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ROLE OF Go-G1 ARREST IN THE INHIBITION OF TUMOR CELL GROWTH BY INTERFERON

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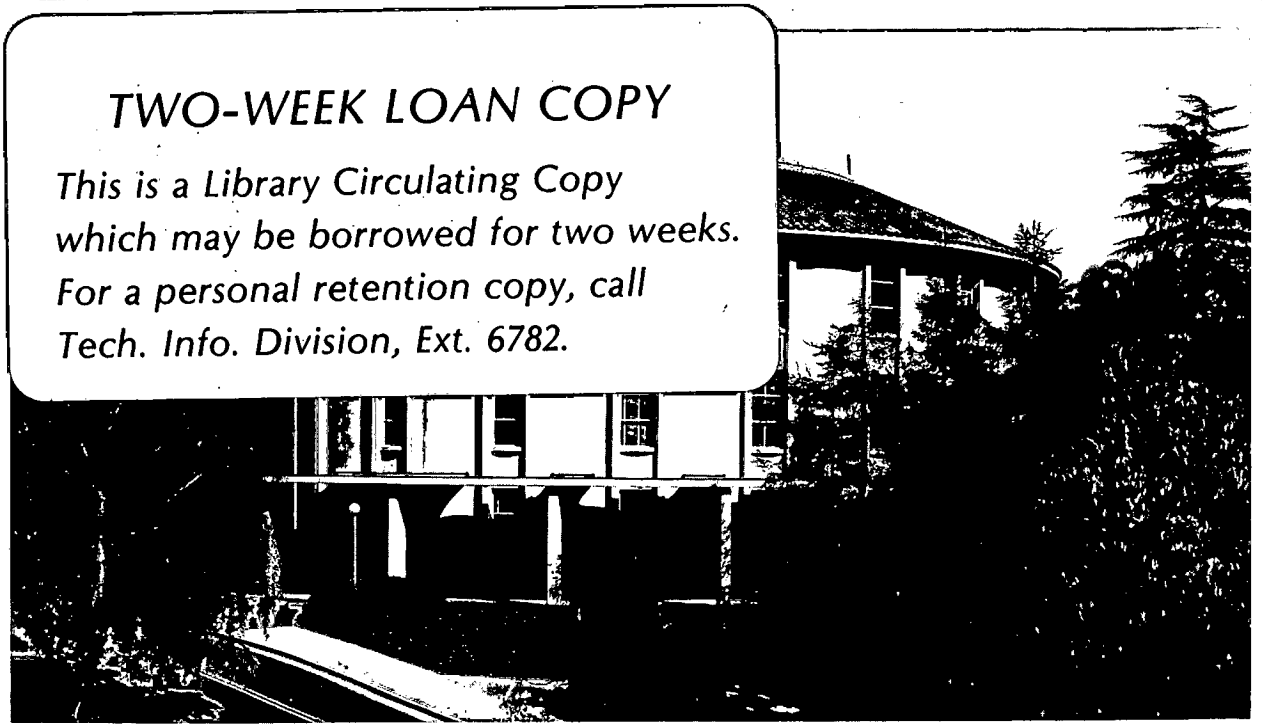

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DISCLAIMER

ROLE OF G_o-G_1 ARREST IN THE INHIBITION OF TUMOR CELL GROWTH BY INTERFERON

(Interferon resistance/melanoma cells/flow cytometry/G_1 arrest)

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Abbreviations: IF, Interferon; FCM, Flow Cytometry; VSV, Vesicular Stomatitis Virus

ABSTRACT

We report here that human leukocyte interferon preparations are capable of influencing the transition of human melanoma cells from the "A" state to the "B" phase. Human melanoma cells that enter a quiescent stage at high cell density are more sensitive to the cytostatic action of interferon than those which continue to proliferate under similar conditions. Cell cycle perturbations caused by interferon in these cells include a decreased transition rate out of G_o-G_1 ("A" state) into S("B" phase), and a prolongation of S. These findings support the idea that some metabolic event is required for progress through the G_o-G_1 phase of the cell cycle, and is susceptible to interferon action.

INTRODUCTION

Interferons are glycoproteins with a number of biologic activities including antiviral, immunoregulatory, and anti-proliferative(1). These properties(especially the anti-proliferative effects) have stimulated interest in interferons as potenial anti-tumor agents in the treatment of human cancer. Interferons have a tumor reducing effect in vivo in osteosarcoma(2) and various other tumors including non-Hodgkin's lymphoma(3). In cell culture, interferons inhibit the proliferation of both normal and malignant cells(4-7). In this report we explore the mechanism of anti-tumor action of interferon by studying its effect on the growth of a number of human melanoma cell lines. Some of these lines behave like normal cells in that they enter a quiescent state(know as G_o, "A", or "R"(8)) at high cell density and with nutrient deprivatiou, while others continue to proliferate under the same conditions.

