

Control of Interferon Synthesis: Effect of Diethylaminoethyl-Dextran on Induction by Polyinosinic-Polycytidylic Acid

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Interferon production in cultures of rabbit kidney cells (RKC) stimulated with 10 to 250 μg of polyinosinic-polycytidylic acid (poly I·poly C) per ml peaked at 3 to 4 hr after the exposure of cells to inducer and rapidly declined thereafter. On the other hand, RKC stimulated with poly I·poly C (10 or 2 $\mu\text{g}/\text{ml}$) in the presence of diethylaminoethyl (DEAE)-dextran (100 or 20 $\mu\text{g}/\text{ml}$, respectively) produced a protracted interferon response, with the release of interferon continuing for over 24 hr. The kinetics of interferon production in RKC stimulated with lower concentrations of the mixture of poly I·poly C and DEAE-dextran were similar to the response produced by poly I·poly C alone (10 to 250 $\mu\text{g}/\text{ml}$). Only the responses that terminated early were paradoxically enhanced by treatment with low doses of actinomycin D or with cycloheximide. Cells stimulated with 50 μg of poly I·poly C/ml showed hyporesponsiveness to a second interferon induction with poly I·poly C when restimulated 7 hr after primary induction. This hyporesponsiveness could be overcome by restimulating with higher concentrations of the poly I·poly C-DEAE-dextran complex. The results are compatible with the hypothesis that the early termination of interferon production and hyporesponsiveness to repeated induction with poly I·poly C are due to a cellular repressor exerting negative control on interferon synthesis, and that the increased cellular uptake of poly I·poly C in the presence of DEAE-dextran may effectively neutralize the repressor. These results also suggested that the often observed different kinetics and the varied effects of inhibitors of ribonucleic acid or protein synthesis on interferon responses in various cells and in cells stimulated with different inducers (such as with viruses as compared with polynucleotides) need not imply the existence of fundamentally different mechanisms of interferon production.

Polyinosinic-polycytidylic acid (poly I·poly C)-induced interferon production in rabbit kidney cells (RKC) has been widely employed as a model system for the study of cellular control mechanisms operating in interferon synthesis. The system is characterized by a rapid synthesis and release of interferon, which peaks in 3 to 4 hr after the exposure of cells to the inducer. Interferon production declines rapidly thereafter; concurrently with the cessation of the interferon response, cells develop a state of hyporesponsiveness to repeated induction (3, 14, 15).

Judicious treatment of induced cells with some inhibitors of ribonucleic acid (RNA) or protein synthesis has been found to interfere with the normal shut-off process of interferon synthesis and to result in a paradoxical enhancement of interferon production. These results suggested that the

early termination of interferon production is due to the synthesis of a regulatory protein (repressor). The paradoxical enhancement of interferon production by some metabolic inhibitors is thought to be the result of a preferential inhibition of repressor synthesis over interferon synthesis (12, 14).

Similar experiments with some other types of cell culture, such as with diploid human cell strains (8, 10; E. A. Havell and J. Vilček, *Bacteriol. Proc.*, p. 195, 1971), largely upheld the results and the conclusions of the experiments in RKC. However, poly I·poly C-induced interferon production in cultures of mouse L cells (11, 14) and chick embryo cells (9) could not be enhanced by treatment with metabolic inhibitors, seemingly suggesting that the control of interferon synthesis

in those latter cells is somehow different from the control exerted in RKC.

The present study was inspired by the fortuitous observation that interferon production in RKC stimulated by the complex of poly I·poly C and diethylaminoethyl (DEAE)-dextran neither displayed the early shut-off phenomenon nor could it be paradoxically enhanced by treatment with metabolic inhibitors. Further experiments offered an explanation of these observations that is fully compatible with the hypotheses advanced earlier on the mechanism of interferon induction by polynucleotides in RKC (10a, 14, 16). Moreover, this study suggested that the differences in the response of poly I·poly C-induced interferon production to metabolic inhibitors, such as observed between RKC and some other types of cells, may not necessarily be due to fundamental dissimilarities in the underlying mechanisms of the control of interferon synthesis, and could be caused by less essential differences in the mode of delivery and uptake of the inducer.

