INTERFERON: EFFECT ON THE SATURATION DENSITY TO WHICH MOUSE CELLS WILL GROW IN VITRO

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Interferon is a protein secreted by animal cells, when stimulated by certain agents, which has the ability to induce in homologous cells a resistance to replication of a wide variety of viruses (1, 2). Moreover, preparations of mouse interferon, both crude and partially purified, have been shown to inhibit the growth of mouse L cells (3) and mouse lymphoid leukemia cells (4) in suspension culture. Recently, crude preparations of mouse interferon have been reported to inhibit the growth in monolayer culture of L, mouse embryo, and mouse kidney cells (5).

The term "density-dependent inhibition" of cell growth has been proposed to describe the termination of animal cell growth in monolayer culture when a certain density of cells per square centimeter of surface is reached (6). The final density to which a given type of cells will grow is called its "saturation density". Some cells are more sensitive to density restriction than others, e.g., viral-transformed mouse 3T3 cells are much less restricted than the original 3T3 cells (7). In this communication the effect of partially purified preparations of mouse interferon on the saturation densities of various mouse cells is reported.

MATERIALS AND METHODS

Cells

Mouse L cells were maintained in suspension culture in Eagle's suspension culture medium (Joklik modified) supplemented with 10% fetal calf serum. The cell concentration was maintained at 10–80 $\times$ 10^4 cells/ml and an equal volume of fresh medium was added every 24 h. Monolayer cultures of L cells were prepared by adding an equal volume of Eagle's minimum essential medium, supplemented with 10% fetal calf serum, to an equal volume (or less) of suspension culture and plating in a 50 mm plastic Petri dish. Mouse 3T3 cells and SV-40-transformed 3T3 (SV-3T3) were a gift to Dr. T. Sugiyama from Dr. H. Green. Polyoma-transformed 3T3 (Py-3T3) was a gift to Dr. T. Sugiyama from Dr. M. Burger. Cultures of 3T3, SV-3T3, and Py-3T3 cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Mouse embryo secondary cultures were prepared from 5-day old primary cultures and maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum. HeLa-O cells were maintained in McCoy's 5A medium (Hsu modified) supplemented with 5% calf serum. LLCMK2 (monkey kidney) cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum. All media were purchased from Grand Island Biological Co., Grand Island, N.Y. For routine maintenance all cells except L were kept in plastic culture bottles, and for growth experiments cells were plated into plastic Petri dishes. In all growth experiments the medium (3 ml) was changed every 48 h until the cell density reached 10 $\times$ 10^4 cells/cm^2; at cell densities above 10 $\times$ 10^4 cells/cm^2 the medium was changed every 24 h. To count the cells the medium was removed by suction and a 0.25% solution of trypsin containing 0.01 M ethylenediaminetraacetate was added to the dish. After 5–10 min at 37°C the suspended cells were counted in a hemacytometer. The experiments shown in Figs. 1 and 2 have been repeated a number of times with the same result. Although the saturation density of a cell in control or interferon-containing medium may vary from experiment to experiment, the difference in saturation density between control and interferon-treated cells remains approximately constant from experiment to experiment.

Interferon

Mouse interferon (L cell) was purified to a specific activity of 1–2 $\times$ 10^7 NIH international reference U/mg.[1] The interferon preparation at this specific activity is not homogeneous when analyzed by electrophoresis on polyacrylamide gel. Interferon antiviral activity was assayed on L cells by the vesicular stomatitis virus plaque reduction method (8). That dilution of interferon causing a 50% reduction in plaques from the control is defined as containing 1 U/ml. 1 U in this assay equals 5 NIH international reference U, and all activities reported in this communication are in NIH international reference units.

RESULTS

Since most research on the effect of interferon preparations on cell growth has been done with

[1] E. Knight, Jr., and W. E. Stewart II. Unpublished results.

cells in suspension culture, experiments were initiated to study the effect of interferon preparations on the saturation densities of various mouse cells growing in monolayer culture. In general, cells were plated at various starting densities (0.05×10^4 to 10×10^4 cells/cm^2), interferon was applied 24–48 h later, and cell counts were made periodically until the cells reached maximum density. Operationally, in these experiments maximum density is that number of cells per square centimeter which does not increase after 24–48 h. The effective concentration of interferon is defined as the lowest concentration which gives the maximum reduction in final cell density. Interferon of specific activity of $1–2 \times 10^7$ U/mg was used in all the experiments reported in this communication.

The effect of interferon on the growth and saturation density of L cells in monolayer is shown in Fig. 1 *a*. L cells in the presence of saturating concentrations of interferon (500 U/ml) grow more slowly, and routinely reach a final density 60–70% that of control cells. Cells grown to maximum density with interferon in the medium have the same appearance, when observed in the light microscope, as maximum density control cells except that the monolayers are less dense. Lindahl-Magnusson, Leary, and Gresser have also reported that preparations of mouse interferon inhibit the growth of L cells in monolayer culture (5). Since their control and interferon-treated cultures did not stop growing and therefore had not reached maximum density, they may have observed the same phenomenon as shown in Fig. 1 *a* but at an earlier stage.

