Cellular Control of Interferon Production and Release After Treatment with Poly I:C Inducer (36537)

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The complex of polyriboinosinic:polyribocytidylic acid, poly I:C (rIn:rCn) readily induces the production of interferon and resistance to viral infection in cell culture (1). Exposure of such treated cells to metabolic inhibitors may either suppress or enhance the production of interferon (2, 3), suggesting that the mechanisms for control of interferon production and its release from cells may be complex.

A preliminary report (4) from these laboratories described the cellular uptake of poly I:C and the production of intracellular interferon. The present report describes the quantitative dynamics of cellular uptake of poly I:C and other polynucleotides by cells, and the production and release of intracellular interferon. Data are presented which indicate that the induction of interferon by poly I:C and the release of interferon from the cells may be independent events.

Materials and Methods. Polynucleotides. The polyriboinosinic (poly I) and polyribocytidylic (poly C) acids were purchased from Miles Laboratories, Elkhart, Indiana. The poly I:C was complexed by mixing the two homopolynucleotides in equimolar concentrations in phosphate-buffered saline solution (PBS, 0.006 *M* sodium phosphate, 0.15 *M* sodium chloride at pH 7.0). Radioactive-labeled poly I:C was prepared by complexing poly I^{14C} (1 μCi/mg), poly C^{14C} (0.5 μCi/mg) or poly C^{3H} (5.7 μCi/mg) with the corresponding unlabeled complementary homopolynucleotide. Complex formation was checked by hypochromic effect and by demonstration of induction of resistance to vesicular stomatitis virus (VSV) in primary rabbit kidney cell culture (PRK). Tritiated inosine diphosphate (IDP^{3H} at 16 μCi/mg and cytidine diphosphate (CDP^{3H}) at 10 μCi/mg were obtained from Drs. J. Zabriskie and C. Rosenblum of the Merck Sharp & Dohme Research Laboratories.

Determination of polynucleotide uptake by cells. Washed PRK cell monolayers were treated with maintenance medium (medium 199 plus 2% agamma calf serum) containing radioactive-labeled polynucleotides or nucleoside diphosphates. At various intervals after incubation at 35°, the cells were washed to remove the free radioactive substance and were treated with trypsin to aid dispersion. The cells were collected by centrifugation, resuspended in 2 ml of maintenance medium, and added to 20 ml of scintillation fluid. Radioactive counts were made in a Packard Tricarb liquid scintillation spectrometer, and counts were corrected for background and cell quenching. Percentage uptake was a determination of the amount of cell-associated label compared to the amount available in the medium.

Preparation of cell-associated interferon. PRK cell monolayers were treated with poly I:C at a concentration of 10 μg/ml, harvested, and washed free of extracellular interferon and inducer by appropriate procedures. The washed cells were suspended in reticulocyte standard buffer (5) (RSB, 10^{-2} *M* Tris, 10^{-2} *M* KCl, 1.5×10^{-3} *M* $MgCl_2$ at pH 7.4) and allowed to swell for 5 min. The cells were then disrupted in a Thomas cell homogenizer and centrifuged to remove cell debris. In some experiments, soybean trypsin inhibitor was added after trypsinization to protect cell-associated interferon from degradation. Omission of this step did not alter the yield of cell-associated interferon.

Analysis of cell extracts by sucrose gradient centrifugation. One half-milliliter

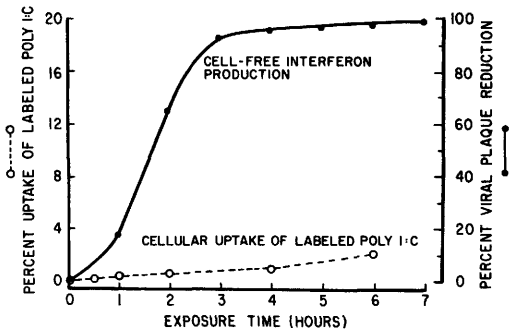

FIG. 1. Poly I:C uptake and production of cell-free interferon upon exposure of primary rabbit kidney cell cultures to 1.05 μg of drug/ml at 35°.

amounts of homogenized cell suspensions were layered onto 15–30% sucrose gradients in RSB buffer and centrifuged at 22.5 × 10^3 rpm for 2 hr in a Spinco SW25 rotor. The gradients were fractionated into 1-ml aliquots which were tested for capacity to inhibit plaque formation by VSV on RK13 rabbit kidney cell monolayers (1), and for ultraviolet absorption at 260 nm. All steps were carried out at 0–4°.

