Production of High-Titered Interferon in Cultures of Human Diploid Cells

EDWARD A. HAVELL AND JAN VILČEK

Department of Microbiology, New York University School of Medicine, New York, New York 10016

Received for publication 24 July 1972

The effect of incubation with interferon prior to the stimulation of interferon production (priming) and of sequential treatment with cycloheximide and actinomycin D (superinduction) on the interferon yield from polyinosinic-polycytidylic acid (poly I·poly C)-induced diploid human foreskin cell cultures (FS-3 strain) was studied. Suitable priming with interferon produced, on the average, about an eightfold increase over the control yield, with a greater increase noted on some occasions when the control interferon yield was very low. Under the optimal conditions carefully defined in our experiments, superinduction produced about a 100-fold increase over the average control yield, resulting in interferon yields of about 10,000 reference units from cultures containing about 10^6 cells. Combined superinduction and priming did not produce yields markedly higher than obtainable by superinduction alone. Essentially similar results were obtained in cultures of human embryonic kidney cells and in FS-3 cells stimulated with other double-stranded polynucleotide inducers. However, stimulation of cells with certain concentrations of a mixture of diethylaminoethyl-dextran and poly I·poly C altered the interferon response; the yield was considerably higher than in cells stimulated with poly I·poly C alone, but it could not be markedly increased further by superinduction.

Previous studies with cultures of rabbit kidney cells demonstrated that polyinosinic-polycytidylic acid (poly I·poly C)-stimulated interferon production could be greatly enhanced by judicious treatment with some inhibitors of cellular ribonucleic acid (RNA) or protein synthesis. A slight enhancement was first noted in cultures that were treated with actinomycin D several hours after exposure to poly I·poly C (19). A more marked enhancement occurred in rabbit kidney cell cultures treated with inhibitors of protein synthesis. The interferon yield was increased about 10-fold in cultures incubated in the presence of cycloheximide during the period of interferon production. To afford such enhancement, the inhibitor could be added prior to, along with, or up to 2 hr after the exposure to poly I·poly C (16). Tan et al. (14) subsequently demonstrated a greater stimulation of interferon production in cultures incubated with cycloheximide only for 3 to 4 hr after poly I·poly C stimulation, with the bulk of interferon production occurring only after the reversal of cycloheximide action. The addition of actinomycin D prior to the removal of cycloheximide caused an additional stimulation of subsequent interferon production. Vilček and Ng (18) showed that such short exposure of

poly I·poly C-stimulated cultures to cycloheximide resulted in a burst of interferon production immediately after the removal of the inhibitor of protein synthesis and a subsequent rapid decline of interferon production; the addition of actinomycin D prior to cycloheximide removal prevented the rapid cessation of interferon production upon the reversal of the inhibition of protein synthesis. The significance of these findings in relation to the cellular mechanisms of control of interferon production has recently been reviewed (11).

It has been recognized that judicious anti-metabolite treatment might provide means for the production of large quantities of human interferon in cell cultures. Preliminary studies have established that the yield of interferon from various diploid human cell strains could be enhanced by treatment with inhibitors of protein synthesis and actinomycin D, applied either separately or in various combinations (3, 6, 8, 10; E. A. Havell and J. Vilček, *Bacteriol. Proc.*, p. 195, 1971). However, the net yields of human interferon reported in these studies were considerably lower than those obtainable from suspension cultures of human peripheral leukocytes (5, 13, 15). Furthermore, no systematic study of the effect of the

numerous variables that influence interferon production and its enhancement by metabolic inhibitors has yet been published.

This paper reports on the establishment of conditions that allow the production of crude preparations of human interferon from diploid cells with titers that exceed 10,000 reference units/2 ml. This material could be used for purification studies and for eventual processing into a preparation for human clinical trials. Furthermore, this work might help to provide additional insight into the cellular control mechanisms of interferon synthesis.

MATERIALS AND METHODS

Cell cultures. A strain of human foreskin cells (FS-3) was derived from neonatal foreskins by the method of Merigan et al. (9). The cells were grown in Eagle's minimal essential medium (MEM) containing 5% heated fetal calf serum. All experiments were performed in cultures grown at 36 C in 60-mm plastic petri plates (Falcon Plastics, Los Angeles, Calif.) in a humidified incubator provided with 5% CO₂. Twenty petri plates were routinely seeded with cells obtained by the trypsinization of a confluent monolayer from one 250-ml plastic tissue culture flask. Plate cultures reached confluency about 6 days after seeding. At this time, the cultures were re-fed with fresh growth medium and incubated without further medium change until used. Most experiments were carried out on 12-day-old cultures. All experiments employed FS-3 cells that had undergone 7 to 13 passages.

