Interferon Induction in Rabbit Cells Irradiated with UV Light

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UV irradiation of a continuous line of rabbit kidney cells (RK13) was used as a tool for the study of the mechanism of interferon induction. Irradiation of cells prior to their exposure to Newcastle disease virus (NDV) resulted in a dose-dependent decrease in interferon production. The inhibition of total cellular RNA synthesis by UV irradiation in uninduced cultures was similar to the inactivation curve of interferon production in NDV-induced cultures. In contrast, the production of interferon with polyinosinate-polycytidylate (poly(I)·poly(C)) paradoxically was enhanced in cells irradiated with a wide range of doses of UV. However, in cells stimulated with poly(I)·poly(C) and "superinduced" by the sequential addition of cycloheximide and actinomycin D, the rate of inactivation of interferon production by UV light was similar to that observed with NDV. These results are not inconsistent with the idea that both poly(I)·poly(C) and NDV stimulate the same interferon gene(s), but indicate that the mechanism controlling its expression may be different for each inducer.

The kinetics of interferon production by cultured animal cells depends upon the type of inducer used. In rabbit kidney cells polyinosinate-polycytidylate (poly(I)·poly(C))-induced interferon production rises rapidly, peaks at 3 to 4 h, and then falls off within a short period of time. In contrast, interferon production by Newcastle disease virus (NDV) is delayed in its initial appearance, but it continues to be produced for a much longer period of time (12, 13). Similar differences have been observed in some other types of cell cultures (2, 11).

We have attempted to elucidate some of the mechanisms underlying these differences through the use of UV irradiation of cells prior to induction. Interferon induction is likely to require the functioning of a cellular gene(s) coding for the interferon protein and, possibly, one or more regulatory genes. UV light, with DNA as its primary target, was chosen as a potentially useful tool for comparing the mechanisms of interferon induction.

It has been previously shown (3-6) that UV irradiation of cells prior to the addition of a viral inducer results in a dose-dependent inhibition of interferon production. We extended these studies to rabbit cells stimulated with NDV or poly(I)·poly(C). Marked differences have been observed in the effects of UV light on the cellular capacity to produce interferon after stimulation with the two inducers. The results suggested that the genetic control of interferon synthesis in rabbit cells stimulated with NDV or poly(I)·poly(C) is quite different.

MATERIALS AND METHODS

Cell cultures. A continuous line of rabbit kidney cells (RK13) was obtained from K. Paucker, Medical College of Pennsylvania, Philadelphia, Pa. Eagle minimal essential medium (MEM) containing antibiotics and supplemented with 10% heated fetal calf serum or 2% gamma globulin-free calf serum was employed as growth medium or maintenance medium, respectively. All experiments were performed in 4-day-old cultures grown at 36 C in 60-mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.) in a humidified incubator provided with 5% CO₂. Each petri dish was routinely seeded with 3.5×10^6 RK13 cells.

Cultures of rabbit embryo cells (REC) were prepared by trypsinization of 15- to 18-day-old rabbit embryos. The cultures, which were used in the assay of interferon, were seeded in growth medium.

Chicken embryo cells, which were employed in the assay of NDV, were obtained by trypsinization of 11-day-old embryos. These cells were grown in MEM containing 5% heated fetal calf serum.

All tissue culture media and sera were purchased from Grand Island Biological Co., Grand Island, N.Y.

Interferon induction. RK13 cells were washed once with phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM phosphate, 0.9 mM CaCl₂, and 0.5 mM MgCl₂·6H₂O; pH 7.4) and incubated at 36 C with the appropriate concentration of the inducer. Poly(I)·poly(C) (supplied by the Infectious Disease Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md.), diluted in MEM to give a final concentration of 300 µg of poly(I)·poly(C) per ml, was first incubated at 37 C for 30 min prior to use. NDV (Hickman strain, originally obtained from E. D. Kilbourne, Mt. Sinai School of Medicine, New York, N.Y.), propagated by intraallantoic inoculation of 11-day-old chicken embryos was diluted in PBS and

exposed to UV light (800 ERG/mm²). It was further diluted in MEM to give a final concentration of 4×10^6 PFU/ml (as calculated from the titer of unirradiated virus). RK13 cells were incubated with 1 ml of poly(I)·poly(C) or 0.5 ml of NDV for 60 min. Thereafter, the plates were washed four times with Earle balanced salt solution, and each culture was refed with 2 ml of maintenance medium.