Measurement of effect of metabolic inhibitors on extracellular interferon production. Monolayer cell cultures of PRK were exposed to 10 μg/ml of poly I:C for 2 hr at 35°, washed free of unbound inducer, and suspended at 10^7 cells/ml in maintenance medium. The cell suspensions were then incubated at 37° in 25-ml Bellco spinner flasks; harvest and replacement of medium was made at hourly intervals. Identical tests were carried out in which suspensions of induced cells were treated with either 10 μg/ml of cycloheximide or 50 μg/ml of puromycin. All the harvested fluids were dialyzed exhaustively to remove inhibitor and were assayed for interferon content by the VSV plaque-reduction assay on RK13 cells.

Determination of the effect of temperature of incubation of treated cells on interferon production. The experiments were carried out as described above for metabolic inhibitors except that the variable factor was the temperature of induction and/or postinduction incubation. In some cases, intracellular interferon was measured after 4 hr of incubation. Prewarming or prechilling of maintenance medium was performed to maintain proper temperatures, and equivalent volume relationships were maintained in the tests for quantitative comparison of the amounts of intracellular and extracellular interferon.

Results. Cellular uptake of polynucleotides and nucleoside diphosphates. Figure 1 shows that there was slow and progressive uptake of labeled poly I:C by PRK cells within the time period measured. Exposure for 2 hr resulted in removal of less than 1% of the available label from the medium indicating that 0.3 μg or less of poly I:C became associated with 10–20 × 10^6 cells. This amount of poly I:C induced a measurable amount of extracellular interferon. A comparison of the uptake of the homopolynucleotides and of nucleoside diphosphates with that of poly I:C is shown in Table I. Except for greater uptake of poly I, no differences were observed. This indicated that the interferon-inducing activity of poly I:C was not due to a preferential association of the inducer with the cells.

Production and characterization of cell-associated interferon. As shown in Fig. 2, intracellular interferon was detected in ex-

TABLE I. Comparative Uptake of Polynucleotides and Nucleoside Diphosphates by Primary Rabbit Kidney Cells.

Substance	Label	Concentration (μg/ml)	Percent uptake after 2-hr exposure
Poly I:C	Poly I^{14C}, poly C^{14C} or poly C^{3H}	1.05	0.5
Poly I	Poly I^{14C}	0.5	3.2
Poly C	Poly C^{14C} or poly C^{3H}	0.5	0.3
IDP[a]	IDP^{3H}	0.5	0.5
CDP[b]	CDP^{3H}	0.5	0.6

[a] Inosine diphosphate.
[b] Cytidine diphosphate.

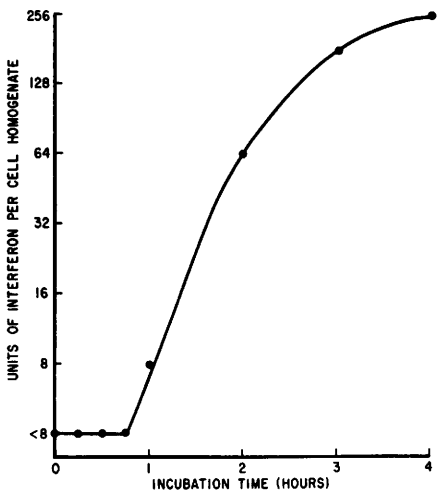

FIG. 2. Induction of cell-associated interferon in primary rabbit kidney cell cultures by poly I:C (10 μg/ml).

tracts of poly I:C-treated rabbit kidney cells within 1 hour after exposure to the drug. Control cultures that were not treated or that were treated with an equivalent amount of poly I or poly C did not produce interferon. The antiviral activity of the cell-associated fraction was identified (data not presented) as interferon based on host cell species-specificity (active against virus in rabbit but not chick cells) and trypsin sensitivity (50 μg/ml crystalline trypsin, 35°, overnight).

Homogenates of poly I:C-treated and untreated cell cultures were fractionated in a sucrose density gradient and the fractions were tested for inhibition of plaque formation by VSV. As seen in Fig. 3, there was no detectable difference in the polysome pattern of an extract of the poly I:C-induced cells compared with that of untreated cells. Nearly all the antiviral activity was retained near the top of the gradient (fractions 26–32) as would be expected for interferon not bound to polyribosomes. However, there was a small but significant amount of the antiviral activity which was retained in association with the polysome-containing fractions 10–24. The presence of polysomes was confirmed by treatment of samples of the extracts with pancreatic ribonuclease (10 μg/ml) for 1 hr prior to sucrose gradient analysis. This caused a shift of all the uv_{260nm}-absorbing material from the polysome region of the gradient (fractions 10–20) into a peak at fraction 24, as is characteristic for dissociation of polysomes to single ribosomes.