Human embryo kidney (HEK) cells were kindly supplied by R. E. Weiss of Abbott Laboratories, North Chicago, Ill. Ampoules containing 2×10^8 HEK cells frozen after two passages were stored in liquid nitrogen. Before use, the cells were quickly thawed and then suspended in medium 199 supplemented with Eagle's basal medium vitamins and amino acids, and containing 5% heated fetal calf serum. The cells were then seeded into 60-mm plastic petri plates (200,000 cells/plate), and the medium was changed every 6th day until confluency was reached on about the 15th day.

All tissue culture media, sera, and Earle's balanced salt solution were purchased from Grand Island Biological Co., Grand Island, N.Y.

Polynucleotides. In all experiments, except where indicated, the poly I·poly C preparation used was obtained from the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md. (The homopolymers employed in this preparation were prepared by P-L Laboratories, Milwaukee, Wis.) Another preparation of poly I·poly C (lyophilized double-stranded sodium salt) was purchased directly from P-L Laboratories. Dilutions of polynucleotide preparations were usually prepared in sterile phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM phosphate, 0.9 mM CaCl₂·6H₂O; pH 7.4) and incubated at 36 C for 1 hr prior to use. Mixtures of poly I·poly C and diethylaminoethyl (DEAE)-dextran (molecular weight, 2×10^6 ; Pharmacia, Uppsala,

Sweden) were prepared in PBS and allowed to incubate at 36 C for 1 hr.

Purified double-stranded ribonucleic acid (RNA) from *Penicillium chrysogenum* (molecular weight, about 2×10^6 ; T_m, 84.5 C) was kindly supplied by Robert F. Bozarth and H. Alan Wood of the Boyce Thompson Institute, Yonkers, N.Y. (20).

Interferon preparations. Sendai virus-induced human leukocyte interferon was a gift of Joseph Valenta, Smith, Kline & French Laboratories, Philadelphia, Pa. When tested in this laboratory, the preparation had a titer of 8,000 reference units/2 ml. Poly I·poly C-induced human interferon was produced in FS-3 cells sequentially treated with cycloheximide and actinomycin D as described in the Results.

Interferon titrations. A modification of the semimicro method described by Armstrong (2) was used. Individual wells of Micro Test II tissue culture plates (Falcon Plastics) were first filled with 50 or 100 μ liters of MEM with 5% fetal calf serum, which had been buffered with sodium bicarbonate (0.15%), N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (13.2 mM), and tricine (6.6 mM) to pH 7.6 and contained 100 units of penicillin, 100 μ g of streptomycin, 100 μ g of gentamicin, and 2.5 μ g of amphotericin B (Fungizone) per ml. To prepare a series of twofold dilutions, 100 μ liters of the titrated sample was added to the first well containing the same volume of medium; serial dilutions were prepared by transferring 100 μ liters of the mixtures with an automatic Eppendorf micropipette (Brinkman Instruments, Westbury, N.Y.). To prepare a series of threefold dilutions, 25 μ liters of the sample was first added to a well containing 50 μ liters of medium, and 25- μ liter portions were then serially transferred from well to well. Each well was then seeded with 50,000 FS-3 cells (obtained by trypsinization of confluent flask cultures) in 100 μ liters of the above medium. After 18 hr of incubation at 36 C in a 5% CO₂ incubator, each well received 1,000 plaque-forming units of the Indiana-type vesicular stomatitis virus in 50 μ liters of the medium described 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 at least 50% protection of cells was considered the end point. In most experiments, each sample was titrated in duplicate. An internal laboratory human standard was included with each titration. This internal standard had been calibrated against a reference human standard (69/19) prepared by the Medical Research Council, Division of Biological Standards, National Institute for Medical Research, Mill Hill, London, England. The calibration of the internal standard against the reference standard was done by the plaque assay in human U cell cultures (13) with the use of 2 ml of each interferon dilution per plate. All interferon titers were corrected to this standard. Therefore, although the actual volume of diluted interferon samples employed in the microplate assay (after adding the cell suspension) was either 150 or 200 μ liters, all interferon yields are expressed per 2 ml. Disregarding of the volume used in the assay,

the sensitivity of the microassay with FS-3 cells was about equivalent to that of the plaque assay on the U cells. The internal laboratory standard calibrated to contain 1,000 reference units in the plaque assay titered, on the average, about 1,000 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

Choice of optimal inducer dose and the effect of priming by interferon. Incubation of cells with interferon prior to stimulation with viral or non-viral inducers has often been shown to increase the interferon yield (12, 15). The effect of such priming with interferon on interferon production with poly I·poly C in two types of cultures is shown in Table 1. An 8-hr preincubation was insufficient to produce priming, but a 16-hr incubation with as little as 4 units of interferon enhanced the yield of interferon in both types of cell culture tested. Priming with 100 units was sufficient to produce maximal enhancement.

