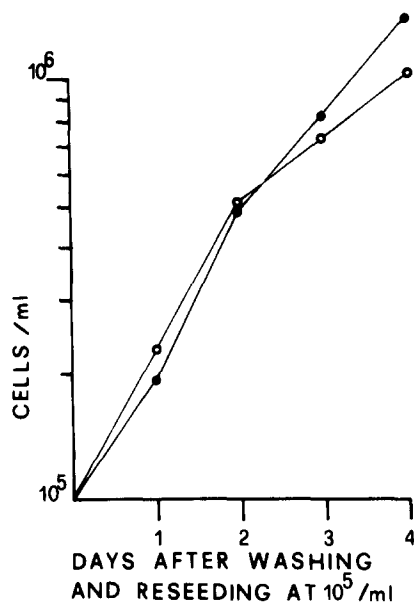


FIGURE 5 Growth curves of untreated cells (●) and cells previously cultivated (for 96 h) in the presence of 1,000 U/ml of interferon (○). Both cultures had been washed thoroughly and reseeded in fresh interferon-free medium.

growth is fully reversible after removal of the compound. It must also be emphasized that reversibility of the effect was obtained even when interferon was washed out at <96 h-intervals.

Despite the observed clear-cut resumption of normal growth potential by cells previously treated with interferon, when checked daily, it was of interest to study in depth the parameters of growth of these cell cultures at a 2-h interval after the removal of interferon. Fig. 6 illustrates the percentage of labeled interphases of the cultures washed free of interferon, to which [³H]TdR was added at the time of seeding, and remained present throughout. After 1 h, 47% of control cells was in S phase, as opposed to only 15% in interferon-treated cultures. Subsequently, control cultures reached the 85–90% plateau at the 6th h. In contrast, interferon-treated cultures showed two plateaus, one which remained at the 50% level from the 2nd to the 8th h, and the other at the 85% level. The unusual steplike behavior of the labeling index of interferon-treated cells only seems in contrast to the kinetic findings shown in Fig. 5. In fact, the latter are rough estimates of daily assessed cell growth, particularly when cell counts are obviously low, as in the instance of those done on day 1. In this case, experimental

errors (expressed by the square root of the actual cell count) are such as to make differences and similarities almost meaningless. The message derived from the data shown in Fig. 5 reads as follows: control and interferon-treated cells, after several washings and reseeding at low density, show an identical growth rate over a 4-day period, indicating that interferon-directed inhibition of growth is reversible. However, the data shown in Fig. 6 represent a much more detailed assessment of growth parameters carried out during the first 12 h after the removal of interferon. They, in fact, describe what happens to cell growth in a time interval (0–12 h) that is obviously overlooked in Fig. 5, and indicate that sizable fractions of cells previously treated with interferon are still held back through the cycle at the early intervals after the removal of interferon.

In another experiment, the evaluation of unlabeled mitoses indicated that most mitoses appearing within the first 2 h were unlabeled in both cultures, representing cells that were in the G₂ phase at the time of reseeding and labeling. As expected, their number declined sharply thereafter.

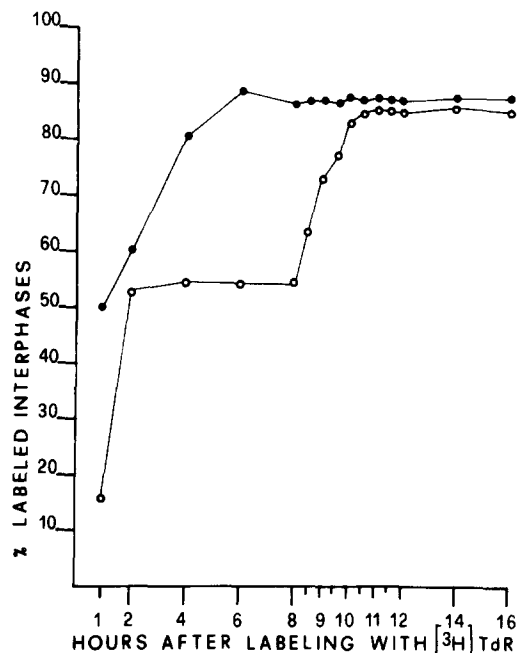


FIGURE 6 Percentage of labeled interphases observed in the continuous presence of [³H]TdR, at different times after washing and reseeding at the density of 10⁵ cells/ml of control cultures (●) and cultures previously cultivated (for 48 h) in the presence of 1,000 U/ml of interferon (○).

ter in control cultures, whereas it did not do so as readily in interferon-treated cultures, which in contrast, showed a peak of unlabeled mitoses at the 8th h, with a subsequent fall to base-line values (Fig. 7).