MATERIALS AND METHODS

Cell cultures. Cultures of RKC were prepared by trypsinization of kidneys from 2- to 4-week-old rabbits. All experiments were done in secondary RKC cultures grown to confluency in 60-mm petri dishes (Falcon Plastics, Los Angeles, Calif.) at 36 C in a humidified incubator provided with 5% CO₂. Eagle minimal essential medium (MEM) with 10% heated fetal calf serum or 2% gamma globulin-free fetal calf serum was employed as growth medium or maintenance medium, respectively. All tissue culture media and sera were purchased from Grand Island Biological Co., Grand Island, N.Y.

Interferon induction. RKC cultures were washed once with Earle balanced salt solution (EBSS) and incubated at 36 C with the appropriate concentration of poly I·poly C (supplied by the Antiviral Substances Program, National Institutes of Health, Bethesda, Md.) or of the mixture of poly I·poly C and DEAE-dextran (molecular weight, 2×10^6 , Pharmacia, Uppsala, Sweden), diluted in warm (37 C) phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM phosphate, 0.9 mM CaCl₂, and 0.5 mM MgCl₂·6H₂O; pH 7.4). The mixture of poly I·poly C (10 µg/ml) and DEAE-dextran (100 µg/ml) in PBS was first incubated for 30 min at 37 C in a water bath; further dilutions of this mixture were prepared in warm (37 C) PBS as required. RKC were incubated with 1 ml of the appropriate inducer for 30 or 60 min, as indicated, and were thoroughly washed with EBSS thereafter.

Interferon titrations. A modification of the semimicromethod described by Armstrong (1) was employed. Individual wells of Micro Test II tissue culture plates (Falcon Plastics, Los Angeles, Calif.) were first filled with 50 µliters of serum-free MEM, buffered with 0.15% sodium bicarbonate, 6.6 mM *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid, and 3.3 mM

tricine to pH 7.6, and containing 100 units of penicillin, 100 µg of streptomycin, 100 µg of gentamicin, and 2.5 µg of Fungizone (amphotericin B) per ml. Portions of 25 or 50 µliters of the interferon samples were then added to the first well. Series of two- or threefold dilutions were then prepared directly in the microplates by serially transferring 25 or 50 µliters with an Eppendorf automatic micropipette (Brinkmann Instruments Inc., Westbury, N.Y.). Each well was then seeded with 40,000 RKC (obtained by trypsinization of primary or secondary cultures) in 100 µliters of MEM containing buffers and antibiotics as indicated above, plus 7.5% heated gamma globulin-free fetal calf serum. After 18 hr of incubation at 36 C in an atmosphere of 5% CO₂, each well was inoculated with 1,000 plaque-forming units (PFU) of the Indiana-type vesicular stomatitis virus in 50 µliters of serum-free MEM with additions as above. Several wells on each plate served as virus controls and cell controls.

The titrations were scored microscopically 24 to 48 hr after virus inoculation, when the virus controls showed complete destruction by the virus. The highest dilution of the titrated sample causing an at least 50% protection of cells was considered the end point. In most experiments, each sample was titrated in duplicate. An internal laboratory rabbit interferon standard was included with each titration. This internal standard had been calibrated against a reference rabbit interferon standard prepared by the laboratory of Monto Ho, University of Pittsburgh, Pittsburgh, Pa., and received from the Reference Reagents Branch, National Institutes of Health, Bethesda, Md. The calibration of the internal standard against the reference standard was done by the plaque assay (16) with the use of 2 ml of each interferon dilution per plate. All interferon titers were corrected to this standard. Thus, although the actual volume of diluted interferon samples employed in the microplate assay was 100 µliters, all interferon yields are expressed per 2 ml. Disregarding the volume used in the assay, the sensitivity of the microplate assay was about half that of the plaque assay. The internal laboratory standard calibrated to contain 1,000 reference units/2 ml in the plaque assay averaged about 500 units in the microplate assay.

Chemicals. Cycloheximide (Acti-dione) was obtained from The Upjohn Co., Kalamazoo, Mich., and actinomycin D was purchased from Calbiochem, Los Angeles, Calif.