The effect of a wide range of inducer doses and of priming with interferon on the interferon yield was studied in the experiments summarized in Table 2. Doses of poly I·poly C below 10 $\mu\text{g/ml}$

TABLE 1. *Priming by preincubation with various concentrations of interferon in two types of human cell cultures*

Concn of interferon during preincubation (units/2 ml) ^a	Interferon yield/2 ml ^b		
	FS-3 cells ^c		HEK cells ^d , 16-hr preincubation
	8-hr preincubation	16-hr preincubation	
0	10	10	9
4	10	40	27
20	10	40	81
100	10	80	81
500	20	80	81

^a Cultures were incubated with the indicated concentration of human leukocyte interferon in 2 ml of MEM with 2% gamma globulin-free calf serum.

^b In culture fluids collected 24 hr after exposure to poly I·poly C.

^c Cultures (12-day-old) were stimulated by incubating for 2 hr with poly I·poly C (50 $\mu\text{g/ml}$ in 2 ml of PBS). The cultures were thoroughly washed thereafter and replenished with 2 ml of MEM with 2% gamma globulin-free calf serum.

^d Cultures (15-day-old) were stimulated with 100 μg of poly I·poly C per ml. Other procedures were as described for FS-3 cells.

TABLE 2. *Interferon yields from primed and unprimed cells stimulated with various doses of poly I·poly C*

Expt no.	Dose of poly I·poly C ($\mu\text{g/ml}$) ^a	Interferon yield/2 ml ^b	
		Control	Primed ^c
1	0.1	5	20
	1.0	20	80
	10.0	40	320
	100.0	40	320
2	10.0	24	108
	50.0	24	108
	100.0	36	324
	250.0	36	324

^a FS-3 cultures (12-day-old) were stimulated by incubation for 2 hr with the indicated concentrations of poly I·poly C in 2 ml of PBS. Thereafter, all cultures were thoroughly washed and replenished with 2 ml of MEM containing 2% gamma globulin-free calf serum.

^b In culture fluids collected 24 hr after exposure to poly I·poly C.

^c Incubated with 100 units of poly I·poly C-induced human FS-3 cell interferon per 2 ml for 16 hr immediately preceding stimulation.

produced inferior yields, but increasing the dose of inducer above 10 $\mu\text{g/ml}$ did not result in a marked enhancement of the yield in unprimed cultures. A dose of 100 $\mu\text{g/ml}$ was chosen for most subsequent experiments because it consistently produced maximal interferon yields in both primed and unprimed cells.

An analysis of the kinetics of accumulation of interferon in the culture fluid showed that in both primed and unprimed cells the onset of interferon production is rapid and the production of interferon is essentially completed by 8 hr after the exposure of cells to poly I·poly C. After this time, there was a drop in interferon activity which was particularly marked in the unprimed cultures (Fig. 1). A similar rapid decline in the activity of accumulated interferon in human cell cultures was observed by Billiau et al. (3). At least in part, this rapid decline may be due to thermal inactivation of interferon, perhaps combined with enzymatic degradation by small quantities of proteases released from the cells.

Effect of treatment with cycloheximide and actinomycin D. On the basis of previous experience with the production of interferon in cultures of rabbit kidney cells, we decided to attempt to "superinduce" the production of human interferon by using sequential treatment with cycloheximide and actinomycin D, as first reported by Armstrong (1) and Tan et al. (14).

FIG. 1. Kinetics of accumulation of interferon in primed and unprimed cultures. Groups of FS-3 cells (12 days old at time of stimulation) were first incubated for 16 hr with 100 units of poly I·polyC-stimulated FS-3 cell interferon in 2 ml of MEM containing 2% gamma globulin-free calf serum (primed cultures) or the same volume of medium alone (unprimed cultures). Both groups were then stimulated by incubating for 2 hr with 100 μ g of poly I·poly C per ml in 2 ml of PBS. All cultures were then thoroughly washed and reincubated with 2 ml of MEM with 2% gamma globulin-free calf serum. At the intervals indicated in the graph, culture fluids were collected from two cultures in each group for interferon titrations.