The transition between the quiescent state and the proliferating phase(B, or S and G_2+M) seems to be a critical step in control of cell growth(8). Cells transformed by viruses have altered regulation of this transition, since agents which inhibit normal cells in the G_o phase fail to affect virally transformed cells in the same manner(9). We have selected human melanoma cell lines whose proliferation appears to be regulated at this restriction point and compared the effect of interferon on these cells with its effect on similar lines which exhibit loss of the restriction point control. Our goals were to determine: (i) if interferon was cell cycle specific, and (ii) whether interferon could differentially influence the proliferation of those cells with the restriction point control versus those that seem to have lost it.

MATERIALS AND METHODS

Interferon Preparations

Human leukocyte interferon(IF) was kindly supplied by Kari Cantell, Central Public Health Laboratory, Helsinki, Finland. The titer of this interferon preparation was six million reference units per milliliter, with a specific activity of 10^6 reference units per milligram of protein.

Cells

Eight human melanoma cell lines, a fibroblastic line(GM37), its SV40 transformed T antigen positive counterpart (GM637), and uncloned Hela as listed in Table 1 were used. Four of the human melanoma cell lines (Hs294T, Hs695T, Hs852T and Hs939T) were initiated and cloned at the Cell Culture Department at the Naval Biosciences Laboratory Oakland, California. The origin, cultural characteristics and cytogenetics of the first 3 lines in Table 1 have been previously described in detail(10). Hs939T was initiated newly at the Cell Culture Department from a mediastinal metastasis of a Caucasian female melanoma patient with no previous history of therapy. The biological properties of the early passage cultures, i.e., passage 5 through 10 show that this line doubles in 24 hours, is pigmented and its karyotype is abnormal(A. Creasey; unpublished observation). M_3, a well characterized melanoma cell line(11) was kindly provided to us by Dr. S. K. Liao, Department of Pediatrics, McMaster University, Hamilton, Ontario, Canada. SH-4 derived from a pleural effusion(12), was obtained from Dr. G. Seman, Department of Virology and Pathology, University of Texas System Cancer Center, Texas Medical Center, Houston, Texas. GM37 and GM637 were supplied by the Human Genetic Mutant Cell Repository, Camden, New Jersey. Hela cells were obtained from the Cell Culture Department of the Naval Biosciences Laboratory, Oakland California.

All the melanoma cell lines and Hela were grown in monolayer cultures in Dulbecco's modified Eagles medium (DME) from Gibco, Grand Island, New York, supplemented with 10% fetal calf serum. GM37 and GM637 were grown in McCoy's and Minimal Essential Media, both containing 2x nonessential amino acids and 2x vitamins from Gibco, Grand Island, New York, and 20% fetal calf serum. All cells were demonstrated to be free from mycoplasma both by scanning and transmission electron microscopy, as well as biological methods (10).

Interferon Resistant and Revertant Cell Lines

Hs294T cells were cloned by plating 100 cells in 24 cm^2

flasks with 250 U/ml IF. Eight of the surviving colonies were picked 3 weeks later and subcultured individually (with 250 U/ml IF). From 8 clones, one (clone 6) was resistant to 10^3 units of IF/ml. After subculturing for 23 weeks with increasing amounts of interferon, this cloned line was resistant to up to 10^6 U/ml of IF. Subsequent passages were then made in the presence of 10^4 U/ml of IF.

Revertants to interferon sensitivity were obtained by subculturing clone 6 cells in the absence of interferon for 8 weeks. After this, growth of the revertant cells was inhibited by 10^3 U/ml.

Effects of Interferon on Cell Lines

Effects on cell growth were measured by counting viable cells. Replicate cultures (5×10^4 cells/35 mm culture dish) were treated with each of the various concentrations of interferon. Ninety six hours later, when control cultures without interferon had a 4 to 6 fold increase in cell number, counts were made using 0.1% trypan blue for viability determination. The effect of interferon on a given cell line was then expressed as percent of control growth.

Antiviral activity was measured using inhibition of vesicular stomatitis virus(VSV) yields(13) in the presence of 250 U/ml IF. VSV yields were quantified on mouse L cells. The log of virus yields in interferon treated cultures was subtracted from that in untreated control.

Cell Cycle Analysis

Cell cycle distributions were determined by flow cytometry as previously described(14). The amount of DNA per individual cell was quantified by staining cells with propidium iodide and passing them, individually, through the beam of an argon-ion laser (Spectra Physics, Mountain View, California) tuned to 514 nm. The pulse of fluorescent light was filtered to reduce scattered exciting light and absorbed by a photomultiplier tube positioned at right angles to the laser beam. The resulting signal was amplified electronically and recorded in the memory of a pulse height analyzer(Northern Scientific, Middletown, Wisconsin). The data in the form of a DNA histogram was analyzed using a computer program as described previously(15). The integration of the area under the curves of the histogram gives the fraction of the population in G_1, S, and G_2+M. Where applicable, the proportion of cells in S was also quantified by pulse labelling for 1 hour with 2.0 uCi/ml ^{3}H-thymidine(10 Ci/mmole; New England Nuclear, Boston, Mass.) followed by autoradiography.

RESULTS

Inhibition of Cell Growth by Interferon

Figure 1 is representative of the dose dependent growth inhibition of human melanoma cells by interferon. Lines with growth reduction of forty percent or less with 10^3 U/ml of interferon, for example Hs695T and Hs294T-clone 6, were considered to be interferon resistant. The inhibition of growth of the melanoma cell lines by interferon was not associated with a decrease in cell viability. This was determined both by trypan blue exclusion and plating cells previously exposed to interferon for colony counts.

