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Kinetics of the Rapid Action of Interferon (39447)

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It was recently found that treatment of cells (under physiological conditions) with interferon for only a few minutes renders them resistant to virus challenge (presumably due to the "antiviral protein"). Thus, this defense mechanism can act more rapidly than does the spread of virus in infected tissues. The purposes of the present study were to examine (a) some of the variables which lead to early-appearing resistance in human cell cultures and (b) some molecular events during this rapid induction of resistance.

Materials and methods. Human leukocyte interferon and human fibroblast interferon were obtained from the Antiviral Substances Program, NIAID, and were produced by methods previously described (3, 7). The interferon-induced antiviral activity was measured by the inhibition of the yield of Sindbis virus hemagglutinin in a single-cycle yield assay (12). Interferon titers are given as reference units per milliliter. Unless otherwise specified, experiments were conducted in tube cultures of human foreskin fibroblasts incubated in a 37° water bath. Culture medium and diluent was Eagle's medium containing 2% fetal bovine serum and antibiotics. At the end of each incubation period, the interferon was removed and the cultures washed four times with Earle's balanced salt solution before challenge with Sindbis virus to determine antiviral activity. All media and wash fluids were prewarmed to 37° and kept warm throughout the experiment.

Results. Effect of temperature on the development and decay of the antiviral state. In the first series of experiments, interferon at room temperature was applied to the human foreskin fibroblast cultures (strain HFS-1). After transfer to the incubator it required 40 to 60 min for the temperature to rise to 37° as determined by an electronic thermometer. As shown in Fig. 1A, application of 5 units/ml of interferon before placing the tubes in the incubator resulted in the initiation of viral resistance between 2 and 4 hr, with the resistance becoming maximum at 6 hr. Thereafter, resistance remained relatively stable as long as the interferon remained in the extracellular fluid (1). Figure 1B shows the effect on established antiviral activity of removal of interferon as compared with its retention. Removal of interferon was preceded by an overnight incubation with interferon to allow the prior development of full antiviral activity. It may be seen that the level of antiviral activity dropped drastically between 10 and 15.5 hr after removal of interferon. In comparison, the replacement of interferon resulted in maintenance of antiviral activity although the level of resistance manifested moderate fluctuation which is characteristic of this system (1; Buckler, C. E., and Baron, S., personal communication, 1970). In most strains of human foreskin cells (including the HR203 strain which was used in the rapid action experiments below) maximum resistance was reached at 6 hr using 3 to 10 units/ml of interferon. However, under the same conditions other human fibroblasts developed maximum resistance at 17 to 20 hr, i.e., the MA 308 human nasal polyp cells (Microbiological Associates) and the continuous line, human U cells (from Dr. K. Cantell).

Similar types of experiments were performed again in human foreskin fibroblast cultures (strain HR203) but with careful temperature maintenance at 37° throughout the experiment. Figure 2 shows the development and decay of antiviral activity after exposure of these cells to 10, 30, or 300 units of human interferon for 5 min before

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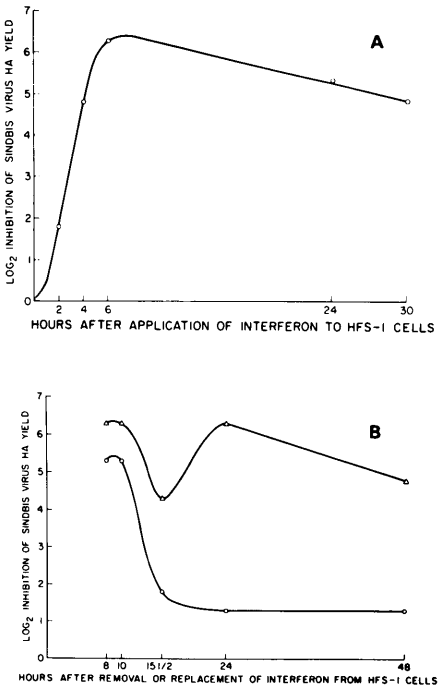

FIG. 1. Development (A) and decay (B) of the antiviral state in human foreskin cultures treated with 5 units/ml of interferon. The interferon was applied to cultures at room temperature before immediate incubation at 37°. In (B) interferon was removed and replaced with medium (O——O) or removed and replaced with the same concentration of interferon (Δ——Δ).

removal and washing. It may be seen that substantial resistance to virus challenge was present at the time interferon was removed (5 min) and that the degree of resistance continued to rise so that between 1 and 8 hr after reaction with interferon resistance was above the measurable level.

In the cultures treated with 10 units of interferon, decrease of resistance was measurable between 8 and 12 hr after removal of interferon and resistance was no longer detectable by the 16th hr. In the cultures treated with 30 or 300 units/ml of interferon, the onset of decreased resistance was delayed until 12 hr after removal of interferon and resistance was similarly delayed.

