Interferon and Cell Division
VI. Inhibitory Effect of Interferon on the Multiplication of Mouse Embryo and Mouse Kidney Cells in Primary Cultures (36047)

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Both crude and partially purified mouse interferon preparations have been shown to inhibit the multiplication of mouse L (1, 2), and lymphoid leukemia, L 1210, cells (3–7) in nonagitated suspension cell cultures. Likewise, crude human leukocyte interferon preparations inhibit the multiplication of human RPMI–1196 cells in suspension cultures (8). On the other hand, it has been reported that interferon does not affect the multiplication of normal chick (9, 10, 11), and human (12, 13) cells in monolayer cultures. These results suggested the possibility that interferon exerts a selective inhibitory effect on the multiplication of transformed cells and does not affect the multiplication of normal cells. It seemed of interest, therefore, to determine concomitantly the effect of mouse interferon preparations on the multiplication of normal mouse cells in primary monolayer cultures and its effect on the multiplication of cells of two established mouse cell lines also grown in monolayer cultures. The results of these experiments are reported herein.

Materials and Methods. Cells. Mouse embryo cells were prepared by trypsinization of IC (Institut du Cancer) or C3H mouse embryos at 12–18 days of gestation, and kidney cells were prepared from 2–3-week-old IC mice. In the experiments to be described, the effect of interferon preparations was determined on primary monolayer cell cultures. The cells were cultivated in Eagle's minimal essential medium (MEM) supplemented with 20% heat inactivated calf serum, 0.6 mg of sodium bicarbonate/ml, penicillin 200 U/ml, and streptomycin 40 μg/ml. Mouse L cells (14) were cultivated in MEM supplemented with 10% heat-inactivated calf serum, 2.7 mg of sodium bicarbonate/ml and antibiotics. Mouse MSV and MSV–IF cells (15), (kindly supplied by Dr. Chany) and human KB cells (kindly supplied by Dr. Girard) were cultivated in the same medium as mouse embryo cells.

Interferon and control preparations. Mouse interferon preparations were obtained from the nutrient medium of monolayer cultures of MSV–Ia cells (15) inoculated with ultraviolet inactivated Newcastle disease virus (NDV) and from the brains of Swiss and IC mice inoculated intracerebrally with West Nile virus (16). Human interferon preparations (generously provided by Dr. Chany) were obtained from the nutrient medium of the amniotic membrane (17), and from leukocyte suspensions (18) inoculated with NDV. The medium of uninoculated cell or tissue cultures, and the brains of uninoculated mice constituted the control preparations. All interferon and control preparations were treated at pH 2 for 18 hr (preparations containing West Nile virus), or 5 days (preparations containing NDV), centrifuged at 30,000 rpm for 1 hr in a Martin Christ centrifuge, Omega (60,000g) and then concentrated 10-fold by forced dialysis.

Assay of interferon. Mouse and human interferon preparations were assayed in duplicate twofold titrations in monolayer cultures of L and BSC cells, challenged with 100 $TCID_{50}$ of vesicular stomatitis virus (19). One mouse interferon unit (as expressed in the text) equals 4 NIH International Reference units and one human interferon unit equals one WHO International Reference unit.

Inactivation of interferon. Trypsin. MSV–Ia interferon and control preparations were incubated with an equal volume of crystalline trypsin in phosphate-buffered saline (final concentration 0.5 mg/ml) for 1 hr at 37°. Iniprol (Choay Laboratories, France)/(100, 000 U) was added to neutralize the enzymatic activity.

Periodate. MSV–Ia interferon and control preparations were dialysed against 0.02 *M*