Table 1 shows the correlation of the cell's distribution in the cell cycle when cultures reached saturation density. The distribution in the cell cycle was determined by FCM and autoradiography. When greater than 90% of the cells had G_1 DNA content at saturation density the cultures were considered blocked in G_o-G_1. G_o-G_1 arrest was confirmed is some cases by following the kinetics of movement through the cell cycle after serum stimulation of cells which had reached saturation density. Melanoma cell lines which reside in G_o-G_1 at saturation density are more susceptible to growth inhibition by interferon than those which are distributed throughout the cell cycle(Table 1). To test if this observation is unique to melanomas, we analyzed the effect of interferon on the growth of a normal human fibroblastic cell line(GM37), its SV40 transformed counterpart(GM637), and on Hela. As shown in Table 1, the observed correlation between behavior at saturation density and interferon sensitivity holds true in these cell lines as well.

Effect of Interferon on Growing Hs294T

Of all the cell lines tested, Hs294T was the most sensitive to the antiproliferative effects of interferon and was used in the detailed cell cycle studies described below. We tested the effect of interferon on actively growing populations of Hs294T cells by exposing the cultures to 250 U/ml interferon beginning 1 day after seeding the cells. The distributions of cells in the cell cycle as a function of time were obtained by FCM and autoradiography and are presented in Table 2. Consistent with Hs294T cell cycle distribution at saturation density, control cells progressed towards residence in G_1 over the 4 day period, as demonstrated by the significant increase in the proportion of cells in G_1 and the decreased fraction in S and G_2+M. However, in the interferon treated cultures, the fraction of the population in S remained essentially constant with a

slight increase in G_1 cells and a decrease in G_2+M.

Constant cell cycle distributions as a function of time after interferon addition could mean either that interferon had no effect on the cell cycle, or that it slowed cells in all phases of the cell cycle. Since the cell number was not increasing at this concentration of interferon (Fig. 1), it is likely that interferon blocks movement through all phases of the cell cycle. If interferon treated cells are moving through the cell cycle, than they should accumulate in M when exposed to the mitotic blocking agent like colcemid. Hs294T cells were treated with interferon(250 U/ml) for 48 hours, with colcemid(0.2 ug/ml) being present for the last 16 hours. FCM results as shown in Table 3 indicate that control cells were trapped behind the colcemid block while almost none of the interferon treated cells were blocked by the colcemid. We conclude that in growing populations interferon impedes the progression of cells through all phases of the cell cycle.

Serum Stimulation of Interferon Treated Cells

The effect of interferon on the progress of serum stimulated G_o-G_1 cells into S was studied. Hs294T cells were grown to high cell density of 3×10^6 cells/cm^2 in fourteen days, medium changed at the 14th day, and then left unchanged for 10 more days. These G_o-G_1 blocked cells were pretreated with 250 U/ml IF for 18 hours, trypsinized and replated at low density in medium containing ten percent serum and 250 U/ml IF. At two hour intervals the proportion of cells in G_1, S, and G_2+M in both the interferon treated and control cultures was obtained by FCM. This data(Fig.2a & b) when plotted against time(Fig.2c & d), showed that: a) it took 14 hours for cells to move into S after release from a G_o-G_1 block in both control and IF treated cultures, b) by 16 hours, twice as many control cells as compared to IF treated(36% vs 18%) had moved into S, and c) the time needed for 50% of the cells stimulated by serum to move into S ($T^S_{1/2}$) was extended by about 4 hours in interferon-treated cultures. The wave of serum stimulated cells moving through S in the control populations had a much tighter distribution than the interferon treated populations. These observations suggest that interferon does not block the serum stimulation of Hs294T; however, it does increase the dispersion in the transit time from the beginning of the serum stimulation to the entrance of cells into S. Cell division in the control cultures, as evidenced by an increase in percentage of cells in G_2+M, commenced between the twentieth and thirtieth hour after release; however, the interferon treated cells had not reached mitosis by 30 hours.

To determine whether pretreatment is a necessary condition for the action of interferon, samples of high density,

nutrient starved Hs294T cells were treated with interferon simultaneously with release into complete medium. After release, control and IF-treated cells were sampled and prepared for FCM every four hours, for 58 hours, to follow movement through more than one cycle. Both control and IF treated cultures(Fig. 3a & b) commenced DNA synthesis after approximately eighteen hours. However, cell division ensued in about 24 hours in control cultures as compared to about 38 hours in the interferon treated ones. These results are consistent with the experiments shown in Figure 2 where cells were pretreated with interferon before serum stimulation, and suggest that pretreatment is not necessary for the interferon effect on serum stimulation. Although the percentage of cells in interferon-treated cultures which eventually entered S are the same as control, the disperion in this movement was markedly different. Specifically, the transition rate of cells into S was decreased in interferon-treated cultures, and their movement through S was prolonged by a minimum of fourteen hours(Fig. 3b). By 58 hours the start of a new division cycle was evident in the control cultures, but not in the IF-treated ones. In this experiment there was no evidence of a start of new movement through S in the presence of interferon.

