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Regulation of Cellular Interferon Production: Enhancement by Antimetabolites*

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Abstract. Cycloheximide, at a protein-inhibitory concentration, when given to rabbit kidney cell cultures that had been exposed either to UV-irradiated Newcastle Disease virus or to a complex of polyinosinic and polycytidylic acids (poly I·poly C), enhanced the production of interferon. The enhancement was greater if, in addition to cycloheximide, the cells were also treated with actinomycin D. On the basis of these findings, a mechanism, consisting primarily of the production of a control protein which normally checks interferon production, is postulated for interferons stimulated by these two substances.

Introduction. The stimulation of interferon production by viruses has been postulated to involve the derepression of an interferon gene.[1] The main evidence for this model is that interferon production is inhibited by actinomycin D, and by inhibitors of protein synthesis. Interferon stimulated by nonviral inducers such as the bacterial endotoxins and synthetic double-stranded RNA appears to be different. Inhibitors of protein and RNA synthesis failed to inhibit the production of interferon in animals in response to these agents.[2–4] Youngner and Hallum postulated[4] that synthetic double-stranded RNA, like endotoxin, stimulated interferon production by the "release" of "preformed" interferon. However, in cell cultures actinomycin D, puromycin, and cycloheximide inhibited the effect of poly I·poly C to different degrees.[5–7] Ho and Ke also found[8] that interferon production by liver slices derived from rabbits injected with poly I·poly C required protein synthesis.

It thus appears that at least in the case of poly I·poly C stimulation of interferon, new protein synthesis is required, and that more than "release" of "preformed" material is involved. But how does one account for the lack of inhibition of interferon formation by antimetabolites in the whole animal, and the finding[7] that the interferon-stimulatory effect of poly I·poly C in rabbit cell cultures could, under appropriate circumstances, actually be accentuated by cycloheximide? We also showed that such accentuation occurred to different degrees in slices of various rabbit tissues.[9] We elaborated on Vilcek's suggestion that in the course of interferon production after exposure to poly I·poly C, a control protein is initiated which checks this production.[7, 9] If this protein is not formed as a result of the addition of antimetabolites at the appropriate time, interferon production is enhanced. This control mechanism may be active

to different degrees in different tissues, which may partially account for the conflicting results in various systems.

In this paper, we find that both cycloheximide and actinomycin D can also accentuate the stimulation of interferon production by an inactivated virus. We present results using both virus and poly I·poly C, and propose that there may be a unitary control mechanism regulating interferon synthesis at the cellular level in response to both of these substances.

Materials and Methods. Cell culture and medium: Monolayer cell cultures were prepared, unless otherwise indicated, from trypsin-treated[10,11] kidneys of weanling rabbits (250–400 g). After primary growth in Povitsky bottles, monolayers were trypsinized, and about 10^6 cells in 5 ml of medium consisting of 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution (LAH) with 4% calf serum, antibiotics, and 0.2% bicarbonates were seeded on 5-cm plastic Petri plates, and incubated for 3 days at 37°C under 5% CO_2.

Interferon inducers: (1) *Virus.* Newcastle Disease Virus (NDV, Herts strain), after four passages at limiting dilutions in African green-monkey kidney cells and one in avian leukosis-free chick embryos (Spafas, Norwich, Conn.), was found free of avian leukosis virus by the COFAL test (Spafas). A virus preparation in 0.85% NaCl, obtained by centrifugation at 2000 × *g* for 10 min and 78,000 × *g* for 2 hr, contained 7.5×10^8 plaque-forming units/0.1 ml as titrated in chick embryo fibroblast cultures. 10 ml was irradiated in a 14-cm glass Petri dish under a 15-W General Electric germicidal UV lamp at a distance of 14 cm for 60 sec. This material, designated as NDV_{uv}, had no residual infectivity as tested in chick fibroblast cultures. For interferon induction, rabbit-kidney cultures were incubated at 37°C for 1 hr with 0.2 ml NDV_{uv}.