It must be pointed out that the observed surge in unlabeled mitoses at the 8th h is highly reproducible. Accordingly, the percentage of labeled mitoses (Fig. 8) increases steadily in control cultures, whereas a pronounced and abrupt decline can be seen at the 8th h in interferon-treated cultures. The data of Figs. 7 and 8 are noteworthy because they provide additional evidence that a sizable fraction of cells previously treated with interferon are apparently delayed in the G_2 phase of the cycle and enter mitosis later than control cells. It is also apparent that the prolonged G_2 phase of these cells, especially evident in the interval between the 2nd and the 8th h (Fig. 7), is responsible for the observed plateau at the 50% value shown in Fig. 6 for the same cell population. It is probably more than a coincidence that this plateau is terminated exactly at the time when the G_2 -delayed cells finally entered mitosis and then proceeded into a new cycle. It should also be pointed out that the curve profile of interferon-treated cells in Fig. 7 differs from, although reminiscent of, those shown for interferon-treated cultures in Figs. 3e and h. In the latter figures, there

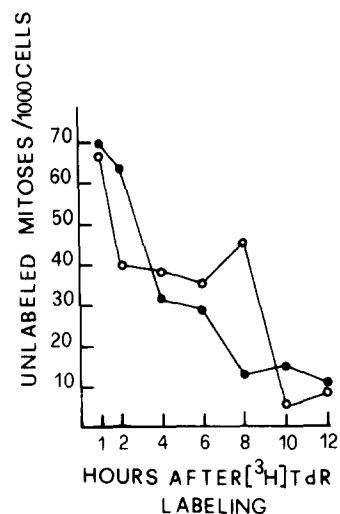


FIGURE 7 Numbers of unlabeled mitoses/1,000 cells observed in the continuous presence of $[^3H]TdR$, at different times after washing and reseeding at the density of 10^5 cells/ml of control cultures (●) and cultures previously cultivated (for 48 h) in the presence of 1,000 U/ml of interferon (○).

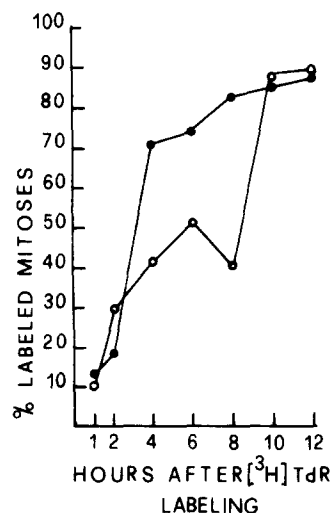


FIGURE 8 Percentage of labeled mitoses observed in the same cell populations illustrated in Fig. 7.

are no detectable peaks of unlabeled mitoses as compared to that shown in Fig. 7. This finding can be explained by the different experimental conditions used: (a) cell cultures described in Fig. 3 were exposed to interferon for only 12–24 h before the addition of the label, whereas those of Fig. 7 had been treated with interferon for 48 h before the drug was washed out. Collyn d'Hooghe et al. (2) also reported interferon effects that were more prominent for longer periods of exposure to the drug; (b) in Fig. 7 the removal of interferon apparently enabled the cells previously treated with interferon to move into a new cell cycle in a quasi-synchronized fashion, whereas the continuous presence of interferon apparently caused a persistent "peakless" delay in the G_2 -M transition.

To rule out that accidental factors (such as a very low mitotic index in cells subjected to extensive washings) could possibly account for the data, mitotic indexes of control cultures and cultures previously treated with interferon were determined. Fig. 9 illustrates the mitotic index of such cultures. The values observed in control cultures showed some fluctuations which could be ascribed to the particular conditions of the experiment, i.e., three washings, reseeding at a low density, stimulation by fresh serum, etc. Still, 5 out of 7 points are very close to the 8.5% value. However, the values of the mitotic index of cultures previously treated with interferon were consistently below those of controls. Data obtained in the experiments illustrated in Figs. 6–9 were tabulated in Table I, where the χ^2 is also shown. *P* values are

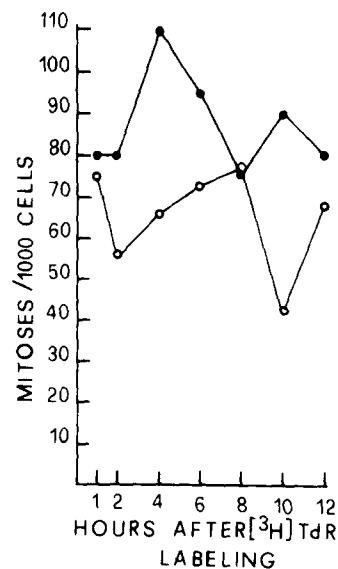


FIGURE 9 Total number of mitoses/1,000 cells observed in the same cell populations described in Fig. 7. The statistical evaluation of these data is included in Table I.

highly significant for all groups of interphases, for mitoses groups at the 4th and 8th h after labeling, and for mitotic indexes at the 4th and 10th h.

