Interferon and Cell Division, I. Inhibition of the Multiplication of Mouse Leukemia L 1210 Cells *In Vitro* by Interferon Preparations

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Abstract. Mouse interferon preparations inhibited the multiplication of mouse leukemia L 1210 cells in stationary suspension cultures. The degree of inhibition was found to correlate with the antiviral titer of the interferon preparations. The factor(s) responsible for inhibition of L 1210 cell multiplication could not be dissociated from interferon by standard physicochemical means.

Introduction. Inhibition of the growth of several transplantable tumors has been observed in mice injected repeatedly with interferon preparations.[1-4] We report here the inhibition *in vitro* of the multiplication of leukemia L 1210 cells by different mouse interferon preparations and present the experimental evidence which suggests that interferon is the active factor. (A preliminary report of some of this work has been published in *C. R. Acad. Sci. (Paris)*, **269**, 406 (1969).)

Materials and Methods. Cells: Leukemia L 1210 arose in a DBA mouse after skin paintings with methylcholanthrene.[5] L 1210 cells adapted to stationary suspension cultures (received through the courtesy of Dr. Thayer) were grown in nutrient medium RPMI 1640 (Gibco) supplemented with 20% heat-inactivated horse serum, L-glutamine 1% (10 mM/ml), penicillin (200 units), and streptomycin (40 γ/ml).

Interferon and control preparations: Mouse interferons were obtained from the nutrient medium of monolayer cultures of murine sarcoma virus-Ia (MSV-Ia) cells[6] and L 1210 cells inoculated with ultraviolet inactivated Newcastle disease virus; the brains of Swiss and IC mice inoculated intracerebrally with West Nile virus[7] and the sera of IC mice inoculated intravenously with Newcastle disease virus or with 50 μg of complexed polyinosinic-polycytidylic acid (poly I·poly C).[8] Human interferons (generously provided by Dr. C. Chany) were obtained from the nutrient medium of the amniotic membrane[9] and from leucocyte suspensions[10] inoculated with Newcastle disease virus. Control preparations consisted of the medium of uninoculated cell cultures or tissues of uninoculated mice. All interferon and control preparations were treated at pH 2 for 18 hr (preparations containing West Nile virus), or 5 days (preparations containing Newcastle disease virus), centrifuged at 30,000 rpm for 1 hr in a Martin Christ centrifuge, Omega (60,000 × *g*), and then concentrated 10-fold by forced dialysis. Mouse brain interferon was purified by batch chromatography utilizing CM Sephadex C 50[11] and MSV-Ia interferon by filtration through a Sephadex G 75 column.[12]

Mouse and human interferon preparations were assayed on monolayer cultures of L- and BSC-cells inoculated with vesicular stomatitis virus.[13,14] One mouse interferon unit (as expressed in the text) equals 4 NIH International Reference units. The specific activity of purified brain and MSV-Ia interferon was 1.2×10^5 and 2×10^5 NIH International Reference units/mg protein respectively.

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Experimental plan: Cells were transferred either to Falcon plastic cell culture tubes (16 × 150 mm) or to 15 ml glass pharmacy bottles, 2 ml/tube or bottle, and placed in a Lwoff incubator air-5% $CO2$ at 37°C. Every 24 hr, cells were counted in a hemacytometer and their viability determined by the trypan blue dye exclusion test. In the figures, each point represents the average cell count of four cultures. Statistical analysis demonstrated that the relative error of a given determination was 8.3% (at 0.95 level of confidence).

Dilutions of interferon or control preparations were added at the time of cell subcultivation to cultures containing $2-2.5 \times 10^5$ cells/ml (final concentration).

Determination of multiplication of vesicular stomatitis virus in L 1210 cells: L 1210 cells (2×10^6 cells) were incubated with 1600 units of MSV-Ia interferon or control preparations for 24 hr. The cultures were inoculated with vesicular stomatitis virus at a multiplicity of infection of 0.005. The cultures were frozen and thawed three times and aliquots were titered on monolayer cultures of L-cells using six cultures per 10-fold viral dilution 48 hr later. A difference of more than 0.5 log_{10} between viral titers was considered significant.

Inactivation of interferon: (a) *Trypsin:* MSV-Ia interferon or control preparations were incubated with an equal volume of crystalline trypsin in phosphate-buffered saline (final concentration 500 μg/ml) for 1 hr at 37°C. Iniprol (100,000 units) was added to neutralize tryptic activity.