(2) *Poly I·poly C.* Homopolymers of polyinosinic and polycytidylic acid (Mann Research Labs.) were made into 1 mg/ml solution in phosphate buffered saline (PBS, pH 7.2), and complexed as described by Field *et al.*[12] before each experiment. To stimulate interferon production, 2 ml of poly I·poly C (at 40 μg/ml of medium) was incubated for 1 hr with each culture.

General procedures, sample collection, and preparation: After exposure to interferon inducers, or an antimetabolite, cultures were washed three times with 5 ml of Hanks' balanced salt solution. They were refed with 2 ml of medium with or without inhibitors. Two or three plates were used for each test. Fluids from cultures were pooled for interferon titration, and results were expressed per 2 ml. Concentrations of antimetabolites used were: cycloheximide (Nutritional Biochemical) 20 μg/ml; puromycin (Nutritional Biochemical), 100 μg/ml and actinomycin D (gift of Merck and Co.), 2 μg/ml. At these concentrations cycloheximide inhibited incorporations of [^{14}C]-amino acids by 90% and its effect was freely reversible after 4-hr exposure. Puromycin inhibited protein synthesis by 93% and actinomycin D inhibited RNA synthesis by 97%. The action of the latter was irreversible. Antimetabolites were removed from samples by dialysis against two changes of about 100 volumes of PBS and once against 100 volumes of LAH, stirring, for 24–30 hr at 4°C. All times are expressed relative to the moment of addition of the inducer, designated "hour 0." Interferon was titrated by inhibition of plaque formation by vesicular stomatitis virus in rabbit kidney-cell cultures, as described[10,11].

Results. Kinetics of interferon production: Two rabbit kidney-culture plates were exposed to NDV_{uv} and two to poly I·poly C for 1 hr. Fluids were harvested from the cultures exposed to poly I·poly C at hourly intervals, and at 2-hr intervals from cultures exposed to NDV_{uv}. After each collection, the cultures were replenished with 2 ml of regular medium. The samples were titrated for interferon and rates of production on an hourly basis computed. Fig. 1 shows that virus-induced interferon was produced between hours 12–24, and that the

rate was maximal around hours 12–16. Poly I·poly C-stimulated interferon was produced much earlier, maximally 2–4 hr after stimulation.

Requirement for protein synthesis: Three sets of duplicate cultures were exposed to NDV_{uv} for 1 hr. Set 1 received cycloheximide in addition. All three sets were washed after 1 hr. Sets 1 and 2 received 2 ml of medium with cycloheximide, and set 3 served as a control. At the end of 24 hr, duplicate samples were pooled and titrated for interferon. Set 1 produced 280 units of interferon; set 2, which contained cycloheximide from hour 1 to hour 24, produced 200 units; and set 3, which was not exposed to cycloheximide, produced 5000 units. Hence, cycloheximide, under these conditions, inhibited interferon production more than 90%, consistent with the prevailing dogma[1] that production of virus-induced interferon requires protein synthesis.