RESULTS

Kinetics of interferon production in cultures stimulated with various concentrations of poly I·poly C and of the poly I·poly C-DEAE-dextran mixture. Interferon production in RKC cultures stimulated with various concentrations of poly I·poly C, ranging from 10 to 250 µg/ml, peaked at 3 to 4 hr after the exposure of cells to the inducer and declined rapidly thereafter. Although RKC stimulated with higher doses of poly I·poly C produced more interferon, the kinetics of interferon release were not strikingly different. How-

ever, cultures of the same batch of RKC exposed to a mixture of poly I·poly C and DEAE-dextran (10 and 100 $\mu\text{g}/\text{ml}$, respectively) produced a markedly different type of response: the rate of interferon production continued to rise until 5 hr after induction and remained high throughout the duration of the experiment (Fig. 1).

To determine whether the protracted interferon response was characteristic of the poly I·poly C-DEAE-dextran mixture, or whether it was essentially a function of the dose of inducer taken up by cells, RKC were stimulated with various dilutions of the mixture of poly I·poly C and DEAE-dextran. A mixture of 10 and 100 μg of poly I·poly C and DEAE-dextran per ml, respectively, produced a response similar to the one observed with the same dose of inducer in the preceding experiment. A five times lower concentration of this mixture also produced a protracted response, but

the rate of decline of interferon production was faster than with the higher dose. Cells stimulated with a 25-fold dilution of the original poly I·poly C-DEAE-dextran mixture produced a response which was quite similar to that produced by poly I·poly C uncomplexed with DEAE-dextran, in that it reached a peak between 3 and 4 hr after induction and rapidly declined thereafter (Fig. 2).

That the protracted interferon response was not the result of a direct action of DEAE-dextran per se on RKC cultures was shown by the results summarized in Fig. 3. RKC cultures were first treated with poly I·poly C for 1 hr, and then were washed and incubated for 30 min with various concentrations of DEAE-dextran alone. Such separate treatment with DEAE-dextran failed to alter the kinetics of interferon production characteristic of poly I·poly C alone.

