3. Hultgren, M. K., Druet, R. L., and Janigan, D. T., Am. J. Pathol. 50, 943 (1967).
4. Janigan, D. T. and Druet, R. L. Am. J. Pathol. 52, 381 (1968).
5. Cohen, A. S., New Engl. J. Med. 277, 522, 574, 628 (1967).
6. Cohen, A. S., Intern. Rev. Exptl. Pathol. 4, 159 (1965).
7. Teilum, G., J. Lab. Clin. Med. 43, 367 (1954).
8. Christensen, H. E. and Hjort, G. H., Acta Pathol. Microbiol. Scand. 47, 140 (1959).
9. Cohen, A. S. and Calkins, E., Nature 183, 1202 (1959).
10. Shirahama, T. and Cohen, A. S., J. Cell Biol. 33, 679, (1967).

Received Oct. 2, 1968. P.S.E.B.M., 1969, Vol. 130.

Effect of Cycloheximide on the Antiviral Action of Interferon (33595)

FERDINANDO DIANZANI, CHARLES E. BUCKLER, AND SAMUEL BARON

U.S. Department of Health, Education and Welfare, Public Health Service, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014

Evidence was provided that interferon (IF) is not directly responsible for the antiviral state in IF-treated cells but rather it acts by inducing the cells to produce one or more substances, perhaps protein(s) which are able to prevent viral growth directly or indirectly. Specifically, IF activity can be prevented by pretreating the cells with actinomycin D or with inhibitors of functional protein synthesis, i.e., puromycin or fluorophenylalanin (FPA) (1–4), suggesting that the development of the antiviral state involves synthesis of messenger ribonucleic acid (mRNA) and proteins. Although this interpretation comes from indirect evidence it will be used as a working model to be critically examined in view of the present findings.

We have used cycloheximide as an inhibitor of protein synthesis to prevent IF activity in mouse embryo (ME) cells. Unexpectedly the cultures treated with IF in the presence of cycloheximide showed substantially the same level of resistance as did the control cultures treated with IF alone. In the present study an attempt was made to explain this apparently anomalous result.

[1] Present address: Institute of Microbiology, University of Siena, Italy. Guest worker at NIH under a fellowship from Italian National Research Council, Group of Experimental Medicine.

Materials and Methods. Cycloheximide (Sigma Chemical Co.) was used at final concentration of 10 μg/ml. Actinomycin D (Merk Sharp Dohme) was used at final concentration of 5 μg/ml unless otherwise specified. DL-*p*-fluorophenylalanine (Mann Research Lab.) was used at final concentration of 80 μg/ml when the drug was dissolved in phenylalanine (PA)–free medium or 600 μg/ml unless specified otherwise, when medium containing 50 μg/ml of PA was used. This dose of FPA has been previously shown to inhibit 70% of the incorporation of phenylalanine^{14}C in the system (5). The reversibility of these inhibitors was confirmed by the full yield of vesicular stomatitis virus (VSV), strain Indiana, from cultures treated with cycloheximide or FPA for 5 hr and then washed free of inhibitor before VSV challenge.

Mouse IF was prepared and assayed as previously described (5), 50 or 100 units/ml were used.

Primary ME tissue cultures were prepared from 15-day-old embryos (NIH strain) using Eagle's minimum essential medium (MEM) supplemented with 10% of fetal bovine serum and antibiotics. During the experiments the concentration of serum was 2%. The level of antiviral resistance was expressed as inhibition of yield of VSV after a

TABLE I. Effect of Cycloheximide on Protein Synthesis and Multiplication of VSV in Mouse Embryo Cells.

Cycloheximide (μg/ml)	Phenylalanine-^{14}C (counts × 100)	Inhibition phenylalanine incorporation (%)	Inhibition VSV yield (%)
0	161	—	—
10	6	96.3	>99.9
100	2	98.8	>99.9

single growth cycle as previously described (5).