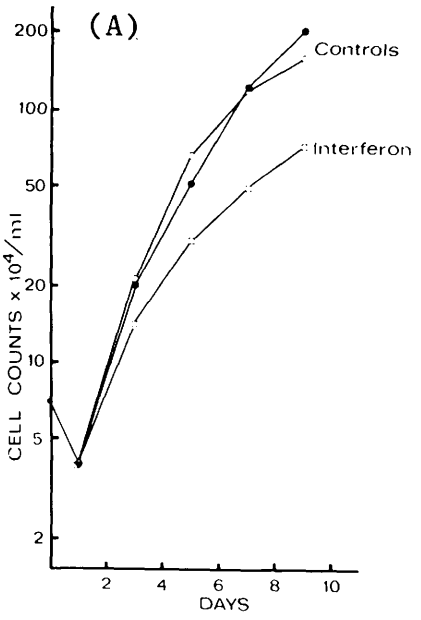

FIG. 1A

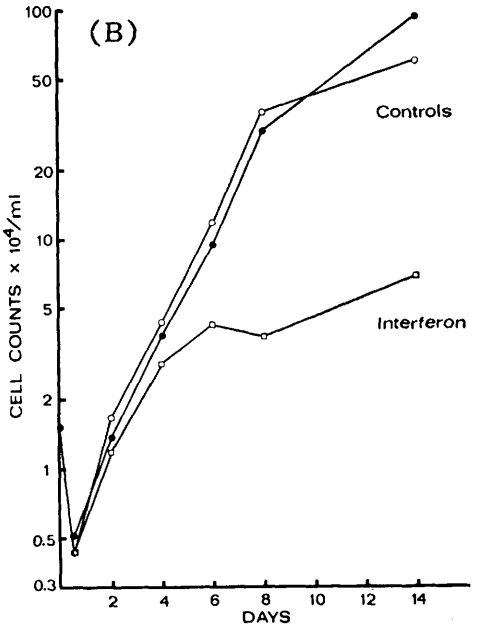

FIG. 1B

Effect of interferon on the multiplication of mouse L cells. A. Cultures seeded with 7.5 × 10^4 cells/ml. B. Cultures seeded with 1.5 × 10^4 cells/ml. ● is nontreated; ○ is mock interferon; □ is interferon.

sodium periodate at 37° for 2 hr. The reaction was terminated by dialysis against 5% glucose for 2 hr at 37° and dialysis was continued against phosphate-buffered saline for 18 hr at 4° (20).

Nucleases. MSV–Ia interferon and control preparations were incubated with 1:10 vol of crystalline RNAase or DNAase (final concentration 10 μg/ml) for 1 hr at 37°.

Heat inactivation. Heat inactivation was performed in a water-bath for 1 hr at 60°.

Experimental plan. Cells were seeded in 50 mm Falcon plastic petri dishes, with 2 ml of medium per dish. Cultures were incubated at 37° in humidified air—5% CO_2 atmosphere for 24 hr. The nutrient medium was discarded and 2 ml of fresh medium containing dilutions of interferon, or control preparations, were added and the cultures reincubated at 37°. Unless otherwise stated, the medium with interferon or control preparations was changed every 3rd or 4th day. Twenty-four hours after seeding and every 2nd day thereafter the following parameters were determined for triplicate cultures of each group:

Cell counts. Cultures were rinsed twice with 2 ml of phosphate–buffered saline (PBS) prior to addition of 2 ml of 0.25% trypsin. Cells were counted in a hemacytometer.

Cell viability. The nutrient medium from the cultures was harvested separately. Trypsin detached cells and cells in the medium were counted in a hemacytometer and their viability was determined by the trypan blue dye exclusion test.

Determination of total DNA. Cultures were rinsed twice with PBS. The DNA was extracted in 7% perchloric acid for 15 min at 70° and measured by the diphenylamine color reaction, slightly modified from Burton (21).

Determination of DNA synthesis. Tritiated thymidine (H^3–TdR) was added at a final concentration of 0.5 μCi/ml (specific activity 15 Ci/m*M*) and the cultures were incubated for 6 hr at 37°. The cell sheets were rinsed twice with cold phosphate–buffered saline and the cells were dissolved in 1 ml of 0.25 *N* NaOH. An equal volume of cold 25% trichloroacetic acid was added. The precipitate was centrifuged at 4° at 9000 rpm for 20 min. The sediment was dissolved in 1 ml of Soluene (Packard) and the radioactivity was measured in a liquid scintillation counter.