Effect of metabolic inhibitors on hourly accumulation of extracellular interferon. Figure 4 shows that suspensions of cells that had been induced by poly I:C continued to release interferon into the medium after removal of residual inducer. The presence of 10 μg/ml of added cycloheximide effected a striking suppression both in the hourly production of interferon and in the total amount of interferon accumulated after 4-hr incubation. The suppression of such appearance of interferon was apparently due to the inhibitory effect of cycloheximide on protein synthesis since the cycloheximide at the concentration used suppressed 97% of the incorporation of ^{14}C-labeled amino acids into trichloroacetic acid-precipitable material prepared from a 10-min pulse-labeled cell suspension. As shown in Fig. 5, this same effect was obtained when puromycin was employed as an inhibitor at 50 μg/ml.

Effect of incubation temperature on production and release of cell-associated interferon. The results obtained in the cycloheximide and puromycin inhibition experiments

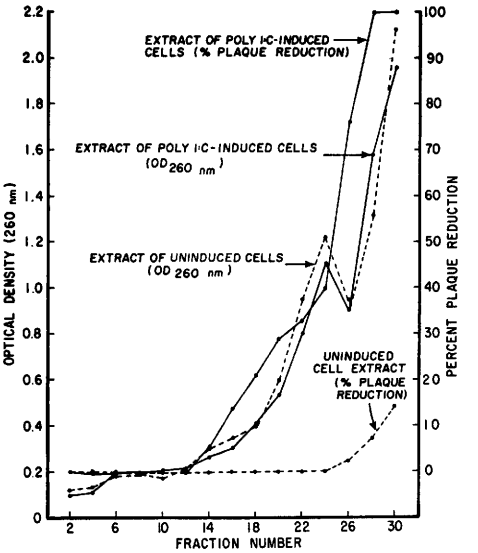

FIG. 3. Sucrose gradient analysis of cytoplasmic extracts of poly I:C-induced and uninduced primary rabbit kidney cells.

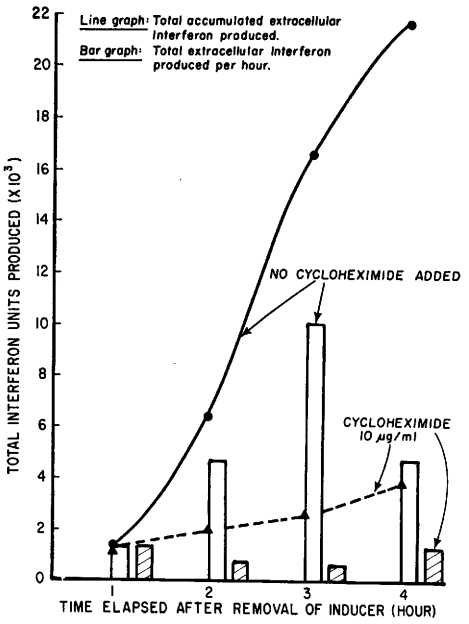

FIG. 4. Effect of cycloheximide on interferon production by primary rabbit kidney cell cultures induced by poly I:C.

suggested that continuous production of interferon by induced cells required parallel production of newly synthesized protein. If active cellular metabolism is required for interferon production, then, lowering of incubation temperature would also be expected to reduce interferon production. Incubation of cells at 0° compared with incubation at 35° caused a greater than 99% reduction in the uptake of ^{14}C-labeled amino acids into TCA-precipitable material prepared from 30-min pulse-labeled cell suspensions. This same low-temperature incubation also caused greater than 90% reduction in the uptake of ^{14}C-labeled uridine into TCA-precipitable material prepared from 90-min pulse-labeled cell suspensions.

As shown in Table II, cells that were exposed to poly I:C for 2 hr at 35° to bring about induction and were then incubated at 35° produced intracellular and extracellular interferon. However, if the cells were placed in an ice bath (0°) immediately after induction, there was no release of interferon. Nevertheless, intracellular interferon had been produced, since homogenization of the cells which were held in the cold for 4 hr yielded significant amounts of interferon. If the cells were placed at 35° after 2 hr in the cold, interferon was readily released into the medium. This indicated that release of preformed interferon was a temperature-sensitive event. If both the induction and the subsequent incubation of cells were carried out at 0°, then neither intracellular nor extracellular interferon was produced, indicating that induction of interferon as well as release of interferon was temperature sensitive. Placing of cells at 35° after treatment with poly I:C at 0° did result in interferon production, but only after a delay of 2 hr.

Discussion. The high-level efficiency of poly I:C as an inducer of interferon was not due to a preferential uptake of the substance by cells. Association of the poly I:C with cells was no greater than that of the noninducing homopolynucleotides and mononucleotides. This confirmed similar observations by Colby and Chamberlin (6) who showed that the efficiency of poly I:C as an interferon inducer in chick embryo cells was not dependent upon its rate of uptake.

