Accentuation of Production of Human Interferon by Metabolic Inhibitors[1] (36122)

MONTO HO, Y. H. TAN, AND JOHN A. ARMSTRONG
Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

Vilcek (1, 2) has shown that the production of interferon by rabbit kidney cells stimulated with poly(I):poly(C) can be enhanced by certain inhibitors of protein synthesis. Accentuated interferon production in the presence of protein inhibitors such as cycloheximide can occur in several different tissues (4) and paradoxically requires concomitant protein synthesis (5). Maximal enhancement is achieved if inhibitors are used sequentially to block the synthesis of a postulated regulatory protein (3). This procedure involves treatment of cells with a reversible inhibitor of protein synthesis during the synthesis of interferon mRNA. Prior to reversal of protein blockade, actinomycin D treatment is initiated to inhibit transcription of the regulatory protein. In this paper we show that human interferon production can also be enhanced by cycloheximide and actinomycin D.

Materials and Methods. Cell cultures. Human embryonic fibroblasts (HEF) were obtained from tissues of an approximately 4-week embryo (about 3 cm long) by treatment with 0.25% trypsin. Cultures were grown in medium consisting of Hanks' balanced salt solution (HBSS) supplemented with 0.5% lactalbumin hydrolyzate, 2 m*M* L-glutamine, Eagle's vitamin mixture, antibiotics, and 5% inactivated (56° for 30 min) newborn calf serum. Subcultures were prepared as needed by treatment of monolayers with 0.25% trypsin and 0.2% EDTA in phosphate-buffered saline (PBS). The cells were centrifuged, resuspended at 2×10^5 cells/ml, and dispensed into suitable culture vessels. Stock cultures were carried in glass prescription bottles but experimental work was performed with cultures in 5-cm plastic dishes or 6-mm microwells (Falcon Microtest II plates) incubated under 5% CO_2. Stock cultures could be stored at room temperature for several weeks.

Interferon inducers. Polyinosinic acid and polycytidylic acid (Mann or P-L) were dissolved in PBS (pH 7.2) at 1 mg/ml and complexed by mixing at 37° immediately prior to use (4). The double-stranded complex [poly(I):poly(C)] was not stored. Newcastle disease virus (NDV, CG strain) was grown in the allantoic cavity of 11-day embryonated hens' eggs. The virus was partially purified by differential centrifugation, suspended in 0.85% NaCl, and irradiated with a 15-W germicidal lamp as previously described (3). The product (NDV_{uv}) was not infective (<1 PFU/0.1 ml) when tested in monolayers of chick embryo fibroblasts, and 0.2 ml of the preparation was used for each culture plate to induce interferon production.

Interferon assay. The microassay of Armstrong, as described for the titration of rabbit interferon (6) was adapted by M. K. Breinig in this laboratory. Human fibroblast cultures in 6-mm wells were treated overnight with dilutions of interferon and then challenged with 100 $TCID_{50}$ of Sindbis virus. Interferon titer was estimated as that dilution of interferon which inhibited cytopathic effects (CPE) by 50% after 24- to 48-hr incubation. An internal standard interferon had a mean titer of 2560 (six assays) and was included in each assay. The International Human Standard 67/87 (80 units) titered 100 units in our hands.

Metabolic inhibitors. Cycloheximide was freshly dissolved in medium as required. Actinomycin D was prepared as a stock solution containing 1 mg/ml and stored in the dark

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at 4°. The inhibitors were removed from the culture dishes and the dishes washed three times (5 ml HBSS per wash) before the cultures were replenished with medium not containing inhibitors. The effect of cycloheximide on protein synthesis was measured by exposing cultures to ^{14}C-leucine (Schwarz Bioresearch, average sp act 222.6 mCi/mmole) for 30 min at 5 μCi/ml/plate as previously described (3). The effect of actinomycin D was measured by exposure of cultures to ^{14}C-uridine (1 μCi/ml/plate) for 30 min. The incorporation of precursors into TCA-precipitable material in the presence of antimetabolites is shown in Table I. Cycloheximide (100 μg/ml) inhibited protein synthesis quite effectively (80–90%) but actinomycin D (20 μg/ml) acted rather slowly in this system.