Results. Effect of interferon on the multi-

plication of L cells. Prior to determining the effect of interferon on the multiplication of "normal" cells in monolayer cultures, it was necessary to determine its effect on cells of an established cell line also maintained in monolayer cultures. As can be seen from Fig. 1A, MSV-Ia interferon (1000 U/ml) inhibited the multiplication of L cells. This inhibition was usually first observed on the 3rd or 4th day after cell seeding, at which time the control cell population had doubled approximately 2–3-fold. The inhibitory effect of interferon was more pronounced in the ensuing few days. In some experiments, cultures were initiated at a low cell concentration *i.e.*, 1.5×10^4 cells per ml, the nutrient medium was not changed, and interferon was not readded. Under these conditions, the inhibitory effect of interferon was also first observed toward the 4th day and became more marked with time (Fig. 1B).

When the nutrient medium was changed every 3rd or 4th day only 1–4% of the cells of both control and interferon-treated cultures incorporated the trypan blue dye during the 10–14 days of the experiment. Interferon treatment was, therefore, not associated with an increased cell mortality.

The continued cultivation of L cells with interferon for 3 months did not result in the selection of a cell population resistant either to the anti-cellular or the anti-viral activity of interferon. (Confluency of cell sheets was attained later in interferon-treated cultures than in control cultures, but treated cultures could be serially passaged by reseeding at a higher cell concentration.)

Effect of interferon on the multiplication of mouse cells in primary culture. Mouse embryo cells. Whereas, reproducible growth curves of L cells were easily obtained, it proved far more difficult to standardize the conditions necessary for optimal growth of mouse embryo cells. The growth rate of these cells was not only lower than that of L cells but varied from one experiment to another. In order to follow the multiplication of the embryonic cells during a sufficiently long period of time it was found essential to seed the cultures at an optimal concentration of cells per ml of medium and to have a sufficiently low concentration of cells per unit surface

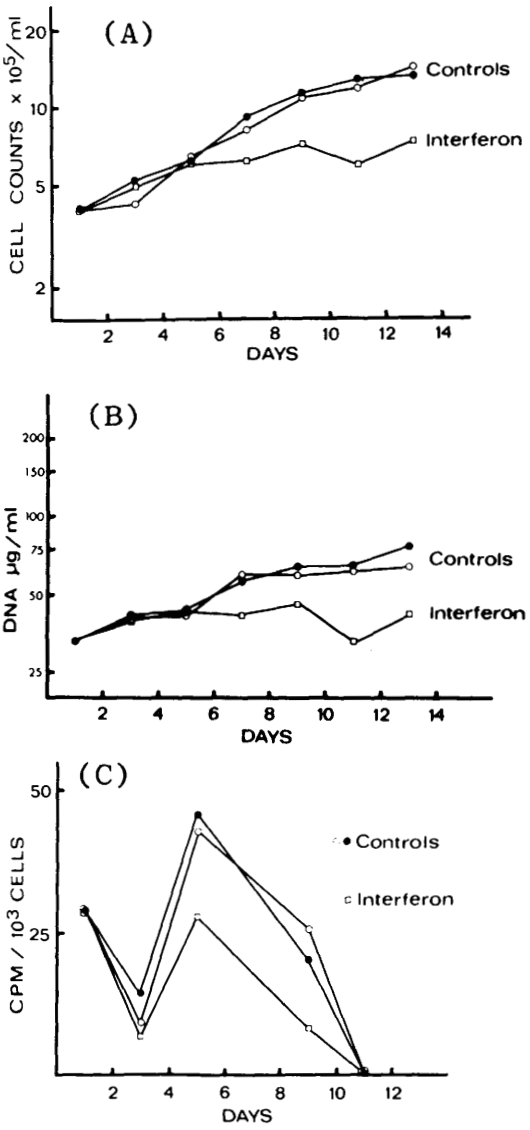

FIG. 2. Effect of interferon on C3H mouse embryo fibroblasts. ● is no treatment. ○ is mock interferon. □ is interferon. A. Effect on multiplication as measured by counting cells in a hemacytometer after trypsinization. B. Effect on total DNA as measured by the diphenylamine test. C. Effect of incorporation of H^3-TdR as measured by pulse labeling.

area in the petri dishes. These conditions were fulfilled by seeding 2 ml of a cell suspension (4×10^5 cells/ml) in 50 mm petri dishes.