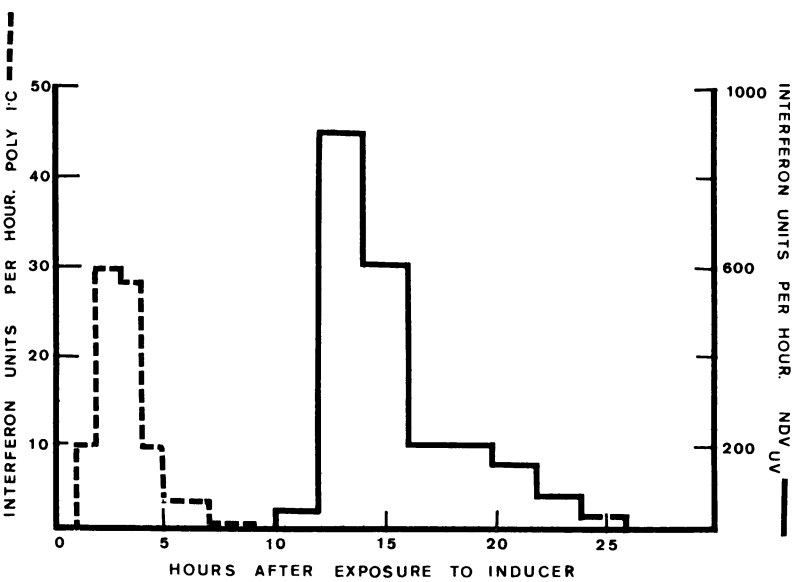

FIG. 1. Kinetics of interferon production after stimulation by NDV_{uv} and poly I·poly C. Rabbit kidney cultures were exposed to 0.2 ml of NDV_{uv} for 1 hr or to 40 μg/ml of poly I·poly C for 1 hr. Culture fluids were collected at hourly intervals for poly I·poly C-treated cultures and at 2-hr intervals for cultures treated with NDV_{uv}. Rates of production per hour are plotted.

To determine if the production of poly I·polyC-stimulated interferon also requires protein synthesis, the effect of puromycin was studied. Cultures were exposed to poly I·poly C for 1 hour and washed before reincubating in 5 ml of regular medium. Puromycin was added to the cultures at hours 1, 2, 3, and 4. Samples for interferon titration were collected at hours 4, 7, and 18. Addition of puromycin at hour 1 and hour 2 inhibited interferon production collected at any of these 3 times by more than 90%. However, later addition of puromycin resulted in less inhibition. Its addition at hour 4 no longer affected interferon production. This effect of puromycin indicates that poly I·poly C induction of

interferon also requires newly synthesized protein, and that this requirement is completed by hour 4.

Enhancement of interferon production by inhibitors of protein synthesis: Since little interferon was produced in the presence of protein inhibitors, shorter exposure periods were tested using NDV_{uv} (Table 1). 3 culture plates were

TABLE 1. *Effect of cycloheximide on production of NDV_{uv}-induced interferon.*

Cycloheximide treatment (hr)	Interferon yield (units) 0–12 hr	During treatment	Total
None	500	...	11,000
12–14	500	100	21,000
12–16	N.D.	100	21,100
12–18	400	300	41,300
12–20	N.D.	300	45,300
12–22	400	500	49,500
12–24	N.D.	600	35,600

Hours refer to time after 1-hr exposure to NDV_{uv}. Interferon yield in units per culture (2 ml). Total refers to amount produced up to 36 hr.

exposed to NDV_{uv} for 1 hr, washed, and incubated in regular medium for 12 hr. At hour 12, culture fluids were collected and all cultures except controls were refed with regular medium containing cycloheximide. At hours 14, 16, 18, 20, 22, and 24, inhibitor was removed from the cultures. Samples were collected for interferon titration at hour 36. There was enhancement of interferon production to about 2–5 times that of controls in all cycloheximide-exposed cultures. This effect increased with longer periods of exposure, up to hour 22. Table 1 also shows that little interferon is made in the presence of cycloheximide, but that its production is rapidly resumed upon the removal of the inhibitor. This is consistent with the requirement for protein synthesis in interferon production and the reversibility of the action of cycloheximide.

Experiments analogous to the above were performed using poly I·poly C (Fig. 2). All cultures were exposed to poly I·poly C for 1 hour. The effect of cycloheximide was tested in five sets of two plates; four were exposed to cycloheximide from hours 0–1, 0–2, 0–3, or 0–4. One set served as a control. At the end of exposure, cultures were washed, refed, and harvested at hour 22. Interferon production during exposure to cycloheximide was <20 units (not shown). After exposure to cycloheximide between hours 0 and 3, a marked accentuation of interferon production occurred: 18,000 units as compared to 500 units in control cultures. Longer exposure (hours 0 to 4) did not cause further accentuation. A similar test was performed by exposing duplicate cultures to puromycin from hour 0 to hour 2 (also Fig. 2). Similar, though less marked, accentuated production (8-fold increase) was observed in puromycin-treated cultures.