(b) *Periodate:* MSV-Ia interferon or control preparations were dialyzed against sodium periodate 0.02 M at 37°C. The reaction was terminated by dialysis against 5% glucose for 2 hr and against phosphate-buffered saline for 18 hr at 4°C.[15]

Rabbit antiinterferon serum: Rabbit antiinterferon serum, prepared by repeated inoculation of rabbits with crude mouse brain interferon preparations induced by West Nile virus was generously provided by Dr. B. Fauconnier.[16] A 1:250 dilution of serum neutralized 8-16 units of mouse brain interferon as assayed on monolayer cultures of L-cells inoculated with vesicular stomatitis virus. Antiinterferon serum and normal rabbit serum were preincubated with L 1210 cells for 1 hr at 37°C and heat-inactivated at 56°C for 30 min prior to utilization. In the experiment reported herein the sera were incubated with MSV-Ia interferon.

Results. Effect of different interferon preparations on the multiplication of L 1210 cells:

Figure 1 illustrates the inhibitory effect of different crude and purified mouse interferon preparations on the multiplication of L 1210 cells. Inhibition of cell multiplication was observed at 24 hr but was more pronounced at 48 hr. Control and human interferon preparations were ineffective.

Correlation between antiviral activity and L 1210 cell multiplication inhibitory activity of interferon preparations: (a) *Linear relationship between inhibition of cell multiplication and interferon titer:* The degree of inhibition of cell multiplication at 48 hr in interferon-treated cultures was directly proportional to the amount of interferon (expressed in antiviral units) present in either crude or purified interferon preparations (Fig. 2). Extrapolation of the line (*dashed line*) to the ordinate intersected the value for the cell concentration observed after cultivation of untreated L 1210 cells for 48 hr (X on the ordinate).

(b) *Comparison of antiviral and cell multiplication inhibitory activities of various fractions of two interferon preparations:* Two different preparations of MSV-Ia interferon and one control preparation were filtered through a Sephadex G-75 column. Figure 3 illustrates the protein content and the antiviral and the cell multiplication inhibitory activities of 10 fractions. A correlation was observed between the antiviral activity of a given fraction and its activity in inhibiting the multiplication of L 1210 cells (expressed as percentage of inhibition compared
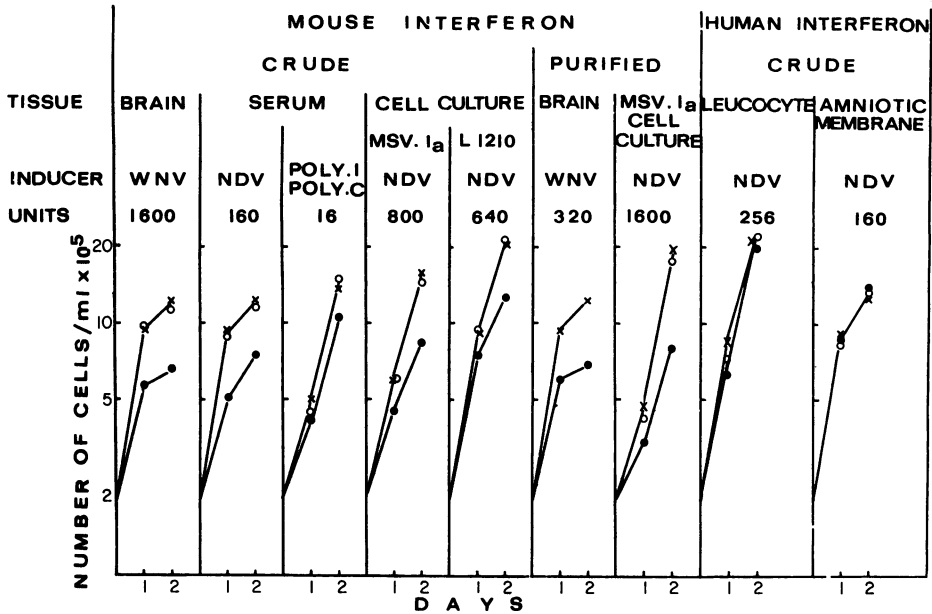
FIG. 1.—Effect of different interferon preparations on the multiplication of L 1210 cells. ×, No treatment; ○, control preparation; ●, interferon.

to the growth of control cultures at 48 hr). In both experiments maximal activities were noted in fraction 5. Most of the protein was recovered in fractions 3 and 4. A slight inhibitory effect on cell multiplication was observed in these fractions of even the control preparation (experiment 1).

(c) *Antiviral activity of interferon preparations as tested in L 1210 cells sensitive and resistant to the cell multiplication inhibitory effects of interferon:* Maintenance of interferon in the nutrient medium of L 1210 cells resulted in the selection of a cell subline resistant to the inhibitory effect of interferon on cell multiplication (Fig. 4). (The origin and characteristics of this subline will be presented in a subsequent publication.)

