PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 112-115 (1976)

Maintenance and Recovery of the Interferon-Induced Antiviral State (39491)

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The paradoxical increase in interferon production following treatment with metabolic inhibitors has led to the hypothesis that a regulatory protein governs the cellular induction of interferon (1-3). There are, however, few reports of similar observations of the effects of inhibitors on the antiviral activity induced by interferon. Potentiation of the antiviral state (AVS) by actinomycin D has been described in L cells (4) and in chick embryo fibroblasts (CEF) (5). Our present experiments describe the effect of cycloheximide treatment on CEF during the decay of the antiviral state following removal of interferon. The level of AVS in chick embryo fibroblasts is indeed dependent upon the presence of interferon in the culture medium. After removal of interferon, however, inhibition of cell protein synthesis by cycloheximide prevents the decay of AVS. Furthermore, after the AVS induced by interferon in CEF has decreased, treatment of cells with cycloheximide leads to the recovery of apparently lost antiviral activity.

Material and methods. Cells and culture media. Primary chicken embryo cells were prepared by trypsination of 10- or 11-day-old embryos, seeded in 60-mm plastic Petri dishes ($5 \cdot 10^6$ cells), and incubated overnight at 36° in a humidified atmosphere with 5% CO_2. Eagle's minimum essential medium (MEM), supplemented with 10 or 2% heated fetal calf serum (FCS), was used as growth or maintenance medium, respectively.

Interferon preparations. Interferon was prepared in confluent "aged" monolayers of CEF (5 days) infected with Sindbis virus (0.1 PFU/cell). After infection, cells were incubated for 30 hr at 37° in serum-free PBS without antibiotics. Medium was then collected, centrifuged at 1000*g*, treated with 2 *N* HCL O_4 to a final pH of 2 (48 hr), and centrifuged for 30 min at 1000*g*; the pH of the supernatant was then adjusted to pH 7 using 5 *N* NaOH and this was finally centrifuged at 100,000*g* for 1 hr. Interferon preparations, as compared to chick standard interferon of the Medical Research Council, exhibited an antiviral activity of 3000 units ml^{-1}.

Highly purified chick interferon (300,000 units ml^{-1}), kindly supplied by Dr. Jungwirth (6), was used for a series of experiments.

Interferon assay. CEF cultures ($5 \cdot 10^6$ cells), in triplicate, were treated with 2 ml of interferon (300 units ml^{-1}) diluted in MEM 2% FCS for 6 or 24 hr according to the experiments. Interferon was removed by three washings with PBS, and cells were infected with Sindbis virus at a m.o.i. of 1 PFU per cell and then incubated for an additional 12 hr at 37°. Cell antiviral state (AVS) was determined by the log inhibition of virus yield 12 hr postinfection compared with untreated cultures. Viral titers were estimated by plaque titration in 60-mm plastic Petri dishes with agarose (0.5%) overlay.

Metabolic inhibitors. Actinomycin D was purchased from the Sigma Chemical Company, St. Louis, Missouri, and cycloheximide was purchased from Calbiochem, Los Angeles, California. Preliminary experiments (unpublished results) have shown that (1) actinomycin D (1 $\mu g\ ml^{-1}$) inhibited by more than 90% RNA synthesis in the CEF and blocked completely the induction of antiviral effect of interferon; (2) cycloheximide (10 $\mu g\ ml^{-1}$) inhibited 90% of the protein synthesis in the CEF; and (3) this inhibition was reversible after three washings of the cells with PBS, whether or not the cells were pretreated with interferon.

Results. Prevention of cellular loss of AVS in CEF by cycloheximide. As shown in Table I, the constant presence of interferon in the extracellular fluid was necessary for the maintenance of the AVS in CEF. At the

TABLE 1. PREVENTION BY CYCLOHEXIMIDE OF CELLULAR LOSS OF THE ANTIVIRAL STATE IN CEF

Pretreatment for 6 hr	Treatment for 18 hr before wash and Sindbis challenge	Yield of Sindbis virus[a] (log)		Reduction of Sindbis virus yield (log)	
Expt. 1					
Medium	None	9.27	9.10[b]	–	–[b]
Interferon[c]	None	5.78	5.36[b]	3.49	3.74[b]
Medium	Medium	9.27	9.20[b]	–	–[b]
Interferon	Interferon	5.68	5.40[b]	3.59	3.80[b]
Interferon	Medium	7.67	7.30[b]	1.60	1.90[b]
Medium	Cyclo[d]	9.25	9.00[b]	–	–[b]
Interferon	Cyclo	5.59	5.24[b]	3.66	3.76[b]
Expt. 2					
Medium	None	9.64		–	
Interferon	None	5.00		4.64	
Medium	Medium	9.64		–	
Interferon	Interferon	5.48		4.16	
Interferon	Medium	7.60		2.04	
Medium	Cyclo	9.60		–	
Interferon	Cyclo	5.48		4.12	