Under these experimental conditions MSV-Ia interferon (1000 U/ml) was repeatedly shown to inhibit the multiplication of mouse embryonic fibroblasts (Fig. 2A). At times, the effect was not evident until the 7th or

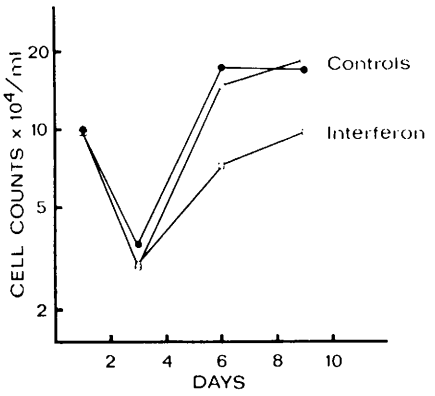

FIG. 3. Effect of interferon on the multiplication of weanling IC mouse kidney cells. ● is no treatment. ○ is mock interferon. □ is interferon.

8th day, depending on the growth-rate of the control cell population. As expected, the same degree of inhibition was also found by determinations of total DNA in parallel cultures (Fig. 2B). DNA synthesis was also decreased in interferon treated cultures (Fig. 2C) although the overall pattern of the curve was similar to that of control cultures.

The cell multiplication inhibitory activity of interferon could be demonstrated in cultures from both C3H and IC mice and from embryos at 12 and 18 days of gestation.

As in the experiments with L cells, interferon treatment was not associated with a significant increase in cell mortality.

Mouse kidney cells. MSV–Ia interferon (1000 U/ml) also inhibited the multiplication of weanling IC mouse kidney cells (Fig. 3).

Correlation between the anti-viral and cell multiplication inhibitory activities of interferon. Physicochemical properties. MSV–Ia interferon preparations were preincubated with RNAase, DNAase, trypsin, sodium periodate, or heated at 60° for 1 hr (see methods and materials). The effect of these treated interferon preparations was determined by cell counts for L cell cultures and by inhibition of H^3–TdR incorporation for mouse embryo fibroblast cultures. (In our experience there was less variability in cell counts after trypsinization of L cell cultures than after trypsinization of mouse embryo cell cultures. Inhibition of H^3–TdR incorporation thus proved a more reliable criterion of interferon action on mouse fibroblasts than cell counts.)

As can be seen in Table I, pretreatment of interferon with trypsin, periodate or heat, resulted in the loss of both antiviral and anticellular activities.

Species specificity. Table II shows the effect of different interferon preparations on the multiplication of mouse L and embryo cells. Both mouse brain and MSV–Ia interferons were effective, whereas the control preparations and human interferon preparations were ineffective.

It was considered of interest to determine whether the anticellular effect of mouse interferon exhibited the species specificity characteristic of its antiviral activity. As can

TABLE I. Effect of Pretreatment of Interferon Preparations on the Anti-cellular and Anti-viral Activities.

Pretreatment of interferon	% Inhibition of multiplication of L cells[a]	% Inhibition of H^3–TdR incorporation in mouse embryonic fibroblasts[b]	Interferon units/ml (anti-viral)
None	36.6	55	1000
RNAase	38.1	55	1000
DNAase	38.5	56	1000
Trypsin	2.7	0	<10
Periodate	4.5	0	<10
Heat 60° 1 hr	1.0	0	<10

[a] Inhibition of L cell multiplication was measured by counting number of cells in quadruplicate cultures 4 days after addition of interferon preparations.

[b] Inhibition of H^3–TdR incorporation was measured by 6 hr pulse labeling of quadruplicate cultures of mouse embryonic fibroblasts 5 days after addition of interferon preparations.

TABLE II. Effect of Different Interferon Preparations on Cultures of L Cells and Mouse Embryonic Fibroblasts.

Sample	Origin of preparation	% Inhibition of multiplication of L cells[a]	% Inhibition of H^3–TdR incorporation in mouse embryonic fibroblasts[b]	Interferon units/ml (anti-viral)
Interferon	MSV–Ia cells	32	60	1000
Interferon	Mouse brain	30	76	1000
Control	MSV–Ia cells	6	0	<10
Control	Mouse brain	3	15	<10
Interferon	Human amniotic membrane	0	0	<10[c]
Interferon	Human leukocyte	NT	2	<10[c]

[a] Inhibition of L cell multiplication was measured by counting number of cells in quadruplicate cultures 4 days after addition of interferon preparations.