In contrast to experiments using pretreatment followed by the continuous application of interferon, pretreatment alone with subsequent removal had no inhibitory effect on transition of cells out of G_1, but rather enhanced the synchrony of movement of cells into S (data not shown).

Addition of interferon at different times after the beginning of serum stimulation was carried out to separate the effects of interferon on the early and late events in the serum stimulation process. Addition of interferon at 14 hours after serum stimulation gave essentially the same cell cycle perturbations observed when cells were treated simultaneously with release from G_o-G_1 block(Fig. 3c). In these cultures, cells began to enter S with a dispersion similar to controls, but traversed S at a rate similar to those cells treated with interferon from the time of serum stimulation. Thus, interferon treatment of cells when they begin DNA synthesis perturbs movement of these cells through S;however, cells that have not committed to make DNA by the time of interferon addition are altered both in their transit time through S and their transition probability per unit time out of G_o-G_1. Treatment of cells in S(+20 hours) as shown in Figure 4 has minimal effect on the rate of incorporation of 3H-thymidine into the population. However, a direct effect of interferon on total 3H-thymidine incorporated per culture is apparent. When interferon was added before the cells entered S both the rate and the total incorporation of 3H-thymidine was reduced.

Relationship of Antiviral and Anticellular Effects of Interferon

The association of the antiviral and antiproliferative activites of interferon in the same molecule has been a subject of controversey in the literature(30,31)). The availability of a number of cell lines with various growth responses to interferon allowed us to investigate this issue. A summary of the ability of interferon to inhibit VSV replication in cells used in this study is shown in Table 1. Most cell lines, except two(Hs294T-clone 6 and GM637), were sensitive to the antiviral action of interferon. Specifically, two to three log reductions in VSV titer were obtained in most cell lines, while almost no reduction or even slight enhancement in VSV multiplication(shown as negative numbers) was observed in others(Table 1). Of interest is the association of the two activities of interferon in some, but not all cell lines. As shown in Table 1, three types of associations were observed:

1) Two cell lines(Hs294T-clone 6 and GM637) resistant to growth inhibition by interferon were also resistant to its antiviral action.

2)Two cell lines(Hs695T and Hela) resistant to growth inhibition by interferon were sensitive to its antiviral action.

3) The remaining seven cell lines were sensitive to both the antiproliferative and antiviral activities of interferon.

DISCUSSION

In this report we demonstrate that human leukocyte interferon preparations are capable of inhibiting the growth of human melanoma cells in culture. The growth of a cell line with interferon appears to be influenced by its behavior at high density. Our experiments show that cell lines that enter G_o–G_1 at saturation density are far more sensitive to the cytostatic action of interferon than those lines which continue to proliferate under similar condition. Although there are many possible explanations for this correlation our data will be discussed in terms of the Smith and Martin model for cell cycling(16). The model states that cells enter an "A" state after mitosis in which their activity is not directed towards replication. Departure from the "A" state into the "B" phase (which consists of S, G_2 + M, and a portion of G_1 is a seemingly random event but one which is governed by an underlying probability function. This transition probability function is determined by the cell type and by environmental conditions. A complete description of the cell lines' growth rate results from the combination of the transition probability function (i.e., rate of progress from "A" to "B"), the duration of the "B" phase, and the rate of cell death. In our case, interferon failed to exhibit cytotoxic effects at doses inhibiting cell growth; however, it affected the growth rate of the melanoma cells by both decreasing their transition probability per unit time and lengthening the "B" phase. Specifically, interferon decreased the transition rate of cells out of G_o–G_1 into S and prolonged S by several hours.

Some of the aforementioned cell cycle perturbations have been shown in interferon-treated, serum starved 3T3 cells(17,18), normal human fibroblasts (7, 19), and some sychronized, virally transformed cells(20). Other conditions previously used to demonstrate cytotoxic effects of interferon, for example, arginine deprivation (21), growth in low serum(22, 23), and aged cells (24-27) may relate to the ability of these treatments to shift cells into G_o–G_1. In our experiments we demonstrated that melanoma cells responsive to the antiproliferative activity of interferon were mainly in the G_o–G_1 state by both (i) the kinetics of their release into S(Figs. 3 & 4) following serum stimulation, including a significant lag of 14 to 18 hours prior to commencement of DNA synthesis and (ii) by the fact that cell division in the stimulated cells was preceded by DNA synthesis(28).

What makes those cells that reside in the G_o–G_1 state vulnerable to the cytostatic action of interferon is unclear and open for speculation. However, our observations with regard to cell cycle behavior at saturation density and susceptibility to inhibition of growth by interferon support the idea that some specially sensitive metabolic event is

required for progress through the G_o-G_1 phase of the cell cycle(29). That specific event may be the target of interferon's cytostatic action in that interferon may influence it in a manner which limits cell cycling. Evidence that interferon affects event(s) in G_1 comes from our data showing that interferon treated cells go slowly through one cycle of division, return to G_1(G_o-G_1), and fail to enter a new cycle. This suggests that interferon influenced the committment step to DNA synthesis.