Contact of cells with poly I:C resulted in the production of intracellular interferon and its release from the cells. Intracellular interferon exhibited the same properties as extracellular interferon, *viz.*, trypsin sensitivity and species specificity. Intracellular interferon could be demonstrated to be present in measurable amount 1 hr after induction. Two hours after induction interferon was found

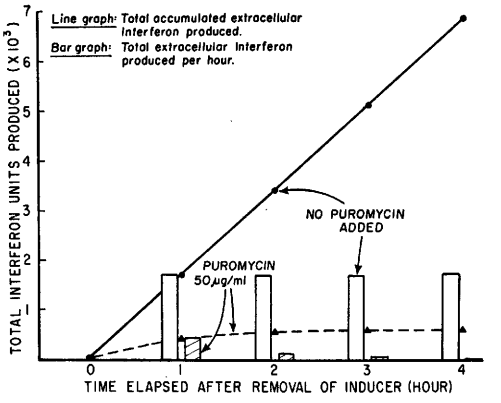

FIG. 5. Effect of puromycin on interferon production by primary rabbit kidney cell cultures induced by poly I:C.

TABLE II. Effect of Incubation Temperature on Poly I:C Induction and Release of Interferon.

Treatment		Interferon titer					
		Extracellular interferon measured hourly					
Induction temperature[a] (°C)	Postinduction temperature[b] (°C)	0	1	2	3	4	Cell-associated interferon[c]
35	35	<8	64	128	1024	256	128
35	0	<8	<8	<8	<8	<8	128
35	0 → 35	<8	<8	—	—	—	—
		—	—	32	2048	128	
0	0	<8	<8	<8	<8	—	<8
0	35	<8	<8	8	16	32[d]	—

[a] Monolayers of primary rabbit kidney cells were exposed to poly I:C (10 μg/ml) for 2 hr at the indicated temperature.

[b] After removal of inducer and suspension of cells by trypsinization, cells were incubated as spinner suspension cultures at the indicated temperature.

[c] Measured at 4 hr.

[d] The 9-hr sample titered 128.

primarily but not totally free of association with polyribosomes. Overall production of interferon as measured in the extracellular fluid was inhibited by cycloheximide, by puromycin, and by reduction of temperature. The process of interferon production and release was separated into two steps by temperature-shift experiments. The first step related to the events after initiation of interferon induction, but prior to the first appearance of interferon within the cells. A similar temperature-sensitive step has been reported by Bausek and Merigan (7) for development of poly I:C-induced resistance in mouse L cells. The second step controlled release of interferon from the cells. This step was temperature sensitive and reversible. This suggested that the release of interferon may be under control of some cellular metabolic event such as active transport, as suggested by Tan *et al.* (8). Further, the continuing production of interferon by cells already induced by poly I:C suggests that there was either continuing synthesis of interferon or continuing enzymatic activation of a precursor of interferon as suggested by Ho and Ke (9).

Summary. Primary rabbit kidney cells released measurable quantities of extracellular interferon within 2 hr after initiation of induction by poly I:C. This occurrence was accompanied by slow uptake of inducer in minute quantity. There was no preferential uptake of poly I:C by primary rabbit kidney cells compared to that of noninducing polynucleotides and nucleotides. Induced cells produced high concentrations of apparently unbound intracellular interferon which appeared to be the source of extracellular interferon. The dynamics of interferon production in primary rabbit kidney cell cultures induced by poly I:C involved two separate steps, *viz.*, the synthesis of intracellular interferon and the release of this interferon into the extracellular medium.

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1. Field, A. K., Tytell, A. A., Lampson, G. P., and Hilleman, M. R., Proc. Nat. Acad. Sci. U.S.A. **61**, 340 (1968).

2. Vilcek, J., Ann. N.Y. Acad. Sci. **173**, 390 (1970).

3. Tan, Y. H., Armstrong, J. A., Ke, Y. H., and Ho, M., Proc. Nat. Acad. Sci. U.S.A. **67**, 464 (1970).

4. Field, A. K., Tytell, A. A., and Hilleman, M. R., Bacteriol. Proc. p. 150, No. V5 (1969).

5. Warner, J. R., Knopf, P. M., and Rich, A., Proc. Nat. Acad. Sci. U.S.A. **49**, 122 (1963).

6. Colby, C., and Chamberlin, M. J., Proc. Nat. Acad. Sci. U.S.A. **63**, 160 (1969).

7. Bausek, G. H., and Merigan, T. C., Virology **39**, 491 (1969).

8. Tan, Y. H., Armstrong, J. A., and Ho, M., Virology **45**, 837 (1971).

9. Ho, M., and Ke, Y. H., Virology **40**, 693 (1970).

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