To determine the optimal dose of cycloheximide for such treatment, cells were stimulated with poly I·poly C and then incubated for 5 hr in the presence of various concentrations of the inhibitor of protein synthesis. Incubation with 50 or 100 μ g/ml produced optimal (about 10-fold) enhancement. The addition of actinomycin D (1 μ g/ml) 30 min before the removal of cycloheximide (50 μ g/ml) produced a further marked enhancement of interferon production (Table 3).

To determine the optimal time of treatment with the inhibitors, cells were stimulated with poly I·poly C and incubated with cycloheximide (50 μ g/ml) for various periods of time. Actinomycin D (1 μ g/ml) was added to each group 30 min prior to the removal of cycloheximide. The results of two independent experiments are shown in Table 4. Treatment with 50 μ g of cycloheximide per ml for 4 hr after a 2-hr exposure to poly I·poly C was sufficient to produce maximal interferon yields.

In another experiment, the time of addition of cycloheximide was varied, but the times of actinomycin D treatment and the removal of the drugs from cultures were kept constant. The addition of cycloheximide later than 3 hr after stimu-

lation with poly I·poly C markedly reduced the interferon yield (Table 5).

The effect of using various concentrations of actinomycin D was studied in the experiment shown in Table 6. Concentrations of 0.25 to 2.0 μ g/ml produced satisfactory results. Lower doses were less effective. It should be noted that treat-

TABLE 3. Interferon yields from cells treated with various concentrations of cycloheximide

Dose of cycloheximide (μ g/ml) ^a	Dose of actinomycin D (μ g/ml) ^b	Interferon yield/2 ml ^c
0	0	36 ^d
10	0	108
25	0	108
50	0	324
100	0	324
50	1	2,916

^a FS-3 cultures (12-day-old) were stimulated by incubating for 2 hr with poly I·poly C (100 μ g/ml) in 2 ml of PBS. The cells were thoroughly washed thereafter and incubated with 2 ml of MEM containing 2% gamma globulin-free calf serum and the indicated concentration of cycloheximide for 5 hr. Thereafter, the cultures were washed again and replenished with drug-free MEM with 2% gamma globulin-free calf serum.

^b Added 30 min prior to removal of cycloheximide.

^c In fluids collected 24 hr after stimulation with poly I·poly C, representing the amount of interferon accumulated from the time of removal of cycloheximide, except as noted below.

^d In this group of cultures, the yield represents the amount of interferon accumulated from the removal of inducer until 24 hr after stimulation.

TABLE 4. Effect of combined treatment with cycloheximide and actinomycin D at various periods after induction^a

Time of treatment with cycloheximide (hr)	Time of addition of actinomycin D (hr)	Interferon yield/2 ml	
		Expt 1	Expt 2
None	None	12	16
2-4	3.5	486	ND
2-5	4.5	972	2,560
2-6	5.5	8,748	5,120
2-7	6.5	2,916	5,120
2-8	7.5	2,916	2,560

^a The times indicated refer to hours after the addition of poly I·poly C to 12-day-old FS-3 cell cultures. The concentrations of cycloheximide and actinomycin D were, in all instances, 50 μ g/ml, respectively. Other details of the procedure were as in the experiment shown in Table 3.

TABLE 5. *Effect of varying the time of addition of cycloheximide^a*

Time of treatment with cycloheximide (hr)	Interferon yield/2 ml
0-6	4,800
1-6	6,400
2-6	6,400
3-6	3,200
4-6	400

^a FS-3 cultures (10-day-old) were stimulated at 0 hr with poly I·poly C (100 μ g/ml) in 2 ml of serum-free MEM. Cycloheximide (final concentration, 50 μ g/ml) was added at the indicated times. All cultures were treated with actinomycin D (final concentration, 1 μ g/ml) at 5.5 hr. The cultures were thoroughly washed at 6 hr and replenished with 2 ml of MEM containing 2% gamma globulin-free calf serum. Fluids were collected 22 hr after stimulation.

TABLE 6. *Effect of combined treatment with cycloheximide and various concentrations of actinomycin D^a*

Dose of actinomycin D (μ g/ml)	Interferon yield/2 ml
0	640
0.0625	1,280
0.125	1,280
0.25	5,120
0.5	5,120
1.0	10,240
2.0	5,120

^a FS-3 cultures (12 days old at time of stimulation) were first incubated with 100 units of human interferon for 16 hr as for the primed group in Table 2. All cultures were incubated with serum-free MEM containing 50 μ g of cycloheximide per ml between hr 2 and 6. Actinomycin D was added at hr 5.5. All other procedures were as in the experiments shown in Tables 3 and 4.

ment with actinomycin D concentrations of up to about 2 μ g/ml after the incubation with cycloheximide produced only minimal or no morphological alterations of cells. Treatment with higher concentrations of actinomycin D (10 μ g/ml or more), however, was toxic and produced inferior interferon yields.