Interferon also inhibits progress of cells through S. It is unclear whether the additional effects of interferon on cell cycling are sequel to events normally initiated in G_1, but suppressed by interferon, or alternatively that interferon has multiple targets of action. Our results (Fig. 4) favor the first explanation, since interferon addition during early S has minimal inhibitory effects on DNA synthesis during that division cycle.

The relatedness of the antiviral and antiproliferative activities of interferon in some cells and not others, suggests that the relationship is mainly dependent on the cell line and not necessarily on the interferon preparations. This observation offers an explanation for the inconsistencies reported in the literature on the relationhip of the two activities to each other (30, 31).

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TABLE 1

RELATIONSHIP OF CELL CYCLE BEHAVIOR
TO INTERFERON SENSITIVITY

Human Cell Lines	Growth Inhibition by Interferon (U/ml) 25	250	1000	Antiviral Activity of Interferon (250 U/ml)	Cell Cycle Position at Confluence[4]
Melanoma					
Hs294T	+[1]	+	+	2.46[3]	G_0-G_1
Hs852T	-[2]	+	+	1.49	G_0-G_1
HS695T	-	-	-	2.10	G1, S, &G2+M
Hs939T	-	+	+	3.47	G_0-G_1
M3	-	+	+	1.65	G_0-G_1
Hs294T-Clone 6	-	-	-	0.52	G1, S, &G2+M
Hs294T-Clone 6 revertant	-	-	+	2.58	G_0-G_1
SH4	-	+	+	3.06	G_0-G_1
Other					
GM37	-	-	+	3.89	G_0-G_1
GM637-SV40 transformed	-	-	-	-0.90	G1, S, &G2+M
HeLa	-	-	-	2.13	G1, S, &G2+M

1. + means greater than 40% reduction in cell numbers as compared to control.

2. - means less than 40% reduction in cell numbers as compared to control.

3. log 10 of virus titer (PfU/0.2ml) without interferon less log 10 of virus titer (PfU/0.2ml) with interferon.

4. Populations with greater than 90% G1 cells were considered blocked in G_0-G_1.

TABLE 2

EFFECT OF INTERFERON ON THE DISTRIBUTION OF GROWING Hs294T CELLS IN THE CELL CYCLE

	Day	Fraction in Phase G_1	S	G_2+M	Fraction in S by Autoradiograph
Control	1	0.430	0.286	0.233	0.39
	2	0.586	0.267	0.148	0.30
	3	0.679	0.192	0.129	0.14
	4	0.836	0.058	0.106	0.07
250 Units	1	0.398	0.412	0.190	0.52
	2	0.523	0.320	0.157	0.34
	3	0.550	0.322	0.128	0.34
	4	0.504	0.375	0.121	0.40

TABLE 3

CELL CYCLE DISTRIBUTION AFTER INTERFERON AND COLCEMID TREATMENT OF GROWING Hs294T CELLS*

Treatment	% of Cells in $S + G_2+M$	
	minus Colcemid	plus Colcemid
Control	53.5	77.9
250 Units IF/ml	42.0	49.8

*Hs204T cells were plated at 5×10^4 cells per dish. Interferon (250 U/ml) was added 24 hours later for 48 hours with colcemid (0.2 µg/ml) being present in the last 16 hours. The percent of cells in $S + G_2+M$ was obtained by FCM. Colcemid treatment altered the ratio of the G_1 peak position to the G_2+M position; therefore, reliable extraction of the proportion of cells in S from G_2+M was not possible.

FIGURE 1

The Effect of Varying Concentrations of Interferon on the Growth of Melanoma Cells.

Twenty four hours following cell seeding (5×10^4 cells/ ml), the indicated amounts of interferon were added to the cultures. Ninety six hours later 0.1% trypan blue excluding cells were counted. The results are expressed as percent of control growth i.e.

$$\frac{\text{cell count with interferon}}{\text{cell count of control}} \times 100$$

Hs294T-clone 6 (△----△) , Hs695T (○-----○), Hs852T (■——■), and Hs294T (●——●).

Figure 4

Determination of time of interferon action during the Go/S interval. High density, nutrient starved Hs294T cells were serum stimulated. Interferon (250 U/ml) was added to test cultures at the time of serum stimulation (0 hours), 8, 16 and 20 hours thereafter. ^{3}H-thymidine (see Materials and Methods) was added at time of interferon addition. Samples ~~were taken at times~~ shown on the ~~abscissa for measurement~~ of ^{3}H incorporation by scintillation counting.