After the effect of interferon on the saturation density of L cells was established, it was of interest to examine its effect on other mouse cells. Mouse 3T3 cells which have a low saturation density, ca. 5×10^4 cells/cm^2, and polyoma and SV-40-transformed 3T3 cells with saturation densities of $30–40 \times 10^4$ cells/cm^2 were chosen for such a study. The effect of interferon, 250 U/ml for Py-3T3 and 500 U/ml for SV-3T3, on the growth and saturation densities is shown in Fig. 1. Three conclusions can be made from these data: (*a*) interferon reduced the saturation densities for Py-3T3 and SV-3T3; (*b*) it decreases the growth rate of both cell types; and (*c*) the saturation density imposed by interferon does not depend on the density at which the interferon is added (compare Fig. 1 *b* to Fig. 1 *c* and Fig. 1 *d* to Fig. 1 *e*). As in L cells, the reductions in saturation densities of Py-3T3 and SV-3T3 are a function of interferon concentration. The effective concentration of interferon as measured by reduced saturation densities is 50 U/ml for Py-3T3 and 250 U/ml for SV-3T3. At 500 U/ml and a specific activity of interferon of 1×10^7 U/mg, the added protein concentration is <0.2 μg/ml.

In contrast to the effect on L, Py-3T3, and SV-3T3 cells, interferon at 500 U/ml does not reduce the saturation density of 3T3 cells (Fig. 2 *a* and *b*) although the growth rate is reduced. Since the saturation density of 3T3 cells was not reduced by interferon it became of interest to examine the effect of interferon on the growth of mouse primary and secondary cultures as well as heterologous established cell lines. Mouse embryo cells were

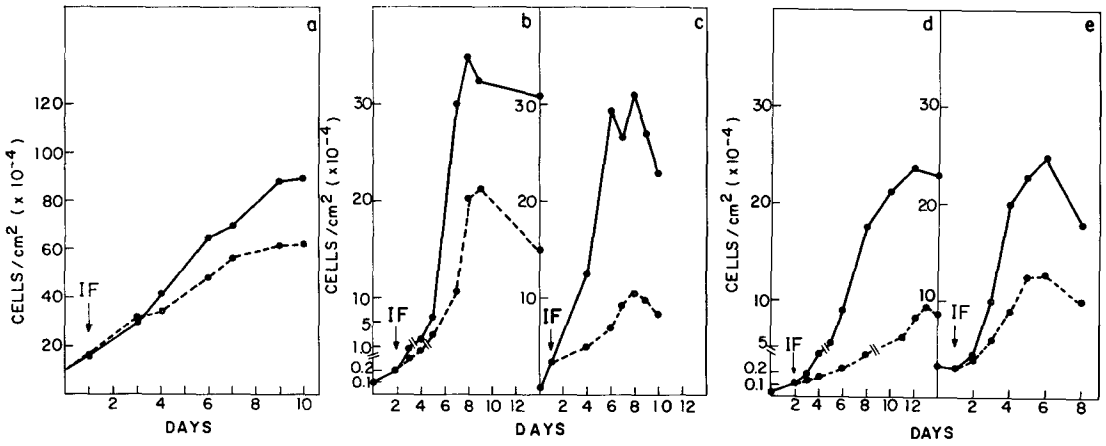

FIGURE 1 The effect of interferon (*IF*) on the saturation densities of L, Py-3T3, and SV-3T3 cells. Each point represents the average of duplicate dishes. Cell counts in duplicate dishes varied no more than 15% from the average. (*a*) L cells; interferon, 500 U/ml. (*b* and *c*) Py-3T3; interferon, 250 U/ml. (*d* and *e*) SV-3T3; interferon, 500 U/ml. • —— • , no interferon; • -- • , interferon.

TABLE I

Viability of 3T3, SV-3T3, and Py-3T3 Grown to Maximum Density in the Presence and Absence of Interferon

Cells	Growth conditions	No. of cells plated 50 mm dish ($\times 10^{-4}$)	Days after plating (No. of cells [$\times 10^{-4}$]) 3	4	5	6	Percent of viable cells after trypan blue*
Py-3T3	Control	5	17			186	95
Py-3T3	IF	5	18			220	92
SV-3T3	Control	5		60		103	93
SV-3T3	IF	5		78		156	84
3T3	Control	5		20			90
3T3	IF	5		19			92

* Trypsinized cells in suspension were mixed with an equal volume of 0.5% trypan blue. Cells were counted in a hemacytometer, and those cells which excluded the trypan blue were scored as viable cells.