It was of interest to determine the effect of the sequential pulse treatment with cycloheximide and actinomycin D on the kinetics of interferon release after the removal of the drugs. Culture fluids from induced cells were collected at various intervals after the removal of the drugs and replenished with the same volume of fresh medium. Interferon production reached a maximal rate within 2 hr, continued at a high rate for about

4 hr, and declined quite rapidly thereafter (Fig. 2). In view of the fact that interferon was found to decay if left in a culture after the period of maximal production (cf. Fig. 1), it appeared that the highest yields would be obtained by harvesting culture fluids about 6 to 8 hr after the removal of the drugs.

Effects of varying the serum concentration in the culture medium. Varying the serum concentration during the period of cycloheximide and actinomycin D treatment (between 2 and 7 hr after exposure to poly I·poly C) had a quite marked effect on the interferon yield in FS-3 cells. Somewhat unexpected was the finding that higher serum concentrations (5% or more) had an unfavorable effect on interferon yields, and serum-free MEM produced the best results. In this experiment (not shown), during the period of actual interferon production, all cultures were incubated with medium containing 2% gamma globulin-free calf serum. On the other hand, omitting serum from

FIG. 2. Kinetics of interferon production after combined treatment with cycloheximide and actinomycin D. A group of FS-3 cultures (12-day-old) was stimulated by incubation for 2 hr with 100 μ g of poly I·poly C per ml, washed, and treated with cycloheximide (50 μ g/ml) and actinomycin D (1 μ g/ml) in serum-free MEM at the indicated times. After the removal of cycloheximide, the cultures were washed and replenished with 2 ml of drug-free MEM containing 2% gamma globulin-free calf serum. At the indicated intervals, fluids were collected; the cultures were washed once with MEM and immediately replenished with the same volume of warm (37 C) fresh medium. Interferon titers were determined in the pooled fluids taken at each interval. 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. (Note that unlike in Fig. 1, yields are not cumulative, i.e., each point in the graph refers to actual interferon production during the respective time period.)

the culture medium during the time of interferon production, i.e., after the removal of cycloheximide and actinomycin D, produced interferon yields that were only about 20 to 25% of the yield from cultures incubated with medium containing 2% gamma globulin-free calf serum. However, other experiments showed that whole serum could be replaced by the human plasma protein fraction (Hyland, Costa Mesa, Calif.), which at the final concentration of 1% produced yields that were equivalent or slightly superior to those obtained with production medium containing various concentrations of gamma globulin-free calf serum or fetal calf serum.

Other variables. The effect of a number of variables was tested in the experiment summarized in Table 7. Cells were stimulated on different days after the cultures were seeded. In cells induced with poly I·poly C and receiving no other treatment, the interferon yield increased with the age of cultures. In view of the extremely low yield in the younger cultures, priming by pre-treatment with interferon was most effective in 9-day-old cultures. Sequential treatment with cycloheximide and actinomycin D produced an interferon yield 10 to 20 times higher than that obtained after priming. Combined priming and treatment with inhibitors caused either only a twofold (and possibly insignificant) further increase or no further increase at all. It thus seems that under the conditions employed the effects of priming and of the treatment with metabolic inhibitors were not more than additive. In terms of the interferon yield per cell, as well as in terms of the net interferon yields obtained, 12-day-old

cultures were superior to cultures stimulated 3 days earlier or 3 days later.

A related experiment was carried out in cultures of HEK cells. Again, the combined effect of priming and inhibitor treatment produced interferon yields that were not more than twofold higher or lower than the yields obtained after inhibitor treatment alone. Generally, the interferon yields were within the ranges of yields usually produced in FS-3 cells under similar conditions (Table 8).

The effect of sequential cycloheximide and actinomycin D treatment was also tested in FS-3 cells stimulated with various polynucleotide inducers. Cells stimulated with double-stranded RNA from *P. chrysogenum* (10 or 100 µg/ml) produced yields comparable to those obtained with two different preparations of poly I·poly C. Poly I·poly C complexed with DEAE-dextran produced more interferon than a higher dose of poly I·poly C alone; however, interferon production by the complex was not significantly increased after treatment with inhibitors (Table 9).