These findings extend the observation that rapid induction of resistance is influenced by the temperature of incubation and the concentration of interferon (5). Specifically, application of 10 or more units/ml of human interferon to human cells, held constantly at 37°, causes rapid development of resistance to virus challenge. The effects of high concentrations of interferon at temperatures below 37° are being studied. The decay kinetics of the established resistance appear to be approximately the same whether the resistance was induced under conditions favoring rapid or slow development (compare Fig. 1B to Fig. 2).

For the experiments below, treatment with 30–300 units/ml of interferon for 1 to 30 min, at 37°, was considered to represent conditions for rapid induction. The level of resistance which develops between 1 and 30 min of treatment is essentially the same (5).

Effect of prior chilling of cells on subsequent development of resistance. It seemed possible that application of cold or room temperature medium to cells might derange cellular metabolism for a prolonged time after rewarming of the cells. This could account for the slower development of resistance by cells exposed to interferon initially at room temperature and subsequently incubated (Fig. 1A) as compared with continuous incubation (Fig. 2). Therefore, development of resistance in cells previously chilled and then warmed was compared to that of unchilled cells. The human cell cultures were initially exposed to ice bath temperatures for 30 min, then warmed to 37° in a waterbath and exposed to 100 units/ml of interferon for 4 min before Sindbis virus

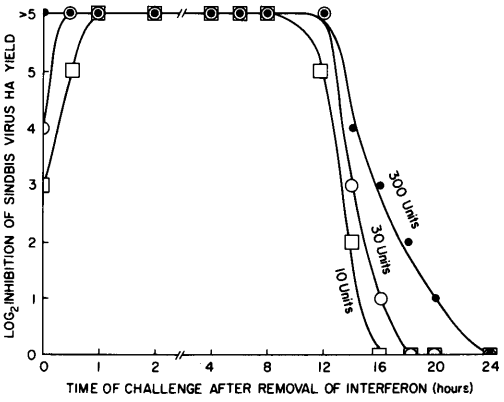

FIG. 2. Development and decay of the antiviral state in human foreskin cultures treated with 10, 30, or 300 units/ml of interferon for 5 min. Interferon, media, and cultures were maintained at a constant 37°.

challenge. The level of resistance (5 $\log_2$ inhibition of yield) induced under this condition of prior chilling was equal to that of control cultures constantly maintained at 37°. This finding mitigates against the possibility that chilling of cells results in prolonged impairment of the ability of cells to respond to interferon.

Possible priming action of the applied interferon. It is possible that the interferon applied for a brief time primes the cells so that the challenge virus induces a more rapid and more effective interferon response (priming) which then inhibits further viral replication (8). To test this possibility, resistance induced by challenge virus was blocked by actinomycin D. Specifically, the cell cultures at 37° were exposed to 300 units/ml of interferon for 20 min, washed four times, incubated at 37° for 1 or 2 hr, and then challenged with Sindbis virus in the presence or absence of 10 μg/ml of actinomycin D. This dose of actinomycin D inhibited 95% of cellular RNA synthesis within 10 min without affecting virus synthesis. As shown in Table I, actinomycin D (which was shown to prevent completely the induction of interferon or the antiviral resistance by challenge virus) did not significantly change the degree of resistance as compared with cultures challenged in the absence of actinomycin D. This finding indicates that the brief exposure of cells to interferon did not function to prime the cells to subsequent production of the antiviral state by the challenge virus.

Kinetics of the actinomycin D effect. The

TABLE I. DEVELOPMENT OF RESISTANCE AFTER 20-MIN EXPOSURE TO INTERFERON UNDER CONDITIONS PREVENTING INTERFERON INDUCTION BY CHALLENGE VIRUS.

Time of challenge after removal of interferon (min)	Actinomycin D present during viral adsorption	Yield of Sindbis virus HA ($\log_2$)
Not treated with interferon	No	5
Not treated with interferon	Yes	5
0	No	1
60	No	<1
60	Yes	1
120	No	<1
120	Yes	<1

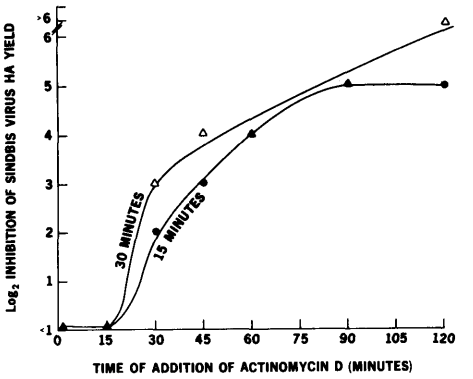

FIG. 3. Actinomycin D timing of the production of the mRNA for the antiviral protein. Cultures were treated with 300 units/ml of interferon at 37° for 15 (○——○) or 30 (△——△) min before washing and incubation for the indicated times before applying 10 μg of actinomycin D and challenging with virus 2 hr later.