[b] Inhibition of H^3–TdR incorporation was measured by 6 hr pulse labeling of quadruplicate cultures of mouse embryonic fibroblasts 5 days after addition of interferon.

[c] Antiviral titer in monkey BSC cells was 1:1000.

NT = not tested.

be seen from Fig. 4, a human amniotic interferon preparation inhibited the multiplication of human KB cells, but the heterologous mouse MSV–Ia interferon was without demonstrable effect.

Effect of interferon on the multiplication of an interferon resistant cell subline, MSV–IF. By long-term cultivation of MSV cells in the presence of interferon, Chany and Vignal (15) were able to select a cell subline (MSV–IF) resistant to the antiviral activity of interferon. Prior to utilization in these experiments, MSV–IF cells were subcultivated 5 times in the absence of interferon.

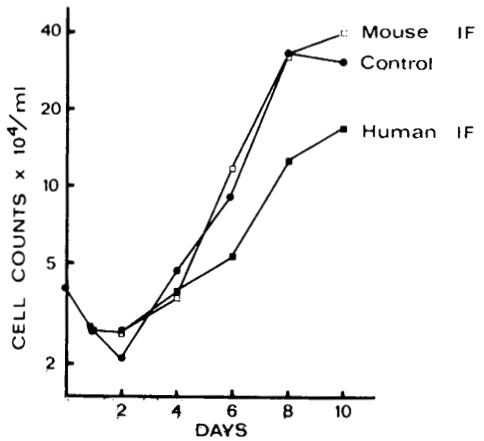

FIG. 4. Effect of human and mouse interferon preparations on the multiplication of human KB cells. ● is nontreated. ■ is human interferon. □ is mouse interferon.

MSV–Ia interferon (1000 U/ml) inhibited cell multiplication of the parent MSV cell line (sensitive to the antiviral action of interferon) but did not inhibit multiplication of cells of the MSV–IF cell line, resistant to the antiviral action of interferon (Fig. 5).

Dose relationship. Titration of the anticellular activity of interferon showed that the minimal concentration of interferon necessary to obtain a 30% inhibition of multiplication of L cells, or a 60% inhibition of H^3–TdR incorporation in mouse embryonic fibroblasts, was approximately 125 U/ml of MSV–Ia interferon and 250 U/ml of mouse brain interferon.

Discussion. The experimental results presented herein demonstrate that mouse interferon preparations inhibited the multiplication of mouse embryo and weanling mouse kidney cells in primary monolayer cell cultures. To the best of our knowledge this is the first report of an inhibitory effect of interferon on the multiplication of "normal" cells. The same interferon preparations also inhibited the multiplication of cells of two established mouse cell lines (L and MSV cells) in monolayer cultures.

Complete purification of interferon has not yet been achieved. However, the factor responsible for the inhibition of cell multiplication fulfilled the various physicochemical

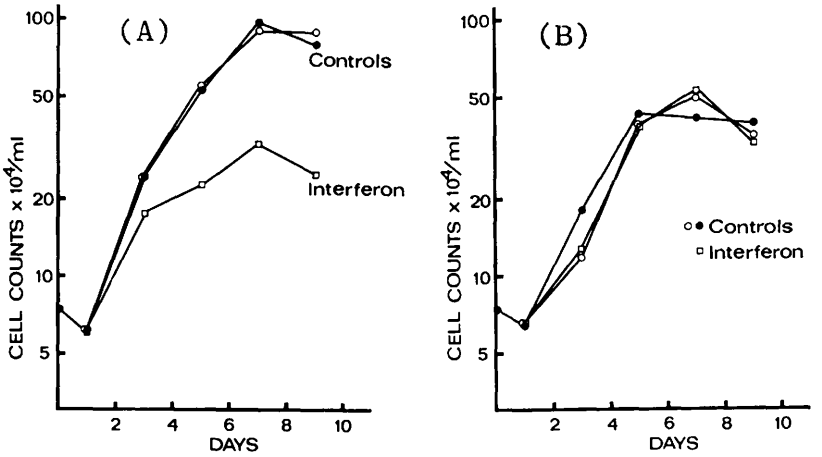

FIG. 5. Effect of interferon on the multiplication of mouse MSV cells. A. Parental MSV cells, sensitive to the anti-viral action of interferon. B. MSV–IF cells, resistant to the anti-viral action of interferon. ● is nontreated. ○ is mock-interferon. □ is interferon.