Effect of actinomycin D on accentuation of interferon production: For NDV_{uv}-induced interferon, an experiment was done as follows (Fig. 3). Eighteen cell cultures were exposed to 0.2 ml of NDV_{uv} for 1 hr, washed, replenished, and incubated till hour 12. In the absence of either cycloheximide or actinomycin, 11,000 units of interferon per culture were produced. Interferon production after exposure to cycloheximide was enhanced to 35,000 units (2nd bar,

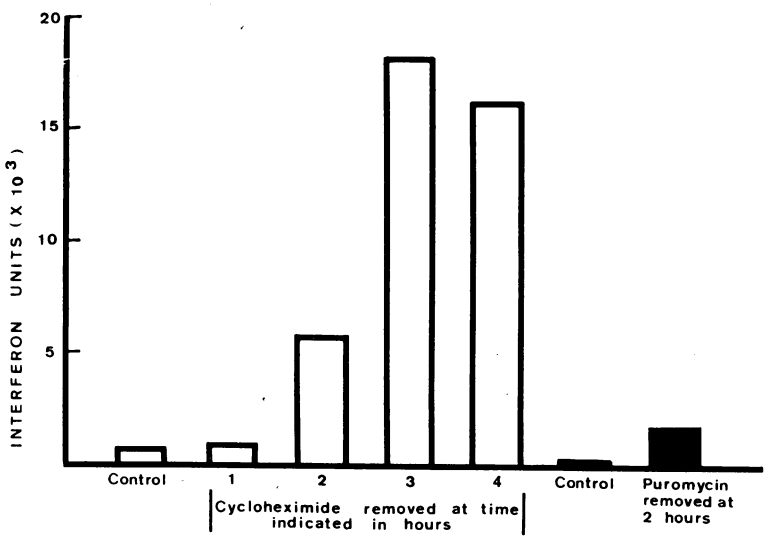

FIG. 2. Enhancement by cycloheximide and puromycin of interferon production stimulated by poly I·poly C. Titers represent amount produced after 22 hr of incubation.