All NDV-induced interferon preparations were dialyzed against 0.2 M KCl-HCl buffer, pH 2.0, for 4 days to inactivate any residual live virus. The samples were next dialyzed, 1 day against PBS and 1 day with MEM. The samples were then centrifuged for 1 h at $100,000 \times g$ (50,000 rpm in a Spinco 65 angle head rotor) to pellet the inactivated virus. The supernatants were used for the titration of interferon.

Conditions of irradiation. A G.E. G15T8 germicidal fluorescent lamp was used for all irradiations. Its calibration by an Eppley Thermopile indicated that it gave a dose rate of 32.3 ERG per mm² per sec at a target distance of 30 cm. The uncovered RK13 plates were irradiated under 2 ml of PBS.

Interferon titrations. A modification of the semi-micro method described by Armstrong (1) was employed. The basic procedure was described in detail previously (14). However, instead of using rabbit kidney cells in the assay system, we employed REC in the same concentration of 40,000 cells per well.

[³H]uridine incorporation. Four-day-old cultures were washed once with warm PBS and irradiated. Maintenance medium (2 ml) was then added, and the plates were reincubated at 36 C. At the indicated time, 20 μ Ci of [³H]uridine (5 mCi/5 ml, 28.5 Ci/mM, lot no. 642-277, New England Nuclear Corp., Boston, Mass.) was added to each plate. Incorporation was stopped by rapidly washing the cells with cold PBS. The cells were dissolved in 2 ml of NaAc-sodium dodecyl sulfate (SDS) buffer (0.01 M NaAc, 0.01 M EDTA, 0.5% SDS, pH 4.8). The protein content of the resulting lysate was determined by using the Lowry procedure (9) on a portion of the lysate. The remainder of the lysate was made to 10% with cold trichloroacetic acid. The precipitates were collected on membrane filters (Millipore Corp., Bedford, Mass.) and washed three times with 10 ml each of 5% cold trichloroacetic acid. Trichloroacetic acid-insoluble radioactivity was measured in a liquid scintillation counter. Specific activities are expressed as counts per minute per milligram of protein.

Chemicals. Cycloheximide (Acti-dione) was obtained from the Upjohn Co., Kalamazoo, Mich., actinomycin D was purchased from Calbiochem, Los Angeles, Calif., and puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

RESULTS

The kinetics of appearance of interferon in unirradiated RK13 cells. The kinetics of appearance of interferon in RK13 cells stimulated with poly(I)·poly(C) or NDV are shown in Fig. 1. NDV-induced interferon production rose gradually and continued for a relatively long period of time, reaching its maximum between

FIG. 1. Kinetics of appearance of interferon in unirradiated RK13 cells stimulated with poly(I)·poly(C) or NDV. Cultures were induced with poly(I)·poly(C) (●) or NDV (○) as described in Materials and Methods. At the indicated intervals, fluids were collected, the cultures were washed once with Earle balanced salt solution and immediately replenished with 36 C, warm, fresh maintenance medium. Interferon titers were determined in the pooled fluids from individual groups. If the interval was more than 1 h, the determined interferon yield was divided by the number of hours that had elapsed from the collection of the preceding sample.

about 10 to 25 h. In contrast, interferon production was rapid after stimulation with poly(I)·poly(C), peaking at 3 to 4 h and then decreasing to undetectable levels by 10 h.