As can be seen from Table 1 interferon inhibited the multiplication of vesicular stomatitis virus in cells of the interferon-sensitive cell line but not in cells of the subline resistant to the effect of interferon on cell multiplication.

(d) *Effect of trypsin, periodate, and heat on the cell multiplication inhibitory activ-*

TABLE 1. *Effect of interferon preparations on the multiplication of vesicular stomatitis virus in L 1210 cells sensitive or resistant to the inhibitory effect of interferon on cell multiplication.*

L 1210 Cells	Expt. 1		Expt. 2		Expt. 3	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
No treatment	5.4*	5.6	5.6	5.6	5.0	5.4
Control preparations	5.4	5.4	NT	NT	NT	NT
Interferon	4.5	5.5	4.1	5.6	4.0	5.5

* Vesicular stomatitis virus yield $\log_{10}$ TC ID$_{50}$/0.1 ml culture fluid at 48 hr.
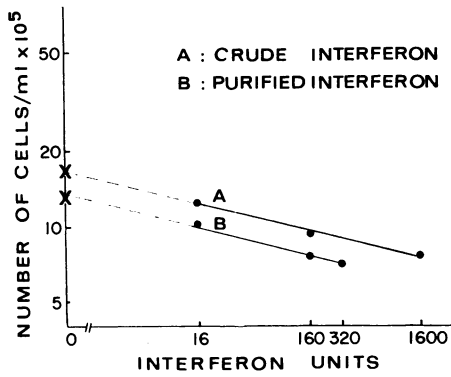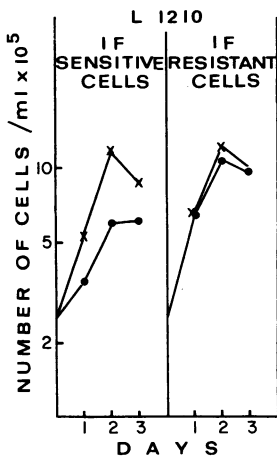NT = not tested.
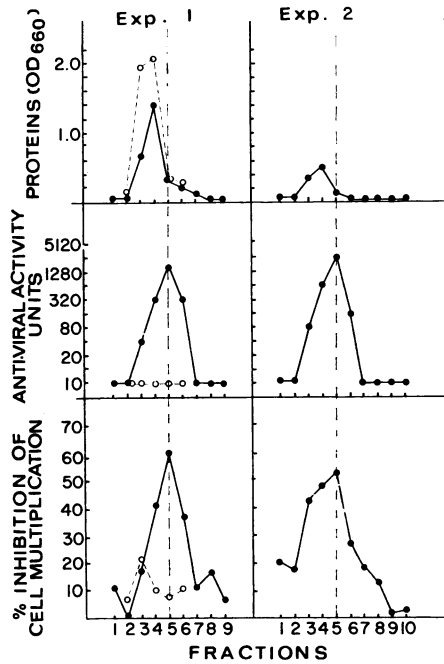
FIG. 2.

FIG. 3.

FIG. 4.

FIG. 2.—Linear relationship between inhibition of cell multiplication at 48 hr and interferon titer. ×, No treatment; ●, MSV-Ia interferon.

FIG. 3.—Comparison of antiviral and anti-L 1210 cell multiplication activities of various fractions of two interferon preparations after filtration through a sephadex column. ○, Control preparation; ●, MSV-Ia interferon.

FIG. 4.—Sensitivity of the parent line and a subline of L 1210 cells to the inhibitory effect of MSV-Ia interferon on cell multiplication. ×, No treatment; ●, interferon (IF).

ity of interferon preparations: Preincubation of MSV-Ia interferon with crystalline trypsin or with sodium periodate[15] reduced the antiviral titer of interferon from 1:1600 to less than 1:5.   Treated interferon preparations did not inhibit L 1210 cell multiplication (Fig. 5).

Heating of MSV-Ia interferon at 60°C for 1 hr reduced the antiviral interferon titer from 1:960 to 1:6, and annulled the cell multiplication inhibitory activity (Fig. 5).