inhibition of viral growth in cultures challenged immediately after a 5-min exposure to interferon does not necessarily mean that the factors responsible for resistance (antiviral protein) are produced by 5 min. Resistance probably develops prior to 4 hr after exposure to interferon since (a) to inhibit virus, resistance must occur before virus replication is complete (6–7) hr for many viruses) and (b) interferon may be effective when applied as late as 2.5 hr after infection (10, 14). To establish this time more closely, the time of production of the mRNA for the resistance factors was determined since it was shown previously that the mRNA is rapidly translated (4). Sets of cultures were treated with 300 units/ml of interferon for 15 or 30 min at a constant 37°. After washing with warm medium, cultures were incubated for varying times before applying 10 μg/ml of actinomycin D. The cultures were then incubated for an additional 2 hr before virus challenge so that any delayed translation of mRNA would have been completed. Since this dose of actinomycin D rapidly and irreversibly inhibits 95% of mRNA production, the development of resistance plotted against the time of application of actinomycin D approximates the kinetics of production of the mRNA for the antiviral protein. It may be seen in Fig. 3 that the addition of actinomycin D, up to 15 min after removal of inter-

TABLE II. EFFECT OF INHIBITON OF PROTEIN SYNTHESIS ON INDUCTION OF THE mRNA FOR THE ANTIVIRAL PROTEIN.

Treatment before addition of actinomycin D	Yield of sindbis virus HA (log_2)	Reduction of sindbis virus HA (log_2)
None	6	—[a]
Cycloheximide	6	0
Interferon	1	5
Interferon and cycloheximide	1	5

[a] Not applicable.

feron, completely inhibited the development of the antiviral state. When actinomycin D was added between 15 min and 2 hr after removal of interferon, there was a gradual development of resistance. Since the interferon had been applied initially for 15 to 30 min and since production of mRNA did not begin for an additional 15 to 30 min, it may be concluded that the first production of the mRNA began between 30 and 45 min after the application of interferon. This requirement for RNA synthesis suggests that the antiviral protein did not exist in a masked form prior to the application of interferon but rather was newly, as well as rapidly synthesized.

Lack of requirement of protein synthesis for transcription of the mRNA for the antiviral protein. It was previously demonstrated (under conditions of slower induction of the antiviral state) that the mRNA for the antiviral protein was directly induced by interferon and did not require the production of a new protein for this derepression (4). This demonstration was based on the production of the mRNA in the absence of protein synthesis and its subsequent translation into the antiviral protein in the presence of actinomycin D. To help determine whether rapid induction of the mRNA for the antiviral protein also did not require the prior induction of a new protein, the cells were treated with 100 units/ml of interferon for 2 hr in the presence or absence of an inhibitor of protein synthesis, cycloheximide (10 μg/ml). Protein synthesis was inhibited by 92%. Cycloheximide inhibition was then reversed by washing the cultures with warm medium, and 10 μg/ml of actinomycin D was added before virus challenge. Since mRNA for the antiviral protein may be produced in the presence of cycloheximide but not later in the presence of actinomycin D, any development of resistance (antiviral protein) would indicate direct induction of the mRNA for the antiviral protein without a requirement for a new, intermediary protein. As shown in Table II, full resistance does develop in the presence of the inhibitor treatment, confirming the lack of requirement for a new, intermediary protein.

Discussion. The results presented here indicate that the rapid development of resistance following interferon treatment was not observed previously (1) for two reasons. First, holding interferon-treated cultures at 4° or at room temperature delays up to 60 min the time at which cell cultures reach incubator temperature for normal metabolic activity. Second, the earlier experiments were performed with levels of interferon below those required for rapid induction. The present findings extend the observation that 10 or more units/ml of interferon rapidly induce resistance. Thus, under conditions of temperature and interferon concentration which mimic those *in vivo* (5), the antiviral action of interferon is extremely rapid.

The kinetics and the molecular events involved in rapid induction of resistance appear to be qualitatively similar to those occurring during slow induction of resistance but the rate is increased. Thus, after brief exposure to interferon, the mRNA for the antiviral protein first appears between 30 and 45 min. If translation of the human mRNA is as rapid as in the mouse system, then the antiviral protein probably is produced shortly thereafter (4). It is noteworthy that, although substantial resistance occurs after very brief exposure to interferon, maximum antiviral activity does not occur until 2 to 4 hr (5).

Studies are underway to help determine whether rapid development of resistance results from the previously observed, cell-bound interferon (2, 6, 9, 11, 13).

The present experimental conditions provide a time period during genetic derepression when the inducer may no longer be present and the induction has not yet oc-

curred. This possibility is being tested. Study of the cellular events between inducer reaction with the cell and the derepression of the cistron might be possible in this system.

Summary. The present study was undertaken to examine some factors which lead to early-appearing resistance in human cells treated with interferon. It was previously shown that two conditions required for rapid development of resistance were continuous maintenance of cultures at 37° and use of more than 10 units/ml of interferon. The decay kinetics of the established resistance appear to be approximately the same whether the resistance was induced under conditions favoring rapid or slow development. With the use of actinomycin D it was shown that the mRNA for the antiviral protein is produced between 30 and 45 min after the first contact with interferon. Ruled out were the possibilities that a priming action of interferon and a newly synthesized intermediary protein were necessary for rapid development of resistance.

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