Effects of UV irradiation on cellular RNA synthesis. Uninduced cells were labeled with [³H]uridine either immediately after exposure to UV or 5 h after irradiation. The effect on RNA synthesis was the same at both time intervals, suggesting that no repair of UV damage occurred within 5 h (Fig. 2).

Effect of UV irradiation on NDV-induced interferon production. Irradiation of cells immediately prior to induction with NDV resulted in a dose-dependent decrease in the amount of interferon produced (Fig. 3). Similar findings have been reported in other types of cell cultures (3-6).

It was important to determine whether irradi-

FIG. 2. Effect of UV irradiation on RNA synthesis in uninduced RK13 cells. Cultures were irradiated with the indicated doses of UV light. Each plate then received 2 ml of maintenance medium. Immediately after exposure to the UV light, half of the cultures were incubated with [3 H]uridine for 30 min. (●), whereas the remainder of the cultures was incubated for 5 h before the labeling with [3 H]uridine (O).

ation was directly affecting the cellular capacity for the production of interferon or whether it was perhaps acting on a viral function necessary for interferon induction. To rule out a direct effect on some virus function, NDV replication was compared in unirradiated and irradiated RK13 cells. Virus multiplication was not suppressed in cultures exposed to doses of UV which either greatly reduced or totally eliminated interferon production (Table 1). Actually, virus multiplication appears to have been facilitated in cultures irradiated with 323 ERG/mm².

This observation is consistent with earlier reports (10).

Effect of UV irradiation on poly(I)·poly(C)-induced interferon. Strikingly different results were obtained when the cells were irradiated immediately prior to induction with poly(I)·poly(C) (Fig. 4). Interferon production in the first 5 h after induction increased in cells irradiated with up to 300 ERG/mm² and then decreased gradually with higher doses of UV.

FIG. 3. Effect of UV irradiation on NDV-induced interferon production. Cultures were exposed to NDV for 1 h and then were replenished with 2 ml of maintenance medium. At 5 h the culture fluids were collected, the cultures were washed once with Earle balanced salt solution and replenished with fresh medium. The culture fluids were again collected at 30 h. Either with or without irradiation, there was no detectable interferon produced during the first 5 h of incubation.

TABLE 1. NDV replication in UV-irradiated RK13 cells^a

Dose of UV (ERG/mm ²)	Yield of NDV (PFU/ml) at hour after inoculation						Interferon yield at 30 h ^b
	1	2	4.5	16	25	52	
0	1.5×10^3	7.1×10^3	2.5×10^4	4.0×10^6	2.9×10^6	2.5×10^6	7,710
323	3.1×10^3	6.3×10^3	2.2×10^4	3.8×10^6	1.0×10^7	1.2×10^6	1,926
1615	2.8×10^3	1.0×10^4	2.7×10^4	2.6×10^6	2.0×10^6	2.2×10^6	96

^a The cultures were irradiated with the indicated dose of UV immediately prior to inoculation with 0.5 ml of a suspension of live Newcastle disease virus (NDV) (100 PFU/cell). The virus was adsorbed at 36 C for 1 h, the cultures were washed four times with warm Earle balanced salt solution and replenished with 2 ml of maintenance medium. Culture fluids for virus assays were collected at the indicated times, and the cultures were washed and refed with fresh medium. Virus yields were assayed on 48-h chicken embryo cell cultures.

^b These values are from a separate experiment shown in Fig. 3.

FIG. 4. Effect of UV irradiation on poly(I)·poly(C)-induced interferon production. Cultures were induced with poly(I)·poly(C). At 5 h after the exposure to inducer, the culture fluids were collected, and the cultures were washed once with Earle balanced salt solution and replenished with fresh medium. The culture fluids were again collected at 28 h. Interferon yields were determined separately in the fluids collected at 5 (●) and 28 h (○).

Unirradiated cultures produced only a small amount of interferon between 5 and 28 h after exposure to the inducer. However, UV irradiation afforded a dramatic increase in interferon production between 5 and 28 h after stimulation. The greatest enhancement of this "late" interferon production (approximately 300-fold) occurred in cells exposed to about 800 ERG/mm². As a result, the total amount of interferon produced in irradiated RK13 cells was also greatly increased over a rather wide range of UV doses.