TABLE I. Effect of Inhibitors on Incorporation of ^{14}C Labels.[a]

Inhibitor	Cone (μg/ml)	Pre-treatment of cultures (hr)	% Inhibition (average of 3 plates)
Cycloheximide	100	½	82
Cycloheximide	100	3½	92
Actinomycin D	20	½	48
Actinomycin D	20	1½	70
Actinomycin D	20	3½	91

[a] Incorporation of ^{14}C leucine was tested in the case of cycloheximide, and of ^{14}C uridine in the case of actinomycin D. Please see Materials and Methods for details.

Experimental protocol. In general, two 5-cm cultures were treated with 1 ml of poly (I):poly(C) (100 μg/ml) or 0.2 ml of NDV_{uv} for 1 hr at 37°. The cultures were washed three times with 5 ml HBSS to remove the inducer and reincubated with 2 ml medium with or without antimetabolites. Fluids were harvested at times indicated and assayed for interferon. Samples of NDV_{uv}-induced interferon were diluted 1:10 in 10% rabbit anti-NDV serum and incubated for 1 hr at 37° prior to assay. In all experiments, the "hour" refers to the time after an inducer was added. Hence "hour 0" refers to the time it was added, "hour 1" refers to 1 hr after it was added.

Results. Effect of cycloheximide and actinomycin D on production of interferon induced by NDV_{uv}. The kinetics of interferon synthesis by cells exposed to NDV_{uv} for 1 hr are shown in Fig. 1. About 32 units were made and peak titer appeared between 2 and 4 hr. The effect of cycloheximide (100 μg/ml) on subsequent interferon production is shown in Table II. Duplicate cultures were exposed to NDV_{uv} and cycloheximide. Little or no interferon was produced in the presence of the inhibitors, and interferon production was measured from the time of removal of the inhibitor until hour 12 when the experiment was terminated. The inhibitor was removed at hours 2, 4, and 5. The medium was harvested for interferon assay at hour 12 (Table II, Groups I, II, III and VII). Interferon yield was enhanced in all cases when compared with the control (Group I). Maximal accentuation was observed with 4-hr exposure to the inhibitor (Group III, 2560 units). The effect of the addition of actinomycin D (20 μg/ml) to cultures treated with cycloheximide is also shown in Table II. This inhibitor has been

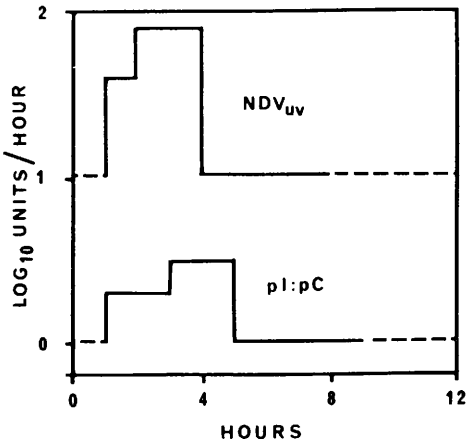

FIG. 1. Kinetics of interferon production by HEF cultures. Two 5-cm cultures were exposed to NDV_{uv} or poly (I):poly (C) (100 μg/ml) from hour 0 to hour 1. Fluids were removed for interferon assay at 1- to 2-hr intervals and replaced with fresh medium. The broken lines indicate interferon titers were below the indicated levels.

TABLE II. Effect of Metabolic Inhibition on NDV_{uv}-Stimulated Interferon.