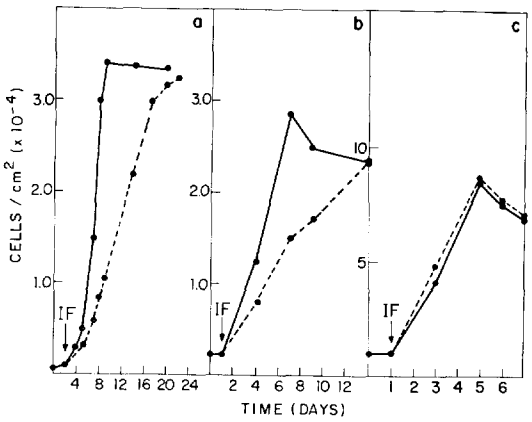

FIGURE 2 The effect of interferon (*IF*) on the saturation densities of 3T3 and mouse embryo secondary cultures. (*a* and *b*) 3T3 cells; • —— •, no interferon; • -- •, interferon, 500 U/ml. (*c*) mouse embryo; • —— • no interferon; • -- •, interferon, 1000 U/ml.

chosen and secondary cultures were prepared from 5-day old primary cultures. The effect of interferon on the growth rate and saturation density of mouse embryo secondary cultures is shown in Fig. 2 *c*. As in 3T3 cells, the interferon does not affect the saturation density of the mouse embryo cells. Mouse interferon does not reduce the growth rate or the saturation density of heterologous cells such as HeLa and LLCMK2.[2]

The reduction in saturation densities of L cells, Py-3T3, and SV-3T3 by interferon probably cannot be attributed to a decrease in cell viability. The data in Table I show that cell viability at the saturation density attained in the presence of interferon is approximately the same as that of the saturation density control cells. The 3T3, Py-3T3, and SV-3T3 cells grown to saturation density in the presence of interferon grow as well as control cells after replating in the absence of interferon. In addition, cells which have reached saturation density in the presence or absence of interferon have the same viability as measured by the exclusion of trypan blue (Table I).

DISCUSSION

The following conclusions can be made from the data presented in this report on the growth inhibitory effect of L cell interferon: (*a*) purified preparations of interferon reduce the saturation densities and the rate of growth of L, SV-3T3, and Py-3T3 cells; (*b*) the effective interferon concentration for maximum reduction in saturation density is different for each of the susceptible cell lines; and (*c*) the purified preparations of interferon are effective in the reduction of the saturation density at "added" protein concentrations of <0.2 μg/ml. The reduction in growth rate and saturation density by interferon preparations are different phenomena. Whether they are caused by the same mechanisms cannot be answered at present.

These results confirm the earlier work of Paucker Cantell, and Henle (3) and extend that of Gresser and co-workers (4, 5, 9). Furthermore, recent reports have shown that mouse interferon reduces the final cell density as well as the growth rate of L1210 cells (9) and mouse embryo and mouse

[2] E. Knight, Jr. Unpublished results.

kidney primary cells (5). It has been suggested that it is unlikely that the growth inhibition is directly related to the antiviral effect of interferon, i.e., different end results are produced by the same mechanism (5). Interferon-treated cells when infected by a virus inhibit the expression of viral genes. It is not known if the expression of viral genes in virus-transformed cells is necessary for cell growth. It is conceivable that interferon has specific binding sites on the surface of the cell and that the two phenomena are "induced" from the same site.

The most interesting question concerning growth inhibition is why interferon reduces the saturation densities of cells which grow to high densities (L, Py-3T3, SV-3T3) but not those which do not (3T3, mouse embryo secondary). Although there is less range for growth in the mouse embryo secondary cultures (ca. three doublings), we have observed no effect of interferon in a number of experiments. Are 3T3 and mouse embryo cells more normal and therefore less susceptible to reduction in saturation density than the virus-transformed 3T3 cells? Although crude preparations of mouse interferon have been reported to inhibit the rate of growth and the final density of mouse embryo and mouse kidney primary cultures (5), it is not clear how that observation relates to that on secondary cultures described in this report. These different observations could be due to either differences in the interferon preparations or to a different response in primary than in secondary cells.

Whether the effect of interferon on cell growth in vitro reported here and earlier by others has any relation to the reported inhibition of growth of murine-transplantable tumors and increased survival of tumor-inoculated mice after administration of preparations of interferon (10) is not clear at present. It should be emphasized that although interferon is designated as the active component in growth inhibition, homogeneous interferon is not yet available. It is therefore still possible that the growth inhibitory and antiviral activities reside in different molecules.

SUMMARY

Partially purified preparations of mouse interferon reduce the growth rate and the saturation densities of L cells and 3T3 cells transformed by either SV-40 or polyoma when grown in monolayer culture. The effects are dependent on the concentration of the interferon in the medium, and the effective concentration is different for different cells. The saturation density of 3T3 cells is unaffected by interferon although they grow at a reduced rate. Mouse embryo cells as well as heterologous cells such as HeLa and LLCMK2 are unaffected by interferon.

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