[a] Yield of Sindbis virus (log PFU ml^{-1}) was estimated 12 hr postinfection, m.o.i. = 1 PFU/cell.
[b] Infection made in the presence of actinomycin D (1 $\mu g\ ml^{-1}$).
[c] Interferon, 300 units ml^{-1}, diluted in MEM 2% FCS. Purified interferon (6) was used in Expt. 2.
[d] Cycloheximide, 10 $\mu g\ ml^{-1}$.
Note. Each value is the mean of at least two separate experiments in triplicate.

concentration used (300 units ml^{-1}), interferon almost completely inhibited the replication of the challenge virus. The antiviral effect was attained after a 5- to 6-hr treatment. If interferon was kept in the medium for a further 18 hr, the AVS remained stable. When interferon was removed at 6 hr and AVS was measured at 24 hr, antiviral activity determined by the log reduction of virus yield decreased by 2 log as may be seen in Table I. Such a decay was, however, prevented by the addition of cycloheximide at 6 hr. These data are compatible with the possibility that at this critical moment cycloheximide blocked the formation of a cellular control protein responsible for the decay of the AVS. In order to prevent the possibility of residual induction of AVS during challenge infection, cells were infected in the presence of 1 $\mu g\ ml^{-1}$ of actinomycin D (Experiment 1b). It should be noted that this addition did not modify the results obtained.

Kinetics of decay of AVS and its restoration by cycloheximide treatment. In further experiments, we explore the effect of cycloheximide treatment during the period of decay of AVS. As shown in Fig. 1, about 5–6 hr after removal of interferon the AVS had rapidly decreased and had almost completely disappeared within 72–90 hr. Similar decay of the antiviral state has been observed in mouse embryo cells (7). Cycloheximide was added at 18, 32, 48, and 72 hr after interferon removal and maintained for 18 hr. Control cells without interferon were also treated in parallel. The inhibitor was then removed, and the cells were washed three times and then infected in the presence of actinomycin D (1 $\mu g\ ml^{-1}$). As shown in Fig. 1, after exposure to cycloheximide, the cells recovered full or partial antiviral activity depending on the addition time of the inhibitor. Blockage of protein synthesis at 18 hr resulted in full recovery of the initial level of AVS at 36 hr (3.50 log inhibition of Sindbis virus yield). At 36 and 48 hr, similar treatment resulted in the restoration 18 hr later of an antiviral activity which reduced virus yield by 2.40 log and 1.40 log. Treatment of cells by cycloheximide-increased interferon activity at 36, 54, and 66 hr resulted in a 1000-, 100-, and 10-fold increase, respectively, as compared to the levels of AVS remaining in cells not exposed to the inhibitor. No effect on the challenge virus yield was observed after reversal of cycloheximide in control cells exposed at parallel time intervals to the inhibitor. Thus, the possibility and efficiency of the rescue of