Poly I·poly C is known to form a complex with

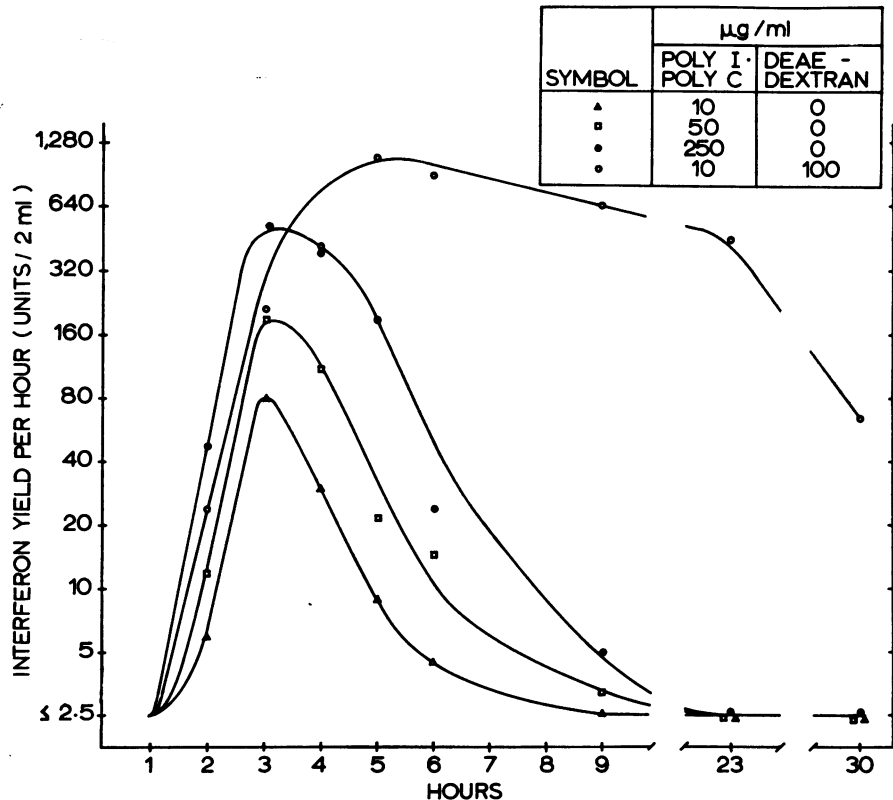


FIG. 1. Kinetics of interferon production in rabbit kidney cells stimulated with various concentrations of poly I·poly C or with a mixture of poly I·poly C and DEAE-dextran. Four groups of cultures were incubated for 1 hr with the indicated concentrations of inducer, washed five times thereafter, and replenished with 2 ml of maintenance medium. At the indicated intervals, fluids were collected, and the cultures were washed once with EBSS and immediately replenished with warm (37 C) fresh maintenance medium. Interferon titers were determined in the pooled fluids from individual groups. If the interval between two samplings was more than 1 hr, the determined interferon yield (units/2 ml) was divided by the number of hours that had elapsed from the collection of the preceding sample.

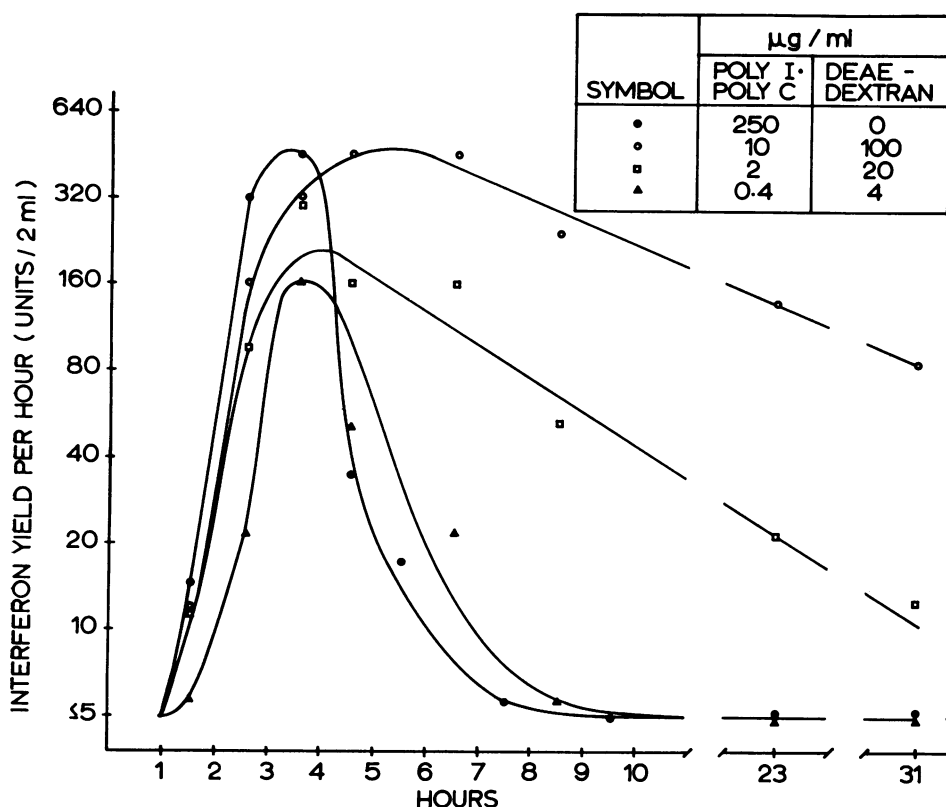


FIG. 2. Kinetics of interferon production in rabbit kidney cells stimulated with various concentrations of the poly I-poly C-DEAE-dextran mixture. Four groups of cultures were incubated for 30 min with the indicated concentrations of the poly I-poly C-DEAE-dextran mixture or for 1 hr with poly I-poly C (250 µg/ml). Other procedures were as in the experiment shown in Fig. 1.