The effects of DEAE-dextran were further investigated by stimulating FS-3 cells with various concentrations of the poly I·poly C-DEAE-dextran mixture. Cultures not subjected to additional treatment produced yields that decreased sharply with the dilution of the inducer complex. Sequential treatment with antimetabolites produced only a slight enhancement over the control yield in cultures stimulated with the highest inducer dose, but produced a relatively increasing degree of superinduction with decreasing inducer doses (Table 10). Essentially similar results were

TABLE 7. Effects of priming with interferon and treatment with metabolic inhibitors at different days after seeding cultures^a

Treatment	Age of culture (days after seeding) and no. of cells/culture					
	9 days, 1.0×10^6 cells		12 days, 1.25×10^6 cells		15 days, 1.3×10^6 cells	
	Interferon yield/2 ml	Interferon yield/ 10^6 cells	Interferon yield/2 ml	Interferon yield/ 10^6 cells	Interferon yield/2 ml	Interferon yield/ 10^6 cells
None.....	0.5	0.5	8	6.4	32	24
Priming ^b	128	128	512	409	256	196
Superinduction ^c	2,560	2,560	10,240	8,352	2,560	1,969
Priming ^b + superinduction ^c	2,560	2,560	20,480	16,704	5,120	3,938

^a All cultures were stimulated by incubating for 2 hr with 100 µg of poly I·poly C/ml. During the period of interferon production, all cultures were incubated with 2 ml of MEM containing 2% gamma globulin-free calf serum.

^b Incubated with 100 units of poly I·poly C-induced human FS-3 cell interferon per 2 ml for 16 hr immediately preceding stimulation.

^c Incubated with cycloheximide (50 µg/ml, in 2 ml of serum-free MEM) between hr 2 and 7. Actinomycin D (final concentration, 1 µg/ml) was added 30 min prior to removal of cycloheximide.

TABLE 8. *Effects of priming and treatment with metabolic inhibitors on interferon production in cultures of human embryonic kidney cells^a*

Treatment	Interferon yield/2 ml	
	Unprimed	Primed ^b
None.....	160	640
Cycloheximide ^c	640	1,280
Cycloheximide + actinomycin D ^{c,d}	10,240	5,120
Cycloheximide + actinomycin D ^{c,e}	10,240	20,480

^a HEK cell cultures (21-day-old) were stimulated by incubating for 2 hr with 100 µg of poly I·poly C/ml. During the period of interferon production, all cultures were incubated with 2 ml of medium 199 containing 2% gamma globulin-free calf serum.

^b Incubated with 100 units of human interferon/2 ml as in Table 7.

^c Treatment with cycloheximide (50 µg/ml) was between hr 2 and 6 in serum-free medium 199.

^d Actinomycin D (1 µg/ml) was added 30 min prior to removal of cycloheximide.

^e Actinomycin D (5 µg/ml) was added 30 min prior to removal of cycloheximide.

recently obtained in cultures of rabbit kidney cells (17).

DISCUSSION

The results of this work corroborate and supplement the conclusions on the control of interferon synthesis made on the basis of studies in which cultures of rabbit kidney cells were used (11). The major characteristics of the poly I·poly C-stimulated interferon responses in rabbit kidney cells and FS-3 cells appear to be the same. Our own work and that of other investigators employing cultures of human diploid cells and poly I·poly C as the interferon inducer (3, 6, 8, 10) thus strongly suggest that fundamental aspects of the control of interferon induction and synthesis in cells of the two species are identical.

Some relatively minor differences were noted between the response of human diploid cells and that of rabbit kidney cells. One difference concerns the optimal conditions of priming with interferon. In contrast to the results in the human cells (Table 1), maximal priming in rabbit cells occurred after a period of incubation with interferon of only 8 hr. Furthermore, the priming effect in rabbit cells was relatively greater if suboptimal doses of poly I·poly C were used for subsequent induction (S. L. Barmak, Ph.D. thesis, New York

TABLE 9. *Effect of combined treatment with cycloheximide and actinomycin D on interferon production with various polynucleotide inducers*

Inducing agent ^a	Interferon yield/2 ml	
	Control ^b	Super-induced ^c
Poly I·poly C (100 µg/ml)...	256	≥ 10,240
Poly I·poly C ^d (100 µg/ml)...	512	10,240
Poly I·poly C (10 µg/ml) + DEAE-dextran (100 µg/ml).....	2,048	2,560
<i>P. chrysogenum</i> RNA (100 µg/ml).....	256	2,560
<i>P. chrysogenum</i> RNA (10 µg/ml).....	256	1,280

^a FS-3 cell cultures were incubated with 2 ml of the appropriate type and dose of inducer in PBS for 2 hr and thoroughly washed.