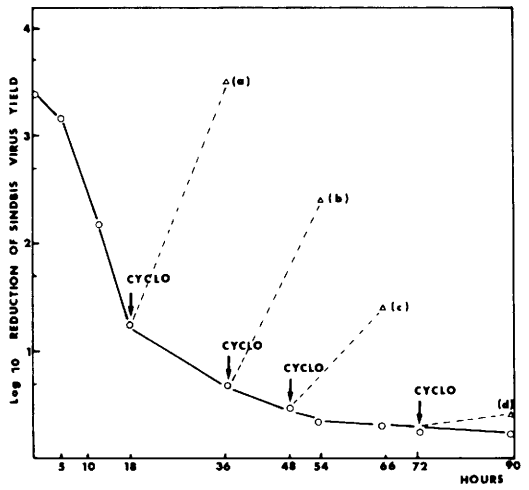

FIG. 1. Time course of decay of the antiviral state and recovery of AVS by cycloheximide in CEF. Induction of the AVS was performed by a 6-hr interferon pretreatment (300 units ml^{-1}). At 0 time, interferon was removed, and cells were washed with PBS and incubated in MEM 2% FCS. AVS was tested at intervals during a 90-hr observation period (○——○). Cycloheximide (10 μg ml^{-1}) was added for 18 hr at 18, 36, 48, and 72 hr. At 36, 54, 66, and 90 hr, respectively (dotted lines a, b, c, d) inhibitor was eliminated and cells were infected. Infection was made in the presence of actinomycin D (1 μg ml^{-1}); AVS is represented by log (PFU ml^{-1}) reduction of yield of Sindbis virus in a 12-hr growth cycle (m.o.i., 1 PFU/cell). Virus yields: in control CEF cells, $1.8 \cdot 10^9$ PFU ml^{-1}; in control CEF cycloheximide-treated cells, $1.5 \cdot 10^9$ PFU ml^{-1}. Each value is the mean of three experiments in triplicate.

the AVS seemed to depend on the degree of its loss in the interferon controls. When the AVS was completely lost (72–90 hr), treatment by cycloheximide no longer had any effect. Similar results were obtained when highly purified interferon (6) was used.

Effect of cycloheximide treatment on cells treated with increasing concentrations of interferon. The effect of cycloheximide treatment was explored in cells incubated with 7.5, 15, 30, 60, and 300 interferon units for 6 hr (Fig. 2). The antiviral effect was estimated immediately (0 hr), 18 hr, and 36 hr after the removal of interferon. A general decrease in interferon activity was observed at 18 and 36 hr. When cycloheximide was added from 0 to 18 hr, the antiviral effect of the various concentrations tested at 18 hr was maintained at the initial level. When cycloheximide was added from 18 to 36 hr after the removal of interferon, cells recovered the antiviral activities corresponding to those initially induced by the various interferon concentrations (0 hr). Thus, both the interferon concentration and the degree of the AVS remaining at a given time determine the level of AVS which can be recovered by an 18-hr exposure of the cells to cycloheximide.

Discussion. The data presented in Table I show that the antiviral state induced by chick interferon decays rapidly within 24 hr if interferon is removed after a 6-hr treatment; on the contrary, the AVS appears completely stable when interferon is kept in contact with the cells during the whole experiment. This observation indicates that interferon acts continuously on the cell to maintain an otherwise transient antiviral state. It is noteworthy that in CEF subjected to a 6-hr interferon pretreatment, antiviral activity can be maintained if, instead of in-

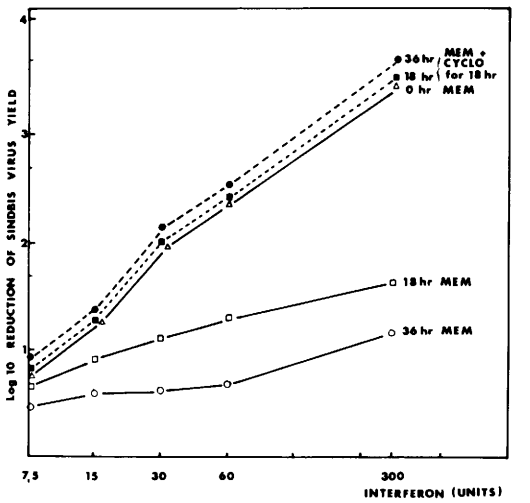

FIG. 2. Maintenance and recovery of the antiviral state by cycloheximide in CEF pretreated for 6 hr with increasing concentrations of interferon. At 0 time, interferon was removed, cells were washed with PBS and incubated in MEM 2% FCS, and AVS was tested (△——△). Subsequently, AVS was estimated at 18 hr in cells incubated in MEM in absence (□——□) or in presence of cycloheximide (10 μg ml^{-1}) from 0 time to 18 hr (■——■) and at 36 hr in cells incubated in MEM in absence (○——○) or in presence of cycloheximide from 18 to 36 hr (●——●). AVS is represented by log (PFU ml^{-1}) reduction of yield of Sindbis virus in a 12-hr growth cycle (m.o.i., 1 PFU/cell). Virus yields: in control CEF cells, $1.2 \cdot 10^9$ PFU ml^{-1}; in control cycloheximide-treated cells, $1.5 \cdot 10^9$ PFU ml^{-1}.

terferon, an inhibitor of protein synthesis such as cycloheximide is added to the cells. These results suggest that concomitant to the induction process of the antiviral state, a cellular control requiring integrity of protein synthesis promotes the degradation and/or inactivation of one of the presumed elements [antiviral protein(s) (AVP) or AVP mRna] which mediate the antiviral state.