DEAE-dextran (4). The formation of such a complex is apparently responsible for the greatly enhanced cellular uptake of poly I-poly C in the presence of DEAE-dextran (2, 5). The protracted interferon production in RKC cultures stimulated with high doses of the poly I-poly C-DEAE-dextran mixture is thus likely to be due to the more efficient uptake of the inducer by cells.

Effects of actinomycin D and cycloheximide on interferon production stimulated by poly I-poly C or poly I-poly C-DEAE-dextran mixtures. RKC cultures were stimulated either with poly I-poly C (50 µg/ml) or with the mixture of poly I-poly C and DEAE-dextran (10 and 100 µg/ml, respectively). Immediately after a 30-min incubation with the inducer, groups of cultures were treated with various concentrations of actinomycin D or cycloheximide. The yield of interferon was determined in culture fluids collected 24 hr after induction. Actinomycin D, at concentrations of 0.1 and 0.3 µg/ml, increased the yield of interferon stimulated with poly I-poly C alone. No such increase

was noted in RKC stimulated with the mixture of poly I-poly C and DEAE-dextran treated with the same doses of actinomycin D. An even more significant difference was found in the effects of cycloheximide on the two interferon responses: the interferon yield in cultures stimulated with poly I-poly C alone was increased about fivefold, but it was decreased in cells exposed to the poly I-poly C-DEAE-dextran mixture (Table 1).

Cultures stimulated with decreasing concentrations of the poly I-poly C-DEAE-dextran mixture produced decreasing yields of interferon, but at the same time interferon production became increasingly sensitive to paradoxical enhancement by actinomycin D or cycloheximide (Table 2).

Hyporesponsiveness to repeated interferon induction in cultures restimulated with various doses of poly I-poly C and of the poly I-poly C-DEAE-dextran mixture. It was shown that RKC stimulated with poly I-poly C (50 µg/ml) produced decreased interferon yields when restimulated with the same dose of inducer. Such hyporespon-