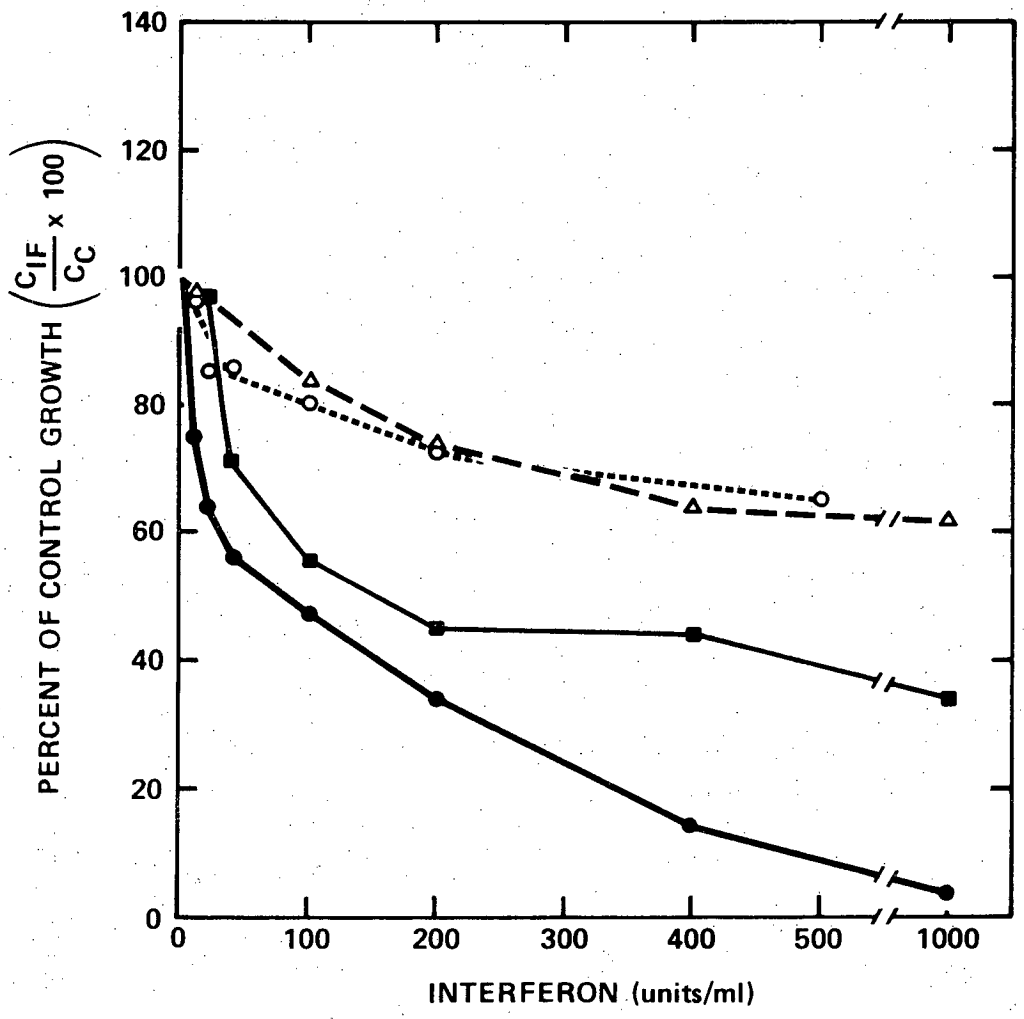
PERCENT OF CONTROL GROWTH $\left(\frac{C_{IF}}{C_C} \times 100\right)$
140
120
100
80
60
40
20
0
0
100
200
300
400
500
1000
INTERFERON (units/ml)

Figure 1

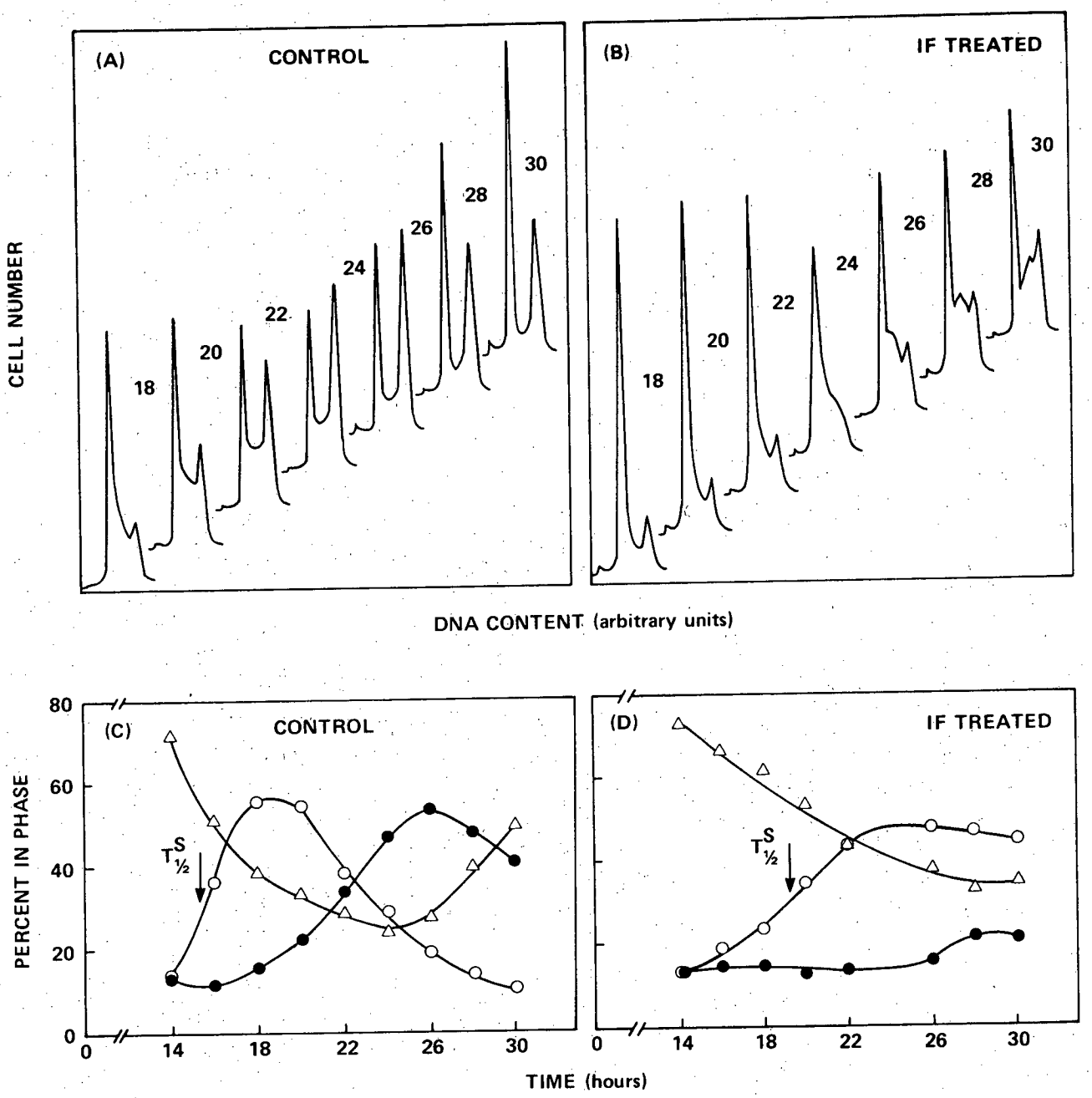
(A)
CONTROL
(B)
IF TREATED
18
20
22
24
26
28
30
CELL NUMBER
DNA CONTENT (arbitrary units)
(C)
CONTROL
(D)
IF TREATED
$T^S_{½}$
PERCENT IN PHASE
TIME (hours)
0
20
40
60
80
14
18
22
26
30