^b After exposure to inducer, cultures were incubated with 2 ml of MEM containing 2% gamma globulin-free calf serum. Culture fluids were harvested 24 hr after stimulation.

^c After exposure to inducer, cultures were incubated with 2 ml of MEM containing 50 µg of cycloheximide per ml for 4 hr. Actinomycin D (final concentration, 1 µg/ml) was added 30 min prior to removal of cycloheximide. The cultures were then thoroughly washed and replenished with drug-free MEM containing 2% gamma globulin-free calf serum. Culture fluids were harvested 22 hr after stimulation.

^d In this group only, poly I·poly C was from a preparation purchased from P-L Biochemicals.

TABLE 10. *Effect of combined treatment with cycloheximide and actinomycin D on interferon production stimulated with various concentrations of the poly I·poly C-DEAE-dextran mixture*

Inducing agents (µg/ml) ^a		Interferon yield/2 ml	
Poly I·poly C	DEAE-dextran	Control ^b	Superinduced ^c
10	100	5,120	7,680
3	30	320	3,840
1	10	80	1,920
0.3	3	15	1,280
0.1	1	< 10	1,280

^a FS-3 cultures (12-day-old) were incubated with 2 ml of the appropriate concentration of the poly I·poly C-DEAE-dextran mixture in PBS for 1 hr and thoroughly washed.

^b As in Table 9, except that culture fluids were harvested 22 hr after stimulation.

^c As in Table 9, except that incubation with cycloheximide was for 5 hr after the 1-hr exposure to inducer.

University, New York, N.Y., 1972). As the mechanism of the priming phenomenon is still not completely understood (12), it is not possible to explain the underlying reasons for these differences between human and rabbit cells.

Another difference concerns the results of the experiment shown in Fig. 2. In a similar experiment in rabbit kidney cells, the rate of interferon production after treatment with cycloheximide and actinomycin D decreased at a slow, steady rate which was thought to reflect the rate of decay of interferon messenger RNA in these cells (18). In contrast, there was a rather abrupt decline of interferon production in FS-3 cells at about 6 hr after the removal of the drugs, suggesting either that (i) the treatment with actinomycin D was insufficient to prevent subsequent synthesis of the repressor which is thought to exert negative control over interferon synthesis (18), or (ii) that a factor required for interferon synthesis is the rate-limiting factor whose depletion results in the rapid cessation of interferon synthesis or release, or both. These points are now being further explored.

There also seems to be a difference in the quantity of interferon units obtainable from cells of the two species: after treatments similar to those described in this paper, rabbit kidney cells yielded up to more than 100,000 reference *rabbit* interferon units per ml in stationary cultures (11, 18). However, this discrepancy may not reflect a real difference in the actual amounts of interferon protein produced under comparable conditions by the two types of cells. The values of reference human and rabbit interferon standards to which interferon yields are related have been assigned rather arbitrarily (4). There are indications that the value of the reference rabbit interferon standard has been set close to the limit of the sensitivity of the usual assay methods for rabbit interferon, whereas the value assigned to the human standards has been chosen more conservatively. Thus, the real difference between the amounts of interferon obtainable by comparable means from human and rabbit cells may be smaller than it seems from a simple comparison of interferon titers.

The results of this work provide the means for producing high-titered human interferon in cultures of diploid cells. The fact that the conditions worked out in cultures derived from foreskin cells also proved suitable to produce high interferon yields in HEK cells suggests that the results of this work may be applicable to other diploid human cell strains. The potency of the best interferon preparations produced in FS-3 or embryonic kidney cells is comparable to that of preparations produced in human leukocyte sus-

pensions stimulated with Newcastle disease or Sendai viruses (5, 13, 15). On a per cell basis, the interferon yield from diploid cell cultures is actually about 10 times higher. The use of diploid cell strains has many obvious advantages over buffy coat leukocytes.

To produce interferon preparations with the high specific activity, it would be advantageous to omit serum from the culture fluid during the period of interferon production. The presence of serum was not required during the period of cycloheximide treatment, the time at which interferon messenger RNA transcription is known to occur (14, 19). On the other hand, the omission of serum during the period of interferon messenger RNA translation reduced the interferon yield. A similar conclusion was reached by Hadházy et al. (7) on the basis of their experiments with human leukocyte suspensions. However, preliminary experiments showed that calf serum in the production medium could be replaced with human plasma protein fraction, which might be employed for the preparation of material safe for clinical use.