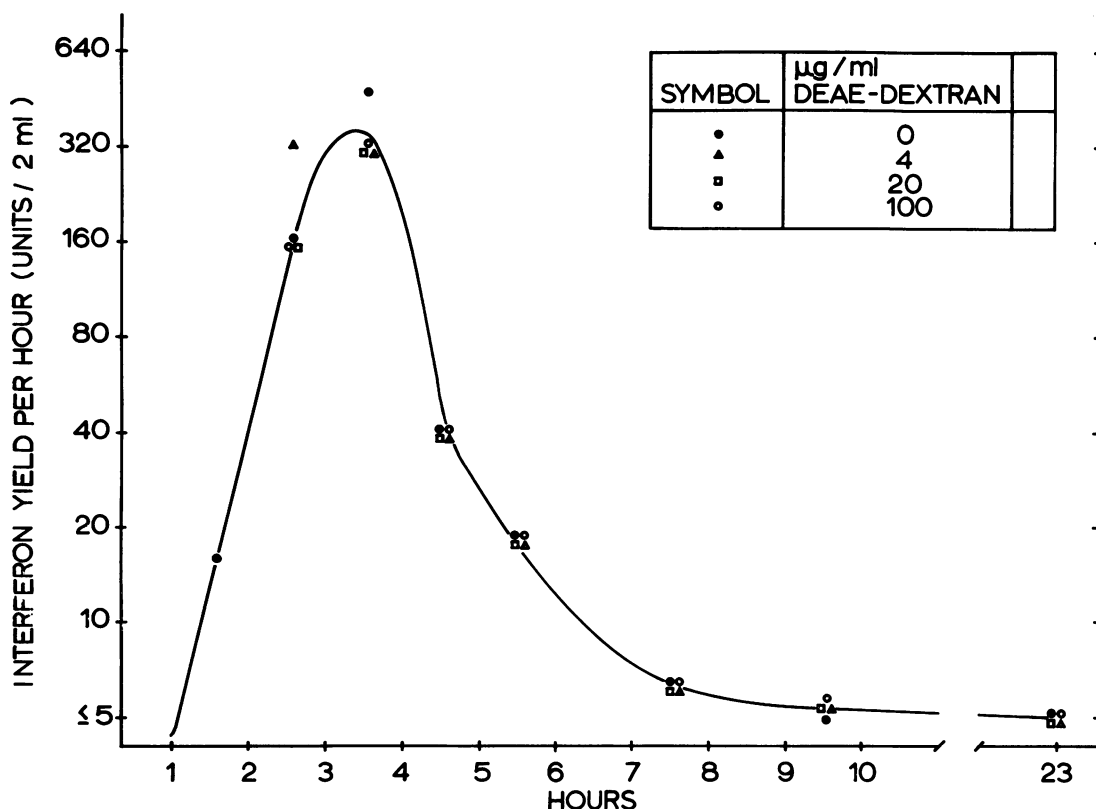


FIG. 3. Effect of DEAE-dextran, added to rabbit kidney cells after induction with poly I-poly C, on the kinetics of interferon production. Cultures were incubated for 1 hr with poly I-poly C (250 µg/ml) and washed five times thereafter. Three groups were then treated for 30 min with the indicated concentrations of DEAE-dextran while one group received no further treatment. Other procedures were as in the experiment shown in Fig. 1.

TABLE 1. Effects of actinomycin D and cycloheximide on interferon production stimulated with poly I-poly C or a mixture of poly I-poly C and DEAE-dextran

Treatment ^a	Interferon yield ^b			
	Poly I-poly C ^c		Poly I-poly C + DEAE-dextran ^d	
	Units/2 ml	Percent ^e	Units/2 ml	Percent ^e
None.....	87	(100)	8,100	(100)
Actinomycin D, 0.1 µg/ml.....	218	250	8,100	100
Actinomycin D, 0.3 µg/ml.....	150	172	4,050	50
Actinomycin D, 1.0 µg/ml.....	16	18	2,025	25
Cycloheximide, 1.0 µg/ml.....	486	558	4,050	50
Cycloheximide, 10.0 µg/ml.....	486	558	1,620	20

^a The indicated concentrations of inhibitors were added immediately after a 30-min exposure to inducer. Actinomycin D was left on the cultures for 30 min; thereafter, the cultures were washed twice and replenished with drug-free maintenance medium. Cycloheximide was maintained in the cultures throughout the duration of experiment.

^b In culture fluids collected 24 hr after exposure to inducer. Fluids from cycloheximide-treated cultures were dialyzed prior to interferon assay.

^c Cultures were incubated with 50 µg of poly I-poly C/ml for 30 min and washed five times thereafter.

^d Cultures were incubated with a mixture of poly I-poly C (10 µg/ml) and DEAE-dextran (100 µg/ml) for 30 min and washed five times thereafter.

^e Percentage of control.

TABLE 2. Effects of actinomycin D and cycloheximide on interferon production stimulated with various concentrations of poly I·poly C and DEAE-dextran

Inducer ($\mu\text{g/ml}$) ^a		Interferon yield ^b						
Poly I·poly C	DEAE-dextran	Control (units/2 ml)	Actinomycin D ^c				Cycloheximide, 10 $\mu\text{g/ml}$ ^d	
			0.3 $\mu\text{g/ml}$		0.1 $\mu\text{g/ml}$			
			Units/2 ml	Percent ^e	Units/2 ml	Percent	Units/2 ml	Percent ^e
10	100	8,747	2,916	33	4,374	50	1,458	16
2	20	1,458	972	66	972	66	1,458	100
0.4	4	162	972	600	2,187	1,345	2,916	1,800
0.08	0.8	18	162	900	1,458	8,100	2,916	16,200

^a Cultures were incubated for 30 min with a mixture of the indicated concentrations of poly I·poly C and DEAE-dextran and washed four times thereafter.

^b In culture fluids collected 24 hr after exposure to inducer.

^c Treated with the indicated concentrations for 30 min immediately after the exposure to inducer. Thereafter, the cultures were washed twice and replenished with drug-free maintenance medium.

^d Cycloheximide was added immediately after the 30-min exposure of cultures to inducer and was maintained in the culture fluids throughout the duration of the experiment. Prior to titrating the interferon content, the harvested fluids were dialyzed to remove the cycloheximide.