As observed in Figs. 1 and 2, cycloheximide treatment, performed during the first 36 hr of the decay of the antiviral state, enables cells to regain their initial antiviral activity. After 36 hr the possibility of recovery following treatment by the inhibitor decreases gradually and disappears at 90 hr. This observation suggests at first that the cellular control protein we postulated here might be subjected to a rapid turnover and might reversibly inactivate one of the metabolic steps, resulting finally in the antiviral state and more likely (in view of our experimental conditions) the ultimate step, i.e., antiviral protein(s) (AVP). In this case, the AVP would not be immediately degraded but would first be converted into an inactive form: in the absence of cell protein synthesis, full reactivation could occur during the first 36 hr after removal of interferon. After 36 hr the AVP would be progressively degraded and the antiviral state only partially restored. Such a mechanism of regulation involving adjustment of the rate of degradation of enzyme activity has been proposed for induced tyrosine aminotransferase in rat liver *in vivo* (8).

Since the effect of cycloheximide must be reversed to permit viral multiplication, in order to test the antiviral state, new antiviral protein(s) may have been rapidly translated from mRna(s) which accumulated during the cycloheximide treatment. A similar mechanism may possibly account for the superinduction of interferon production following treatment by cycloheximide (3). Yet, it has been found that in chick cells treated by interferon, cycloheximide prevented the development of antiviral activity (9), presumably by allowing degradation of unstable mRna (10). The possibility of a new induction of mRna(s) for AVP(s) occurring after removal of cycloheximide, during challenge infection, could also be considered. This is unlikely in view of the results reported in Table I. Experiment 1b, and Fig. 1. In these experiments, indeed, maintenance and recovery of AVS are observed in cells where, after cycloheximide treatment, infection was made in the presence of actinomycin D (1 μg ml^{-1}) in order to block cellular RNA synthesis. Thus, although direct evidence is missing, in our system, cycloheximide might act more by preserving the activity of AVP than by increasing the amount of mRna available for its translation.

Summary. The antiviral state in CEF decays rapidly after the removal of interferon. Inhibition of cell protein synthesis by cycloheximide prevents this decay. Even after the decay of the antiviral state the addition of cycloheximide to the cells is followed by recovery of antiviral activity. The treatment by cycloheximide does not act by increasing the direct antiviral effect of various concentrations of interferon but rather by blocking the synthesis of a postulated regulatory protein which could reversibly inactivate the antiviral protein or its expression.

We thank Professors C. Chany and A. Kirn for constant interest and advice, Carol Girard for help in the preparation of the manuscript, and Suzanne Thouault for technical assistance. This study was supported by a grant from D.R.M.E.

1. Vilcek, J., Rossman, T. F., and Varacalli, F., Nature (London) **222,** 682 (1969).
2. Tan, Y. H., Armstrong, J. A., Ke, Y. H., and Ho, M., Proc. Natl. Acad. Sci. USA **67,** 464 9(1970).
3. Vilcek, J., and Havell, E. A., Proc. Natl. Acad. Sci. USA **70,** 3909 (1973).
4. Chany, C., Fournier, F., and Rousset, S., Nature New Biol. **230,** 113 (1971).
5. Lab, M., and Koehren, F., Ann. Inst. Pasteur **122,** 569 (1972).
6. Jungwirth, C., Horak, I., Bodo, G., Lindner, J., and Schultze, B., Virology **48,** 59 (1972).
7. Baron, S., Buckler, C. E., Levy, H. B., and Friedman, A. M., Proc. Soc. Exp. Biol. Med. **125,** 1320 (1967).
8. Grossman, A., and Boctor, A., Proc. Natl. Acad. Sci. USA **69,** 1161 (1972).
9. Field, A. K., Lampson, G. P., and Tytell, A. A., Life Sci. **6,** 1483 (1967).
10. Friedman, R. M., and Sonnabend, J. A., *in* "Symposia Series in Immunobiological Standardization" (F. T. Perkins and R. H. Regamey, eds.), Vol. 14, pp. 125–130. Karger, Basel (1970).

Received December 17, 1975. P.S.E.B.M. 1976, Vol. 153.