To determine whether this increase was due to newly synthesized interferon, puromycin was added to cultures during the induction period with poly(I)·poly(C) and during the entire subsequent incubation. At the concentration employed in the experiment (50 µg/ml), puromycin inhibited interferon production in both irradiated and unirradiated cultures (Table 2).

Effect of UV on poly(I)·poly(C)-stimulated interferon production in "superinduced" cells. It has been shown that judicious treatment of rabbit kidney cells with metabolic inhibitors resulted in a marked enhancement of interferon production (12, 13, 16). Poly(I)·poly(C)-stimulated interferon production in RK13

cells could also be greatly "superinduced" by sequential treatment with cycloheximide and actinomycin D.

After 1 h of incubation with poly(I)·poly(C), RK13 cells were washed and replenished with medium containing cycloheximide. After an additional 2 h of incubation, actinomycin D was added to each plate. Thirty minutes later the cells were washed and reincubated with drug-free maintenance medium. The dose response of such superinduced cells to the UV light is shown in Table 3. If irradiation was performed imme-

TABLE 2. The effect of puromycin on poly(I)·poly(C)-induced interferon production in irradiated cultures^a

Dose of UV (ERG/mm²)	Puromycin (µg/ml)	Interferon yield	
		5 h	29 h
0	0	605	<35
	50	<30	<30
872	0	4,260	4,865
	50	<30	<30

^a Irradiated and control unirradiated cultures were exposed to poly(I)·poly(C) in the presence or absence of puromycin. The inducer was removed from the cultures after 1 h as described in Materials and Methods. Puromycin was re-added to the appropriate cultures with the maintenance medium and kept in the cultures throughout the duration of the experiment. The procedure for determining 5-h and 29-h interferon yields was as described in Fig. 4. All culture fluids were dialyzed prior to the interferon assays.

TABLE 3. Effect of UV irradiation on "superinduced" poly(I)·poly(C)-stimulated interferon production^a

Dose of UV (ERG/mm²)	Interferon yield from cultures irradiated	
	Before induction	3.5 h after induction
0	173,500	173,500
161	35,300	144,800
323	11,900	93,000
485	6,000	173,500
808	2,340	115,800
1,615	555	62,500

^a All cultures were exposed to poly(I)·poly(C) for 1 h, washed, and replenished with maintenance medium containing 25 µg of cycloheximide per ml. Actinomycin D (5 µg/ml, final concentration) was added to all cultures 3 h after the beginning of exposure to poly(I)·poly(C). All cultures were washed at 3.5 h and replenished with 2 ml of drug-free maintenance medium. Interferon yields were assayed from culture fluids collected 24 h after exposure to inducer.

diately prior to the addition of poly(I)·poly(C), the interferon titer decreased linearly with increasing doses of UV. However, if the irradiation of the superinduced cells was not performed until after the treatment with actinomycin D, then the yield of interferon remained essentially unaltered, except in cells irradiated with the highest dose of UV employed.

A graphical comparison of the effects of UV irradiation of cells prior to induction on the interferon yields from NDV-induced cells or superinduced poly(I)·poly(C)-stimulated cells is shown in Fig. 5. The curves show basically the same linear inactivation of interferon production by the UV light, with the superinduced cells being even somewhat more sensitive to the irradiation.

DISCUSSION

UV irradiation of RK13 cells produced a dose-dependent decrease in NDV-induced interferon production. Similar observations of the decrease in viral-induced interferon production after irradiation of the cells and prior to induction were reported by others (3-6). The doses of irradiation used did not have an adverse effect on the ability of the RK13 cells to support the replication of live NDV, suggesting that the

effect of the irradiation was on the cellular capacity to produce interferon per se and not upon a function of the inducing virus.