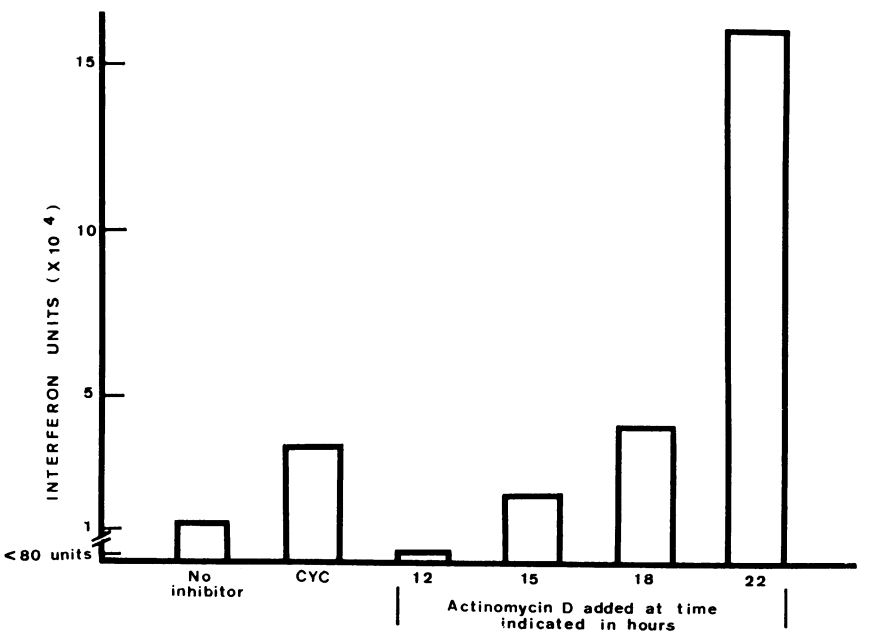

FIG. 3. Effect of actinomycin D on the accentuation of interferon production stimulated by NDV_{uv}. All cultures except the ones labeled "no inhibitor" received cyclohexamide from hour 12 to hour 24 after induction with NDV_{uv}. Actinomycin D was added for 1 hour. Samples for interferon titration were collected at 36 hr.

left to right). The addition of actinomycin at hours 12, 15, 18, and 22 resulted in the production of <80, 20,000, 40,000, and 160,000 units. It appears that actinomycin D added at hour 12—when the cultures were beginning to produce interferon (Fig. 1)—strongly inhibited interferon production, indicating the transcription of interferon mRNA at about this time. When actinomycin D was added somewhat later, at hour 15, thus permitting some RNA synthesis, the interferon yield was 57% of the control (bar 4). Allowing the RNA synthesis to proceed further by adding actinomycin D at hour 18 permitted 114% of the control. Surprisingly, when actinomycin D was added at hour 22, it caused a further marked increase over the control values of cultures not treated with any antimetabolite or treated with cycloheximide alone.

Essentially the same test was repeated with cultures stimulated with poly I·poly C for 1 hr (Fig. 4). Six groups of two cultures each were used. All except

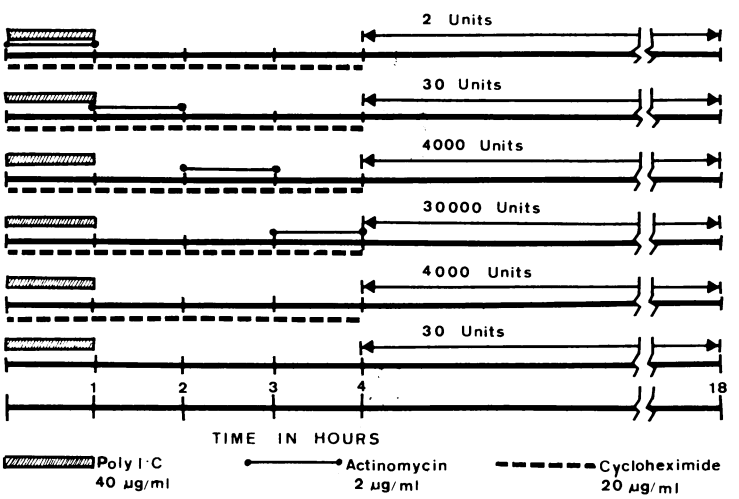

FIG. 4. Effect of actinomycin D on the accentuation of interferon production stimulated by poly I·poly C. Five sets of cultures were exposed to cycloheximide (top five lines). A control set (line 6) received no inhibitors.

one received cycloheximide from 0 to 4 hr. Of the five groups exposed to cycloheximide one served as a control and four were treated for 1 hr at hours 0, 1, 2, and 3 with actinomycin D. All cultures were washed at 4 hr, replenished with medium, and incubated for 18 hr before titrating for interferon.

In the absence of cycloheximide and actinomycin (bottom line), 30 units were produced between hours 4 and 18, and 250 units in total (not shown). Exposure to cycloheximide (second from bottom) accentuated production to 4000 units. Early exposure to actinomycin D (hour 0 to hour 1, top line) essentially eliminated interferon production (<1% of control). By allowing mRNA synthesis to proceed for 2 hr before adding actinomycin D, interferon production was restored to 100% of the control value. When actinomycin was added at hour 3, it caused further marked accentuation of interferon production. Similar results were obtained with puromycin although the accentuation was less marked.