^e Percentage of control.

siveness was shown to develop concurrently with the cessation of interferon production and to last for about 48 hr after the first exposure of cells to the inducer (3, 15; S. L. Barmak, Ph.D. thesis, New York University, 1972).

In the experiment summarized in Table 3, RKC were first stimulated with poly I·poly C (50 $\mu\text{g/ml}$) and were restimulated 7 hr later with various doses of poly I·poly C either complexed or uncomplexed with DEAE-dextran. The interferon yields obtained on the second stimulation were compared with yields from control, previously unstimulated cultures exposed to the same doses of inducer. The degree of hyporesponsiveness decreased with increasing doses of poly I·poly C used for restimulation. Hyporesponsiveness was not detected in cultures restimulated with 2 or 10 μg of poly I·poly C per ml in the presence of a 10-fold excess of DEAE-dextran.

DISCUSSION

The kinetics of interferon production in RKC stimulated with the poly I·poly C-DEAE-dextran complex were different from those observed with poly I·poly C alone. Stimulation with various concentrations of the polynucleotide uncomplexed with DEAE-dextran produced an interferon response that peaked at 3 to 4 hr after induction and declined quite rapidly thereafter. On the other hand, cells stimulated with 2 or 10 μg of poly I·poly C per ml in the presence of a 10-fold excess of DEAE-dextran produced a much protracted response, with interferon being released into the culture fluid for more than 24 hr.

As noted earlier (12, 14), interferon production

stimulated with poly I·poly C was paradoxically enhanced by treatment with certain concentrations of actinomycin D or by incubating stimulated cultures in the presence of cycloheximide. On the other hand, the production of interferon stimulated with high concentrations of the poly I·poly C-DEAE-dextran complex was suppressed by similar treatment with actinomycin D or cycloheximide.

These findings raised the question of whether interferon induction by the poly I·poly C-DEAE-dextran complex was perhaps qualitatively different from the interferon response elicited by poly I·poly C alone. Further experiments revealed that interferon production in response to lower concentrations of the poly I·poly C-DEAE-dextran complex showed similar characteristics to interferon production after the stimulation with poly I·poly C alone: it ceased early after induction and it could be paradoxically enhanced by treatment with actinomycin D or cycloheximide.

The findings support the previously suggested notion that a relationship exists among the early shut-off of interferon production, the paradoxical enhancement of interferon synthesis by metabolic inhibitors, and the hyporesponsiveness to repeated interferon induction by poly I·poly C (15). The early cessation of interferon production has been attributed to a labile repressor protein that exerts a negative control on interferon synthesis. The paradoxical enhancement of interferon production by metabolic inhibitors is the result of protracted release of interferon and is believed to be due to preferential inhibition of repressor synthesis over interferon synthesis (12, 14). Hypo-

TABLE 3. *Effect of stimulation with various concentrations of poly I·poly C and of the poly I·poly C-DEAE-dextran mixture on hyporesponsiveness to repeated interferon induction*

Inducer ($\mu\text{g/ml}$) ^a		Interferon yield ^b		
Poly I·poly C	DEAE-dextran	Control cultures ^c (units/2 ml)	Restimulated cultures ^d	
			Units/2 ml	Percent ^e
250	0	192	128	66
50	0	144	80	55
10	0	72	20	27
10	100	2,880	3,824	132
2	20	1,440	1,424	98
0.4	4	576	128	22
0.08	0.8	288	32	11

^a Cultures were incubated for 1 hr with a mixture of the indicated concentrations of poly I·poly C and DEAE-dextran and washed five times thereafter.

^b In culture fluids collected 24 hr after exposure to inducer.

^c At 7 hr prior to stimulation, these cultures were incubated with PBS for 1 hr, washed, and then incubated in maintenance medium until stimulation.

^d At 7 hr prior to restimulation, these cultures were incubated with poly I·poly C (50 $\mu\text{g/ml}$) for 1 hr, washed three times, and replenished with maintenance medium; 6 hr later, the cultures were washed twice and restimulated by incubating with the appropriate inducer for 1 hr. Thereafter, the cultures were washed four times and replenished with maintenance medium. Culture fluids collected 7 hr after primary stimulation contained 192 units of interferon per 2 ml. The interferon yields given are after subtraction of the residual amount of interferon (16 units/2 ml) produced by control unstimulated cultures during the same experimental period.