The inhibition of RNA synthesis by UV irradiation of cells not exposed to an interferon inducer, as shown in Fig. 2, was similar to the UV inactivation curve of interferon production in NDV-induced cells. Thus the decrease of interferon production in NDV-induced cells after UV irradiation is likely to be the direct result of the effect of irradiation on the expression of the interferon gene.

A strikingly different effect of the UV light was observed when poly(I)·poly(C) was used as the inducer. In place of the decrease, there was a marked stimulation in the amount of interferon produced in cultures irradiated with a wide range of UV doses. The bulk of this increase was due to the enhancement in the production of interferon appearing between 5 and 28 h, i.e., at a time when interferon production in unirradiated cultures had virtually ceased. Yet, interferon produced in irradiated cultures was likely to be newly synthesized because the inclusion of puromycin suppressed interferon production in UV-treated cultures.

Poly(I)·poly(C)-induced and viral-induced interferon production were previously shown to exhibit differences in sensitivities to inhibitors of RNA and protein synthesis, with viral-induced interferon production being generally more susceptible to the action of metabolic inhibitors than interferon production stimulated with poly(I)·poly(C) (7, 8).

The key to the interpretation of the observed action of UV irradiation on the poly(I)·poly(C)-induced interferon response may be found in the results obtained with "superinduced" cells. It has been postulated that the inclusion of cycloheximide in the culture medium during the initial hours of the exposure of rabbit kidney cell cultures to poly(I)·poly(C) leads to the stabilization of interferon mRNA (15). Presumably, in the absence of cycloheximide (or of another inhibitor of protein synthesis) translation of the interferon mRNA, synthesized as a result of the action of poly(I)·poly(C), is prematurely terminated by a repressor protein. The inclusion of cycloheximide is thought to prevent repressor synthesis while allowing the synthesis and accumulation of interferon mRNA. The latter can be efficiently translated after the reversal of cycloheximide action. Treatment with actinomycin D prior to the removal of cycloheximide action serves to prevent repressor synthesis after the reversal of the inhibition of protein synthesis (12, 16).

Under optimal conditions, "superinduction" can result in about a 100-fold increase in inter-

FIG. 5. Effect of UV irradiation on the interferon yields from NDV-induced cells (○) or "superinduced" poly(I)·poly(C)-stimulated cells (●). The data shown in the graph were derived from the results shown in Fig. 3 and Table 3.

feron yields over those produced in cells exposed to poly(I)·poly(C) in the absence of metabolic inhibitors, suggesting that only a fraction of the interferon-producing potential of cells is expressed after exposure to poly(I)·poly(C). It seems that UV irradiation of cells prior to exposure to poly(I)·poly(C) also results in an effect akin to superinduction. This effect of UV light could be due to the preferential inhibition of repressor synthesis over interferon synthesis. One expected result of such action would be the absence of the rapid shut-off of interferon production around 5 h after induction, which is thought to be the result of repressor action (13). Indeed, irradiated cells continued to produce large amounts of interferon between 5 and 28 h after exposure to inducer (Fig. 4).

The idea that UV irradiation of cells prior to induction with poly(I)·poly(C) leads to a preferential inhibition of repressor synthesis also finds support in the observed effects of UV light on interferon production in cultures sequentially treated with cycloheximide and actinomycin D. Under these conditions repressor synthesis is thought to be eliminated, and the cellular potential to produce interferon may be fully expressed. Therefore, the effect of UV irradiation on interferon production in superinduced cells should reflect more truly the action of UV light on the expression of the interferon gene(s) alone. This prediction was borne out: in superinduced cells interferon production decreased linearly with the dose of UV applied (Table 3 and Fig. 5).

These results are not inconsistent with the idea that both poly(I)·poly(C) and NDV stimulate the same interferon gene(s), but that the mechanisms controlling its expression may be different for each inducer. It is plausible that whereas poly(I)·poly(C)-induced interferon production is under negative control of the translational repressor, this repressor is either completely absent or it plays a much less important role in virus-induced interferon synthesis.

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