Discussion. Interferon production stimulated by either UV-irradiated NDV or poly I·poly C was limited in the presence of cycloheximide or puromycin, showing that new protein synthesis was necessary. However, the removal of the protein inhibitor was followed by accentuation of interferon production. We believe that this accentuation occurred because cycloheximide or puromycin permitted the accumulation of mRNA for interferon while delaying the onset of the control mechanism which normally shuts off interferon production.

Early treatment with actinomycin D almost eliminated subsequent interferon production, showed that interferon mRNA was probably accumulating. But perhaps our most significant finding was that late treatment with actinomycin D caused further enhancement of interferon production stimulated by either NDV_{uv} or poly I·poly C (Figs. 3 and 4), which points to the elimination of a normal mechanism.

We proposed the following model of interferon induction:

Step 1: An inducer (NDV_{uv} or poly I·poly C) triggers the synthesis of an mRNA for interferon. Transcription commences within an hour after exposure of cells to poly I·poly C (Fig. 4). In the NDV_{uv} model, transcription may not begin until between hours 10 and 12, when interferon first appears, since actinomycin D added at hour 12 virtually eliminated interferon production (Fig. 3). The reason for termination of transcription is unknown, but it may be the unavailability of the inducer. *Step 2: Translation of interferon mRNA.* Protein synthesis is required for interferon production, presumably translated from mRNA produced in Step 1. Although some interferon is made in the presence of protein inhibitors (e.g., Table 1), it seldom exceeds 1–5% of the amount made after the blockade is lifted. The protein made may be interferon, a "preinterferon",[8] or an unidentified essential protein. *Step 3: A protein made in Step 2 initiates transcription of an mRNA involved in termination of interferon production.* The existence of this RNA is revealed in the experiments in which actinomycin D, added after the transcription of RNA described in Step 1 is complete, further enhanced interferon production (Figs. 3 and 4). *Step 4: Translation of control protein.* The RNA from Step 3 is translated and a labile control protein is made. We believe the control substance is a protein because its partial inhibition under cycloheximide blockade enhances interferon production (Table 1, Fig. 2). Direct evidence for this is still lacking. *Step 5: Termination of interferon production.* The control substance inhibits interferon production at a stage after transcription—probably translation (Step 2), but perhaps by inactivating interferon mRNA or inhibiting conversion of a precursor, preinterferon,[8] into active interferon.

This model may be compared with that of Vilcek,[13] who concluded that interferon induced in rabbit kidney cells after stimulation by poly I·poly C or NDV_{uv} was of two types. One type was newly synthesized and the other, which was cycloheximide-resistant, was thought to be "preformed." We have not found it necessary or desirable to postulate two types of interferon. In fact, we suggest that stimulation of interferon production by poly I·poly C and by viruses may represent one mechanism. If so, the long latent period preceding interferon production in NDV_{uv} stimulated cultures (Fig. 1), may represent the time

required for the synthesis of double-stranded viral RNA.[5] How endotoxin stimulation of interferon fits into the picture remains to be seen.

The presence of a regulatory mechanism explains the well-known "one-shot" characteristic of interferon induction.[14] We referred to the possibility that this mechanism may be more active in some tissues than others.[9] Chany[15] reports a cell line which is repeatedly reinducible. One would predict that pretreatment with exogenous interferon would activate the control mechanism and inhibit induction of interferon. This is well known to be true of virus induction of interferon,[16] and both Paucker[17] and Younger and Hallum[18] report that interferon treatment depresses the response of cells to poly I·poly C.

We wish to thank our colleague Dr. Bosko Postic for preparing the UV-irradiated Newcastle Disease virus, and Miss Lucille Ople for her excellent technical assistance.

Abbreviations: Poly I·poly C, complex of polyinosinic and polycytidylic acids; NDV, Newcastle disease virus; NDV_{uv}, UV-irradiated Newcastle disease virus.

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‡ Postdoctoral fellow of the Canadian Medical Research Council.

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