The effect of cycloheximide on protein synthesis has been studied by determining the rate of incorporation of PA-^{14}C (0.2 μCi/ml) into acid precipitable labeled material in ME incubated at 37° for 30 min in PA-free medium. Counting of radioactivity was performed in a Packard Tri-Carb scintillation counter.

Results. Inhibition of protein synthesis by cycloheximide. Two different concentrations of cycloheximide, 10 and 100 μg/ml, were added to triplicate samples of ME tissue cultures. After 1 hr at 37°, incorporation of PA-^{14}C was determined. Moreover, the effectiveness of cycloheximide on multiplication of virus was investigated in one set of tubes challenged with VSV in the continued presence of cycloheximide. The results (Table I) show that both concentrations of cycloheximide strongly inhibit protein synthesis and VSV growth.

Effect of cycloheximide and FPA on the development of interferon-induced antiviral activity. In Table 2 are reported the results of a representative experiment of the effect of treatment of ME cells with a mixture of IF and cycloheximide or FPA. The ME cells were treated with a mixture of IF and inhibitor for 5 hr and challenged with VSV (25 plaque-forming units/cell) after removal of inhibitor and IF. As shown in Table II development of resistance was prevented by FPA but resistance did develop in the presence of cycloheximide.

These results show that the development of resistance (antiviral protein) occurred under conditions of 96% inhibition of protein synthesis by cycloheximide. The possibility that the 4% residual protein synthesis during cycloheximide treatment was sufficient to support synthesis of substantial amounts of antiviral protein was tested by combined treatment of ME with interferon, cycloheximide, and FPA. Any antiviral protein synthesized under these conditions would be nonfunctional due to the 70% incorporation of FPA as evidenced in line 2 of Table II. As shown in the last 2 lines of Table II, resistance did develop when the cells were washed free of interferon, cycloheximide, and FPA and then challenged with VSV. This finding indicates that the antiviral protein was produced after removal of cycloheximide and interferon.

TABLE II. Effect of Combined FPA and Cycloheximide on Development of Interferon-Induced Antiviral Activity.

Treatment for 5 hr before wash and VSV challenge	Yield of VSV ($\log_{10}$)	Reduction of VSV yield ($\log_{10}$)	Reduction of VSV yield (%)
Medium[a]	6.4	—	—
Interferon	5.6	0.8	84
Interferon + FPA[b]	6.6	0.0	0
Interferon + cycloheximide[c]	5.4	1.0	90
Interferon + FPA + cycloheximide	5.6	0.8	84
Medium + FPA + cycloheximide	6.4	—	—

[a] Phenylalanine-free medium.
[b] FPA = 80 μg/ml in phenylalanine-free medium.
[c] Ten μg/ml.

TABLE III. Effect of Treatment of Mouse Embryo Cells with Interferon and Cycloheximide Followed by Replacement with Actinomycin D.

Treatment for 5 hr before addition of actinomycin D[a]	Yield of VSV (log_{10}) after wash, refeeding with actinomycin D, incubation for 3 hr and challenge with VSV	Reduction of VSV yield (log_{10})	Reduction of VSV yield (%)
Expt. 1			
None	6.2	—	—
Interferon[b]	3.9	2.3	99.5
Interferon + cycloheximide	4.2	1.8	98.4
Expt. 2			
None	6.3	—	—
Interferon	4.2	2.1	99.2
Interferon + cycloheximide	4.4	1.9	98.7
Expt. 3			
None	6.4	—	—
Interferon	4.2	2.2	99.4
Interferon + cycloheximide	4.7	1.7	98.0

[a] 0.2 ml of 5 μg/ml added to 1 ml volume in each tube for ½ hour before wash.
[b] 100 units/ml.