Figure 2

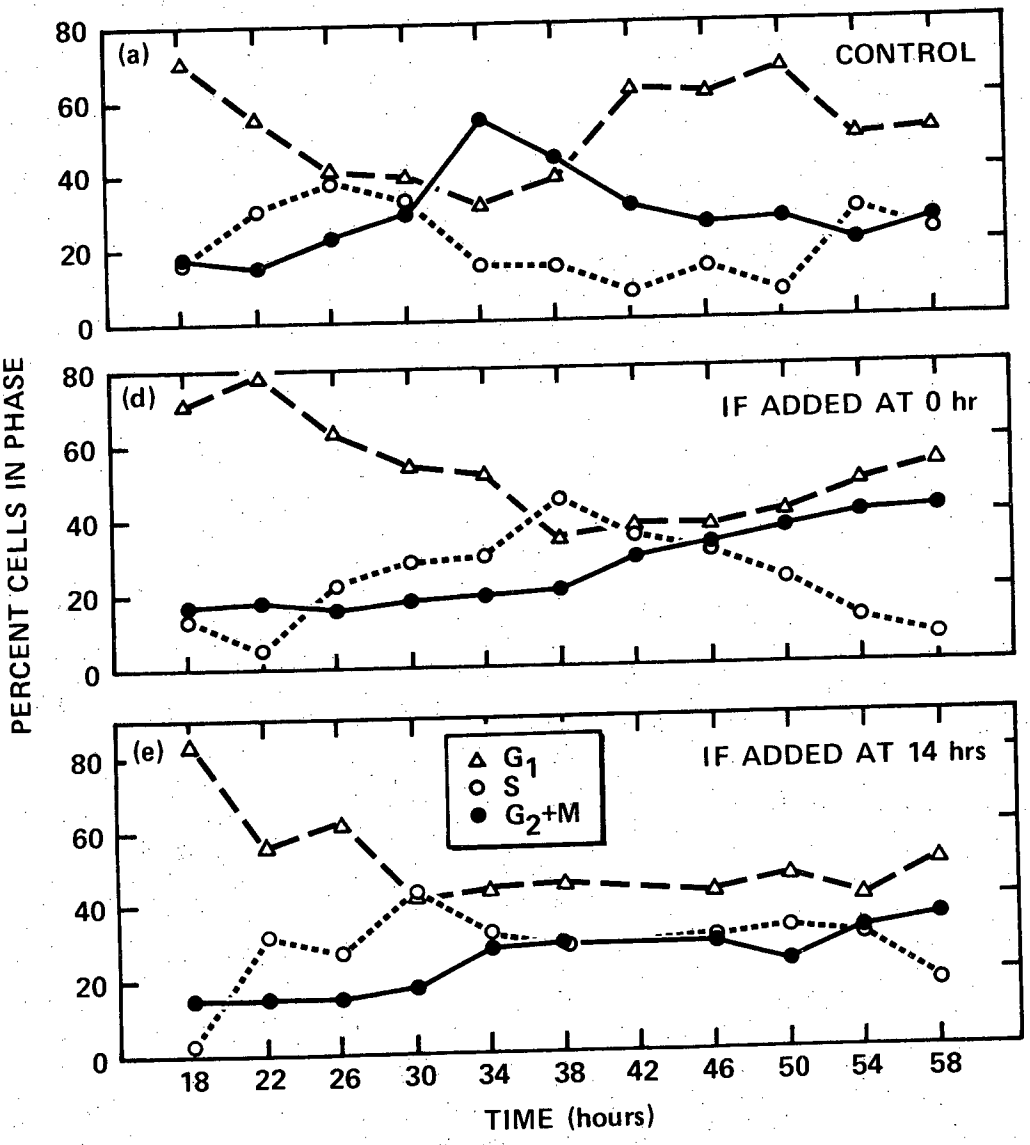
(a)
CONTROL
(d)
IF ADDED AT 0 hr
(e)
IF ADDED AT 14 hrs
G_1
S
G_2+M
PERCENT CELLS IN PHASE
TIME (hours)
0
20
40
60
80
18
22
26
30
34
38
42
46
50
54
58