In another series of experiments, the labeling indexes, the percentages of unlabeled mitoses, and the mitotic indexes were determined in control cultures and in interferon-treated cultures that were washed free of interferon, reseeded in interferon-free medium, and labeled with [^3H]TdR 12 and 24 h later. In contrast to the data illustrated in Figs. 6-9, no differences were observed between the two sets of cultures with respect to every parameter tested (data not shown for the sake of brevity).

Karyotypes of control cells, interferon-treated cells, and cells previously treated with interferon were determined to ensure that the observed interferon effects were not due to selective processes. The chromosomal mode and spread, as well as the percentage of polyploidy, were not found to be significantly affected in cultured cells both during treatment with, and after removal of, interferon.

TABLE I
Statistical Analysis of Data Shown in Figs. 6-9

Time after seeding <i>h</i>	Culture group	No. interphases			No. of mitoses			Mitotic index (Fig. 9) Total no. of		
		UI* (Fig. 6)	LI	χ^2 ‡	UM (Fig. 7)	LM (Fig. 8)	χ^2	Inter-phases	Mitoses	χ^2
1	C	926	913	477.76	140	21	0.41	1,839	161	0.4
	IF	1,557	294		134	16		1,851	150	
2	C	691	1,149	29.6	129	31	2.71	1,840	160	9.53
	IF	876	1,014		80	31		1,890	111	
4	C	350	1,430	298.18	64	157	29.72	1,780	221	24.5
	IF	863	1,005		77	55		1,868	132	
6	C	386	1,435	283.59	59	122	8.89	1,821	181	4.32
	IF	884	973		71	74		1,857	145	
8	C	371	1,478	320.57	27	124	56.16	1,849	151	0.0001
	IF	891	968		91	61		1,859	152	
10	C	382	1,437	11.35	31	150	1.11	1,819	181	38.9
	IF	320	1,597		10	73		1,917	83	
12	C	322	1,517	12.32	23	138	0.159	1,839	161	2.67
	IF	249	1,617		17	117		1,866	134	

* UI, Unlabeled Interphases; LI, Labeled Interphases; UM, Unlabeled Mitoses; LM, Labeled Mitoses; C, Control; IF, Interferon Treated.

‡ χ^2 distribution, as the minimum level of significance, we chose $P < 0.002$, that equals, with 1° of freedom, 10.827.

DISCUSSION

The data presented in this paper demonstrate that mouse interferon markedly decreases the rate of growth of Friend leukemia cells (Fig. 1). Although it had been previously reported that interferon does not inhibit the growth of Friend leukemia cells, the lack of effect was probably due to the fact that lower concentrations of the compound in the range of 100–220 U/ml per 10^5 cells (13, 25) were employed. Instead, it was necessary to expose Friend leukemia cells to at least 500 mouse units of interferon/ml per 10^5 cells to observe the inhibition of cell growth (Fig. 2). A large majority of Friend leukemia cells differentiate and synthesize hemoglobin upon exposure to 1–2% dimethyl sulfoxide (vol/vol) (5, 19). The effect of interferon administration upon growth and differentiation of dimethyl sulfoxide-stimulated Friend leukemia cells has been described elsewhere.

Briefly, when interferon is administered on day 1 to cultures seeded in the presence of dimethyl sulfoxide, cell growth is still depressed but to a lesser extent, whereas heme and hemoglobin synthesis are inhibited two- and ninefold, respectively (23). Globin messenger RNA accumulation in cells treated with interferon plus dimethyl sulfoxide is reduced twofold, whereas globin synthesis is almost completely blocked under conditions where the overall protein synthesis is not affected (24).

In the present studies the evidence that the inhibition of growth of Friend leukemia cells is attributable to interferon rather than to unknown contaminants of interferon preparations was as follows: (a) growth inhibition was observed with interferon preparations regardless of the degree of partial purification. Although Gresser's preparations had 10^6 or more U/mg protein and Dianzani's preparations had 10^5 or 10^4 U/mg protein, the effects they produced were indistinguishable; (b) mouse interferon proved equally effective regardless of the cell type used as a producer of interferon (brain, fibroblasts, ascites cells) and regardless of various inducers (West Nile virus or Newcastle Disease virus); (c) rabbit heterologous interferon or mock interferon from uninduced mouse cultures was ineffective (data not shown for heterologous interferon).