Development of resistance in ME treated with interferon and cycloheximide followed by replacement with actinomycin D. The antiviral protein which is produced by cells after removal of interferon and cycloheximide could result from (a) cell-bound interferon which is not removed by the washing procedure and which stimulates messenger RNA leading to antiviral protein, or (b) formation of the proposed mRNA in the presence of cycloheximide and interferon and rapid translation to antiviral protein after washing. To distinguish between these possibilities IF and cycloheximide were used to pretreat ME. Actinomycin D was added at the time of removal of interferon and cycloheximide, to prevent the possible formation of mRNA after removal of cycloheximide and interferon. Specifically, after 5 hr of pretreatment, 5 μg of actinomycin D was added and after 30 min more the cultures were carefully washed and refed with medium containing 1 μg/ml. of actinomycin D. Three hr later the cultures were challenged with VSV. The results are presented in Table III.

Resistance to VSV developed after removal of IF and cycloheximide and replacement with actinomycin D. Control sets demonstrated that actinomycin D was able to completely inhibit the development of IF-induced resistance when applied at the same time as IF. This finding excludes the possibility that the hypothesized mRNA for antiviral protein was produced after removal of IF and cycloheximide. The result favors interpretation (b) above; i.e., in the presence of interferon and cycloheximide, the mRNA for the antiviral protein is transcribed and is rapidly translated after removal of cycloheximide.

Translation of the proposed mRNA for antiviral protein in the presence of FPA. Additional experiments were performed to confirm the interpretation that the proposed mRNA is translated after removal of interferon and cycloheximide. These experiments also served to determine whether or not the mRNA was stable during treatment of ME with FPA. The general plan was to induce mRNA only and then allow translation in the presence of FPA. Specifically ME cultures were treated with IF, cycloheximide, and FPA to induce mRNA (Fig. 1). After 1.5, 4, or 5.5 hr different sets of tubes were washed 3 times and refed with plain medi-

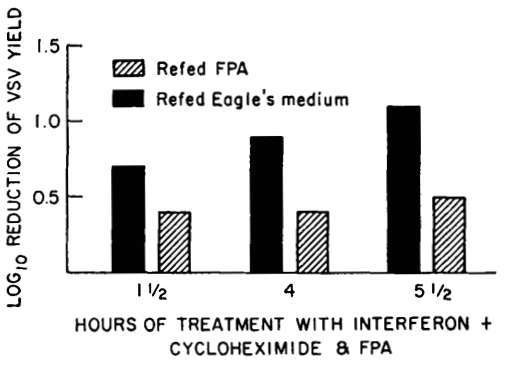

FIG. 1. Effect of treatment of mouse embryo cells with interferon, cycloheximide, and FPA followed by replacement with only FPA: FPA (1000 μg/ml) in Eagle's medium + 2% fetal bovine serum; shaded bar, refed with FPA; solid bar, refed with Eagle's medium.

um; other groups of tubes were washed and refed with FPA containing medium. After 3 hr at 37° the tubes were washed again to reverse FPA and challenged with VSV. As shown in Fig. 1, consistently less resistance developed when the mRNA was translated in the presence of FPA as compared with the absence of FPA. This result indicates that functional translation of the mRNA is prevented by FPA applied after removal of cycloheximide. It also suggests that the hypothesized mRNA is unstable in the presence of FPA alone since the preestablished mRNA did not induce resistance between the time of removal of FPA and the time of VSV challenge.

Discussion. On the basis of the experimental results, the failure of cycloheximide to inhibit development of resistance in IF-treated ME cells is consistent with the interpretation that a mRNA for antiviral protein is produced during the phase of protein inhibition (Table III). After reversal of cycloheximide and resumption of protein synthesis the mRNA becomes available for translation into antiviral protein. However, the antiviral protein must be synthesized rapidly to account for its inhibition of the simultaneous virus challenge. This concept of the effect of cycloheximide is consistent with its mode of action. It has been observed that cycloheximide inhibits transfer of amino acids from soluble RNA to polypeptides thereby leaving polysomes largely intact. When the drug was removed, protein synthesis resumed at normal rate in a very short time (6–8). These observations indicate stability of mRNA in the presence of cycloheximide. In our opinion the main conclusion of this study is that formation of an informational RNA appears to be separated in time from the production of its proposed antiviral protein. This finding strongly supports the working model that interferon exerts its antiviral activity by newly inducing an antiviral protein (1–4).