^e Percentage of control.

responsiveness to repeated induction by poly I·poly C, which appears concurrently with the cessation of interferon production (15), is also likely to be attributable to the action of this repressor. The repressor apparently controls interferon production by suppressing its synthesis at a post-transcriptional level, most likely by directly binding to the interferon messenger RNA (16). A similar mechanism was earlier implicated in the control of steroid hormone-induced enzyme synthesis in animal cells (13).

It has been suggested that the first step in interferon induction by polynucleotides involves the binding and neutralization of the repressor protein by the inducer (10a). According to this as yet speculative notion, a basal level of repressor

is thought to be continuously synthesized in uninduced cells; the rate of repressor synthesis would be stepped up after interferon induction and the increased repressor concentration would in turn result in the cessation of interferon synthesis and hyporesponsiveness to repeated induction.

The observations made in this study could be explained by the increased cellular uptake of inducer, resulting in a more efficient binding of the repressor in cells stimulated with higher concentrations of the poly I·poly C-DEAE-dextran complex. Efficient neutralization of the repressor could account for the lack of the early shut-off, as well as for the failure to increase interferon production by treatment with metabolic inhibitors. As long as the cellular concentration of inducer is sufficient to neutralize the repressor, interferon synthesis could continue, and inhibition of repressor synthesis by metabolic inhibitors would not be expected to promote interferon production. The overcoming of hyporesponsiveness to repeated induction by higher concentrations of the poly I·poly C-DEAE-dextran complex could be explained on the same basis: by the uptake of enough inducer to neutralize the increased concentration of repressor believed to be present in hyporesponsive cells.

We have also considered a somewhat different explanation for the dissimilar responses produced by poly I·poly C or low concentrations of the poly I·poly C-DEAE-dextran mixtures as compared to the response obtained with high doses of the complexed inducer. It was shown earlier that primary mouse kidney cell cultures are composed of heterogeneous cell populations which differ in their response to stimulation with poly I·poly C or Newcastle disease virus (11). It cannot be ruled out that RKC cultures may also have contained heterogeneous cell populations, with one type of cells that could be stimulated to produce the early response, and another cell type characteristically producing a later and protracted interferon response that could be stimulated only with high doses of the poly I·poly C-DEAE-dextran complex. However, experiments similar to the ones described in this paper, performed in cultures of a serially passaged human diploid cell strain and of a continuous line of RKC (RK 13) which are not composed of markedly heterogeneous cell populations, yielded results quite comparable to those in RKC (E. A. Havell, L. Mozes, and J. Vilček, unpublished data). Thus, cell heterogeneity does not seem to be a likely basis for the observed differences in the interferon responses.

That the presence of DEAE-dextran greatly enhances the rate of cellular uptake of poly I·poly C was demonstrated by other investigators (2, 5).

Whether the polycation actually increases the amount of inducer that penetrates across the cell membrane or only increases its rate of attachment to the cell surface has not been determined. It is also not clear what role, if any, the increased resistance of the poly I·poly C-DEAE-dextran complex to nuclease degradation (4, 6) plays in promoting inducer uptake and interferon production.

Earlier studies failed to demonstrate paradoxical enhancement of poly I·poly C-induced interferon production by inhibitors of RNA or protein synthesis in cultures of L cells (11, 14) or chick embryo cells (9), and it was concluded that the control of interferon production in these cells is probably different from the control in RKC. However, as these studies employed high concentrations of the poly I·poly C-DEAE-dextran complex, the differences may, in fact, not have been due to fundamental dissimilarities in the control of interferon synthesis but, rather, to different conditions of induction. Our results also suggest that the observed differences in the kinetics, and in the degree of suppression or enhancement by various metabolic inhibitors, of the interferon responses produced by viruses or by poly I·poly C (7, 9, 15, 17) need not imply that fundamentally different mechanisms operate in interferon production stimulated by viruses or isolated polynucleotides.

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