The data illustrated in Fig. 3 are of particular interest because they show that: (a) no differences between control and interferon-treated cultures were observed when cells were simultaneously

treated with interferon and labeled with [3 H]TdR (panels *a*, *b*, *c*); (b) when cells were labeled 12 h after exposure to interferon, it was possible to demonstrate a marked derangement of growth parameters of interferon-treated cultures, i.e., lower labeling index (panel *d*), more prolonged persistence of unlabeled mitoses (panel *e*), and lower mitotic indexes (panel *f*) than in control cultures. Therefore, it is apparent that in the Friend leukemia cell system there was a lag between the time of interferon administration and the time at which its effects were detectable. This time interval appeared to be approximately equal to the length of Friend leukemia cell cycle. Studies are in progress to investigate whether there is a correlation between the time of administration of interferon and a specific phase of the cell cycle. (c) Significant differences in the growth parameters between the two sets of cultures under study were also observed when cells were labeled 24 h after exposure to interferon, but did not appear to be additive during the course of the experiment. However, it is evident, that interferon treatment caused a prolongation of the length of the G_2 phase of the cell cycle, as a sizable fraction of unlabeled mitoses could still be observed 8 h after labeling of cultures that had been exposed to interferon for 12 and 24 h (panels *e*, *h*, respectively). This indicates that these cells were already beyond the S phase at labeling time, but they were kept from proceeding to mitosis, and therefore remained in the G_2 phase for ~6 h.

These data are also confirmed by pulse-labeling experiments carried out to determine the generation time and the duration of each phase of the cell cycle of control and interferon-treated cultures (Fig. 4). The length of the G_2 phase of interferon-exposed cells was doubled as compared to that of control cells, the generation time of which was 4 h shorter than in interferon-treated cells, in keeping with recent findings by Collyn d'Hooghe et al. (2), who exposed mouse mammary tumor cells to interferon.

Analysis of the division cycle of cells which had been washed free of interferon and labeled with [3 H]TdR at the time of cell reseeding, revealed striking differences as compared to control cultures, despite the fact that growth curves of the two cell populations, determined by daily cell counts, were indistinguishable. The labeling indexes curve for interferon-treated cultures was biphasic, suggesting a quasi-synchronized flow of these cells through the cell cycle (Fig. 6). The

different number of labeled interphases of control cultures vs. cultures previously treated with interferon 1 h after removal of interferon (47 vs. 15%) is in accord with the prolongation of the duration of G_1 observed in interferon-treated cells (legend to Fig. 4). It is also worth noting that the biphasic character of the labeling indexes curve for interferon-treated cultures is also strongly suggestive of another fraction of this cell population being arrested in the G_2 phase. The peak of unlabeled mitoses observed at the 8th h after removal of the compound, cell reseeding, and [3H]TdR-labeling, provides additional evidence for a G_2 block (Fig. 7). The sharp surge in the percent of labeled interphases from the 8th to the 10th h (studied in detail at $\frac{1}{2}$ -h intervals in Fig. 6) is also suggestive of a synchronized flow of this fraction of the population.

Although the above data indicated that the G_2 -halted fraction of the interferon-treated cell population eventually went through mitosis and entered the S phase, it was not conclusively proven that these cells were still fully viable. The separation of these cells from the rest of the population by velocity sedimentation in a bovine serum albumin gradient should resolve this issue. Blocks in the G_2 phase of the cell cycle of ascitic cells have been reported as compatible with resumption of normal growth potential (1, 6).

None of the findings described in Figs. 6-9 were observed when [3H]TdR was added at 12 or 24 h after removal of interferon and reseeding of cultures. This indicates that after a time interval, approximately one cell cycle long, the great majority of the cells did fully recover from interferon-directed derangements of cell cycle parameters.

This observation, if taken together with the one cell cycle interval observed between interferon administration and the detectability of its effects, is suggestive of quantized cell cycles possibly being involved in the expression of interferon action.

Interferon exerted a strong inhibitory effect on growth of Friend leukemia cells inoculated into irradiated, histocompatible DBA/2 mice (22). Because supralethal irradiation is markedly immunosuppressive, this is seemingly due to a direct inhibition of the multiplication of the leukemic cells themselves, rather than to *strictu sensu* host immunosurveillance mechanisms. The results of the in vitro experiments presented herein may be relevant to the effects observed in

vivo. Interferon treatment may result in a progressive lengthening in the intermitotic time of Friend leukemia cells in vivo (with increased cell loss). This increase in leukemic cell generation time may also allow host cells such as macrophages, known to be radioresistant, to deal more effectively with growing leukemic cells and increase cell elimination. In this respect, it is probably more than coincidental that macrophages are involved in host antigrft reactions elicited against the products of hemopoietic histocompatibility genes (16), the expression of which is enhanced following transformation by Friend leukemia virus (3, 20, 21).

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