More difficult appears the evaluation of the effect of FPA on production and stability of the theoretical mRNA for antiviral protein. However, a speculative interpretation may be attempted on the basis of the following considerations. FPA does not arrest RNA synthesis and also does not arrest protein synthesis but rather is incorporated into developing polypeptide chains, thereby rendering them nonfunctional (9). Thus in the presence of FPA, interferon should be able to induce the mRNA for the AVP which should then give rise to nonfunctional AVP. Indication that the mRNA for the antiviral protein is short lived in the presence of FPA comes from the failure of preestablished mRNA in ME to produce AVP after treatment with FPA (Table II). A short half-life for this mRNA during normal translation in the absence of FPA has also been observed (10). Control experiments showed that any fallacious protein that might be formed in the presence of interferon and FPA does not inhibit subsequent development of resistance after removal of FPA but retention of interferon. Therefore, the failure of interferon to induce resistance in the presence of FPA may be directly attributed to formation of fallacious AVP. Failure of resistance to develop after removal of combined interferon and FPA may be due to the relative instability of the hypothesized mRNA for AVP in the presence of FPA. In addition, the evidence that mRNA for AVP is synthesized in the absence of protein synthesis and is translated when production of additional RNA is inhibited by actinomycin D indicates that the production

of proposed antiviral protein is directly induced by IF without synthesis of newly induced, mediating proteins. It remains to be determined whether these findings will apply to the cells of other species.

Summary. Mouse embryo cultures treated with interferon in the presence of the inhibitor of protein synthesis, cycloheximide, showed substantially the same level of resistance as did the control cultures treated with interferon alone. In contrast fluorophenylalanine (FPA) prevented the development of resistance. When cells were treated for 5 hr with a combination of interferon, cycloheximide, and FPA, washed and then challenged with virus, antiviral resistance did develop. Mouse embryo cultures treated with a combination of interferon, cycloheximide and FPA to induce mRNA and then washed and refed with FPA-containing medium developed consistently less resistance than did cultures refed with medium alone. These findings were interpreted to indicate that in the presence of interferon and cycloheximide, mRNA for the antiviral protein (AVP) may be transcribed, and it is then rapidly translated after removal of cycloheximide. It also suggests that the hypothesized mRNA is unstable in the presence of FPA alone. Actinomycin D, added at the time of removal of interferon and cycloheximide did not prevent the development of antiviral resistance. This finding that the proposed mRNA for AVP is synthesized in the absence of protein synthesis and it is translated when production of additional RNA is inhibited by actinomycin D indicates that the formation of proposed AVP is directly induced by interferon without synthesis of newly induced, intermediary proteins. Taken together these findings support the working model that interferon exerts its antiviral activity by newly inducing an antiviral protein.

1. Taylor, J., Biochem. Biophys. Res. Commun. **14**, 447 (1964).

2. Friedman, R. M. and Sonnabend, J. A., Nature **203**, 366 (1964).

3. Lockart, R. Z., Jr., Biochem. Biophys. Res. Commun. **15**, 513 (1964).

4. Levine, S., Virology **24**, 229 (1964).

5. Baron, S., Buckler, C. E., Levy, H. B., and Friedman, R. M., Proc. Soc. Exptl. Biol. Med. **125**, 1320 (1967).

6. Ennis, H. L. and Lubin, M., Federation Proc. **23**, 269 (1964).

7. Willems, M. and Penman, S., Virology **30**, 355 (1966).

8. Colombo, B., Felicetti, L., and Baglioni, C., Biochem. Biophys. Res. Commun. **18**, 389 (1965).

9. Munier, R. and Cohen, G. N., Biochem. Biophys. Acta **31**, 378 (1959).

10. Dianzani, F., Buckler, C. E., and Baron, S., "The Interferons" (G. Rita, ed.), Academic Press, New York (1968).

Received Oct. 7, 1968. P.S.E.B.M., 1969, Vol. 130.