Group	Cycloheximide (duration of exposure from hour 0 to hour)[a]	Actinomycin D		Interferon yield (units)
		Time of addition (hr)	Duration of exposure (hr)	
I	None	None	None	40
II	2	None	None	480
III	4	None	None	2560
IV	4	0	1	160
V	4	1	1	160
VI	4	3	1	2000
VII	5	None	None	1280
VIII	5	3	1	2560
IX	5	3	2	8000
X	5	4	1	640

[a] Hour 0 refers to time of addition of inducer.

shown to cause a further marked accentuation interferon response in rabbit cells when added at the optimal time to cultures treated with cycloheximide (3). In this system maximal enhancement was observed when actinomycin D was added at hour 3 and kept in the cultures for 2 hr in the presence of cycloheximide (Group IX). One hour of exposure to this inhibitor is not sufficient to abolish RNA synthesis in HEF cultures (Table I) and was, therefore, less effective in accentuating interferon production (Table II, Groups VIII and X). This type of accentuation is presumably caused by inhibition of transcription of mRNA for a protein which normally terminates interferon synthesis (3).

Effect of cycloheximide and actinomycin D on production of interferon induced by poly (I):poly(C). The kinetics of interferon synthesis by cells exposed to poly(I):poly (C) (100 μg/ml) are shown in Fig. 1. Human cells often respond rather poorly to poly(I):poly(C) (7) and in this experiment only 14 units were produced with peak titers at 3 to 5 hr. The effect of duration of exposure to cycloheximide on interferon production is shown in Table III. It can be seen that interferon production subsequent to exposure to cycloheximide increases with duration of exposure up to at least 3 hr (Group III). This exposure period accentuated production almost 10-fold.

The addition of actinomycin D (20 μg/ml) concurrently with poly(I):poly(C) or 1 hr later completely inhibited the interferon response in cultures exposed to cycloheximide for 4 hr (Table III, Groups IV and V). However, if the addition of actinomycin was delayed until hour 2 or hour 3, accentuation was observed. In the latter case 900

TABLE III. Effect of Metabolic Inhibitors on Poly(I):Poly(C)-Stimulated Interferon.

Group	Cycloheximide (duration of exposure from hour 0 to hour)[a]	Actinomycin D		Interferon yield (units)
		Time of addition (hr)	Duration of exposure (hr)	
I	None	None	None	32
II	2	None	None	192
III	3	None	None	300
IV	4	0	1	<4
V	4	1	1	<4
VI	4	2	1	600
VII	4	3	1	900

[a] Hour 0 refers to time of addition of inducer.

units were obtained (Group VII). This represents a 28-fold accentuation.

Discussion. The purpose of this investigation was to determine if sequential blockade of protein and RNA synthesis would enhance the production of human interferon as is the case for rabbit interferon (3). Experiments reported here show that cycloheximide accentuates the subsequent interferon response of cells stimulated with poly(I):poly(C). Actinomycin D, added early, will inhibit interferon completely and if added later will cause some further enhancement of production in cells treated with cycloheximide (Table III). Actinomycin D is only a moderately effective inhibitor of RNA in this system (Table I) and, accordingly, it is not as effective in accentuation interferon response in HEF cultures as it is in rabbit kidney cells.

The system of induction of interferon by NDV_{uv} used in the present paper is different from that previously described for rabbit cells (3). The CG strain of NDV causes extremely rapid production of interferon in HEF cultures (Fig. 1). On the other hand, with the Herts strain in rabbit cells, there was a delay of 10–12 hr between exposure to the NDV_{uv} and the appearance of extracellular interferon (3). This delay may reflect the time required to make the active inducer, perhaps double-stranded RNA, from viral precursors, presumably the uv-damaged +-strand RNA in the virion. The accelerated response with the CG strain may be due to the self-annealing property observed in certain strains of NDV (8).

We conclude that the regulation of interferon production in HEF is similar to that in rabbit kidney cells, although, owing to our inability to terminate RNA synthesis rapidly, it is harder to demonstrate, and that useful accentuation can be achieved by the use of inhibitors. Hence, the appropriate combined use of metabolic inhibitors should substantially increase interferon production in human cells, and may be of eventual importance industrially. Further improvements in the methodology is to be anticipated; for example, a more effective method for controlling RNA synthesis in this system may be devised.

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