and biological criteria of interferon, and its activity could not be dissociated from the antiviral activity of interferon. Thus, (i) mouse interferon preparations derived from brain or from cell culture (induced by 2 different viruses) proved effective in inhibiting the multiplication of murine cells, whereas the corresponding control preparations were ineffective. Preparations of human interferon derived from 2 different tissues, using the same interferon inducer (NDV), did not inhibit the multiplication of mouse cells. (ii) Conversely, mouse interferon preparations did not inhibit the multiplication of human KB cells, which were however, inhibited by human interferon preparations. (iii) Heating of mouse interferon for 1 hr at 60°, or treatment with trypsin or periodate, markedly reduced both the antiviral and the anticellular activities of interferon. (iv) Interferon preparations inhibited the multiplication of MSV cells which are sensitive to the antiviral action of interferon, but the same interferon preparations did not inhibit the multiplication of cells of a subline, MSV–IF, resistant to the antiviral action of interferon (15).

In considering the results of a number of experiments on the effect of interferon on the multiplication of cells in primary monolayer cultures, two conditions seemed of special importance in demonstrating an inhibitory effect: (i) the duration and the rate of exponential growth of the cell population and (ii) the concentration of interferon. Thus, it was necessary to define the optimal seeding concentration of mouse embryo and mouse kidney cells per ml of nutrient medium and the optimal cell density per unit surface area to ensure continued cell growth over several days. It may be that a certain time must elapse either for interferon to act and/or for the effect to become evident. The time of appearance of the inhibitory effect on cell multiplication also seemed to be closely related to the cell growth rate and was observed earlier in cultures in which cells were multiplying rapidly than in cultures showing a less rapid growth rate. We should also emphasize that in most experiments L and mouse embryo cells were cultivated in nutrient medium containing 1000 U of interferon per ml. Titration of the anti-cellular activity revealed that the minimal interferon concentration was approximately 125–250 U/ml. These considerations may explain in part the failure of other investigators to observe an inhibitory effect of interferon on the multiplication of human (12, 13) and chick (9, 10, 11) cells in primary cultures. In their experiments, the observation period may have been too brief, or cultures seeded at too high a cell concentration may have reached the saturation density before an inhibitory effect could be observed; or the interferon concentration may have been too low. However, it is also possible that the difference in results between the

inhibitory effect of interferon on mouse embryo cells and the lack of interferon effect on human and chick embryo cells as reported by other investigators may reflect an inherent difference in the behavior of these cells. In this regard, it is of interest to note that "spontaneous" transformation is often observed in cultures of mouse embryo cells (22) and rarely observed in cultures of human and chick embryo cells (23, 24).

If interferon is the responsible factor, the question arises whether the inhibitory effect of interferon on cell multiplication was mediated by its antiviral action. To explain the effect of interferon on this basis implies that all the different primary cell cultures utilized in over a year of experimentation were infected with viruses, and that these viruses influenced cell multiplication. This seems unlikely to us. In view of our present findings and those previously described (3–7) it seems more reasonable to suggest the following interpretation. Either the mechanism of action of interferon responsible for the inhibitory effect on cell multiplication is different from that responsible for inhibition of viral replication, or, both effects are the result of a common mechanism of action. In either case these findings suggest that under certain experimental conditions interferon is not exclusively an antiviral substance.

Summary. Mouse interferon preparations inhibited the multiplication of mouse embryo and weanling mouse kidney cells in primary monolayer cell cultures. A comparable inhibitory effect was also observed on the multiplication of cells of 2 established mouse cell lines. Both the anti-cellular and the anti-viral activities were markedly reduced by pretreatment of the interferon preparations with trypsin, sodium periodate or by heating at 60° for 1 hr. Mouse interferon preparations did not inhibit the multiplication of human KB cells. Human interferon preparations inhibited the multiplication of KB cells, but did not affect the multiplication of mouse cells. Mouse interferon preparations also inhibited the multiplication of mouse MSV cells, sensitive to the anti-viral action of interferon, but did not affect the multiplication of cells of a subline, MSV–IF, resistant to the anti-viral activity of interferon.

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