Figure 3

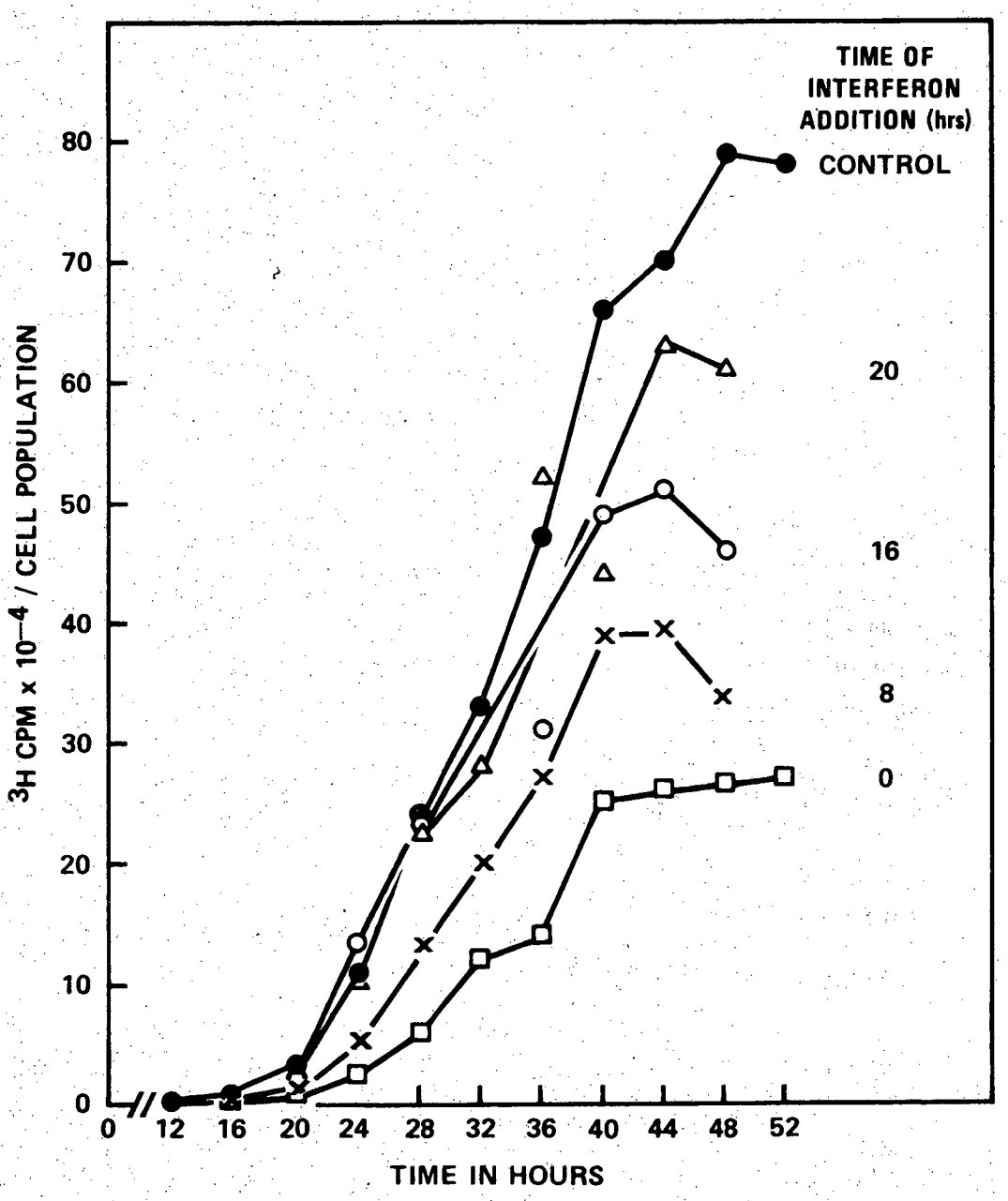
TIME OF INTERFERON ADDITION (hrs)
CONTROL
20
16
8
0
80
70
60
50
40
30
20
10
0
^{3}H CPM x 10^{-4} / CELL POPULATION
0
12
16
20
24
28
32
36
40
44
48
52
TIME IN HOURS

Figure 4

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