After the submission of this paper for publication, we received a related manuscript by A. Billiau, M. Joniau, and P. DeSomer (*J. Gen. Virol.*, *in press*). The results of their study concerning the effects of priming with interferon and antimetabolite treatment on interferon production in human diploid cells are in good agreement with the results presented in this paper.

ACKNOWLEDGMENTS

We thank Fermina Varacalli and Juan Casanova for expert technical assistance.

This investigation was supported by Public Health Service contract NIH-NIAID-70-2169 and by a grant from the Irwin Strasburger Memorial Medical Foundation. J. V. is the recipient of Public Health Service career development award 5-K4-AI-38784.

LITERATURE CITED

1. Armstrong, J. A. 1970. Control of interferon production in rabbit cells treated with poly I:C. *J. Gen. Physiol.* 56(2): 97s-98s.
2. Armstrong, J. A. 1971. Semi-micro, dye-binding assay for rabbit interferon. *Appl. Microbiol.* 21:723-725.
3. Billiau, A., M. Joniau, and P. DeSomer. 1972. Production of crude human interferon with high specific activity. *Proc. Soc. Exp. Biol. Med.* 140:485-491.
4. Conference on Standardization of Interferon and Interferon Inducers. 1970. Recommendations of the Conference, p. 326-328. *In* F. T. Perkins and R. H. Regamey (ed.), Symposium Series on Immunobiological Standardization, vol. 14. Karger, Basel.
5. Falcoff, E., H. Lévy, J. Colin, and C. Chany. 1965. Estimation du poids moléculaire de l'interféron produit par les globules blancs humains. *C. R. Acad. Sci.* 260:5405-5407.
6. Finkelstein, M. S., G. H. Bausek, and T. C. Merigan. 1968. Interferon inducers *in vitro*: difference in sensitivity to inhibitors of RNA and protein synthesis. *Science* 161:465-466.
7. Hadházy, G. Y., H. Strander, and K. Cantell. 1969. Serum

- requirement for interferon production by suspended human leucocytes: studies on action of serum. *J. Gen. Virol.* 5:351–358.
8. Ho, M., Y. H. Tan, and J. A. Armstrong. 1972. Accentuation of production of human interferon by metabolic inhibitors. *Proc. Soc. Exp. Biol. Med.* 139:259–262.
9. Merigan, T. C., D. F. Gregory, and J. K. Petralli. 1966. Physical properties of human interferon prepared *in vitro* and *in vivo*. *Virology* 29:515–522.
10. Myers, M. W., and R. M. Friedman. 1971. Potentiation of human interferon production by superinduction. *J. Nat. Cancer Inst.* 47:757–764.
11. Ng, M. H., and J. Vilček. 1972. Interferons: physicochemical properties and control of cellular synthesis. *Advan. Protein Chem.* 26:173–241.
12. Stewart, W. E., L. B. Gosser, and R. Z. Lockart, Jr. 1971. Priming: a nonantiviral function of interferon. *J. Virol.* 7:792–801.
13. Strander, H., and K. Cantell. 1967. Further studies on the production of interferon by human leukocytes *in vitro*. *Ann. Med. Exp. Biol. Fenn.* 45:20–29.
14. Tan, Y. H., J. A. Armstrong, Y. H. Ke, and M. Ho. 1970. Regulation of cellular interferon production: enhancement by antimetabolites. *Proc. Nat. Acad. Sci. U.S.A.* 67:464–471.
15. Tovell, D., and K. Cantell. 1971. Kinetics of interferon production in human leukocyte suspensions. *J. Gen. Virol.* 13:485–489.
16. Vilček, J. 1970. Metabolic determinants of the induction of interferon by a synthetic double-stranded polynucleotide in rabbit kidney cells. *Ann. N.Y. Acad. Sci.* 173:390–403.
17. Vilček, J., S. L. Barmak, and E. A. Havell. 1972. Control of interferon synthesis: effect of diethylaminoethyl-dextran on induction by polyinosinic-polycytidylic acid. *J. Virol.* 10:614–621.
18. Vilček, J., and M. H. Ng. 1971. Post-transcriptional control of interferon synthesis. *J. Virol.* 7:588–594.
19. Vilček, J., T. G. Rossman, and F. Varacalli. 1969. Differential effects of actinomycin D and puromycin on the release of interferon induced by double stranded RNA. *Nature (London)* 223:682–683.
20. Wood, H. A., and Bozarth, R. F. 1972. Properties of viruslike particles of *Penicillium chrysogenum*: one double-stranded RNA molecule per particle. *Virology* 47:604–609.