(e) *Effect of rabbit antiinterferon serum on the cell multiplication inhibitory activity of interferon preparations:*   Preincubation of 1600 units of MSV-Ia interferon with a 1:10 dilution of antiserum for 1 hr at 37°C resulted in a partial neutralization of the inhibitory effect of interferon on cell multiplication (Fig. 6).
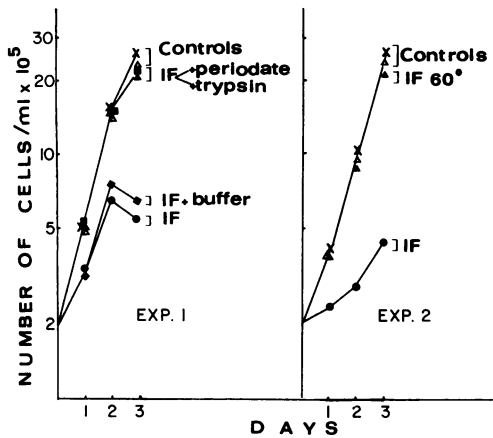
FIG. 5.—Effect of trypsin, periodate and heat on the L 1210 cell multiplication inhibitory activity of MSV-Ia interferon.

Expt. 1. ×, No treatment; △, trypsin (and iniprol); ▲, trypsin and interferon (and iniprol); ■, periodate and interferon; ◆, buffer and interferon; ●, interferon alone.

Expt. 2. ×, No treatment; △, control preparation heated; ▲, interferon heated; ●, interferon.
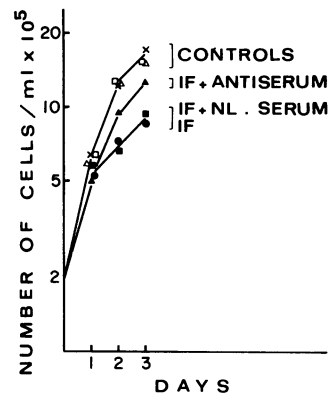
FIG. 6.—Effect of rabbit anti-interferon serum on the cell multiplication inhibitory activity of MSV-Ia interferon. ×, No treatment; □, normal serum alone; △, antiserum alone; ■, normal serum and interferon; ▲, antiserum and interferon; ●, interferon alone.

Discussion. Paucker, Cantell, and Henle reported that crude interferon preparations inhibited the multiplication of mouse L-cells in suspension cultures.[17] Other investigators, unable to confirm these findings, attributed the occasional inhibitory effects to "noninterferon contaminants."[18] Recently, Paucker and Golgher have confirmed their earlier observations utilizing purified interferon preparations.[19] Cantell has observed a similar inhibition of cellular multiplication in interferon-treated RPMI—1196 cell cultures.[20]

The experimental results presented herein suggested that interferon was the factor responsible for the inhibition of L 1210 cell multiplication: (1) All preparations of *mouse* interferon, regardless of the tissue of origin, and the interferon inducer employed (viral or nonviral) proved inhibitory for L 1210 cells. Partially purified brain or cell-culture interferon utilizing two different techniques of purification were equally effective. Control preparations proved ineffective. Preparations of human interferon derived from two different tissues, using the same interferon inducer, Newcastle disease virus, did not inhibit cell multiplication. (2) The cell multiplication inhibitory activity was directly proportional to the dose of interferon employed—as expressed in antiviral units. (3) After filtration of two interferon preparations through a Sephadex column, a good correlation was observed between the antiviral and anticell multiplication activities of the different fractions. (4) Interferon preparations inhibited the multiplication of vesicular stomatitis virus in L 1210 cells sensitive to the cell multiplication inhibitory action of interferon but did not inhibit the multiplication of this virus in a subline of L 1210 cells resistant to this effect. (5) Heating of interferon for

1 hr at 60°C, or treatment with trypsin, periodate, or antiinterferon rabbit serum diminished the antiviral activity of interferon and its effect on cell multiplication.

Since even the most purified interferon preparations are still highly impure,[21,22] it is not possible to state with certainty that interferon is indeed the responsible factor. This problem will eventually be resolved either by testing chemically pure interferon, when available, or by demonstrating a dissociation in the antiviral and anticell multiplication activities of interferon preparations.

If interferon is the responsible factor, two hypotheses may be suggested to explain its mode of action: (1) The L 1210 cells utilized in these experiments contain numerous type A and type C viral particles. If the multiplication of L 1210 cells is related to the presence of these particles it is possible that the inhibitory effect of interferon preparations on cellular multiplication was mediated in some manner by its antiviral property. (2) The effect of interferon on L 1210 cell multiplication may be independent of its effect on viral multiplication. In this regard, interferon preparations were shown to exert an inhibitory effect on chlamydia,[23,24] plasmodium berghei malaria,[25] and toxoplasma.[26] This hypothesis therefore suggests that inhibition of viral replication is only one expression of the effect of interferon on some animal cells.

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