JOURNAL OF VIROLOGY, Jan. 1972, p. 85-89
Vol. 9, No. 1

Inhibition of Protein Synthesis in L Cells Infected with Vesicular Stomatitis Virus

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Received for publication 30 August 1971

The inhibition of protein synthesis in L cells by vesicular stomatitis virus (VSV) requires the synthesis of new protein subsequent to virus infection. However, two mechanisms may be involved in the inhibition of cell protein synthesis by VSV: an initial, multiplicity-dependent, ultraviolet-insensitive inhibition and a progressive, ultraviolet-sensitive inhibition.

Two mechanisms have been described by which animal viruses inhibit host cell macromolecular synthesis. One mechanism involves shut-off of host cell synthesis in the absence of new protein synthesis, implying that a structural component of the infecting virion is responsible for the inhibition. Such a mechanism has been demonstrated with vaccinia virus infection of HeLa cells (4). The second mechanism of inhibition of host cell macromolecular synthesis requires protein synthesis. For example, during mengovirus infection of L cells, host protein synthesis is inhibited only if protein synthesis is allowed to occur in the early stages of infection (1). At present, it is not possible to distinguish whether a new protein specified by the virus acts as the inhibitor or whether virus infection derepresses the synthesis of a cellular protein which serves as the inhibitor.

Huang and Wagner (3) reported that vesicular stomatitis virus (VSV), a single-stranded ribonucleic acid (RNA) virus, inhibits RNA synthesis in Krebs-2 cells soon after infection. With ultraviolet (UV)-irradiated virus devoid of infectivity, they showed that a "preformed toxic component" of the virion was responsible for the inhibiton of cellular RNA synthesis in the Krebs-2 system. Yamazaki and Wagner (8) reported that at high multiplicities of VSV the shut-off of host protein synthesis in primary rabbit kidney cells is caused by an infecting virion component rather than by a newly synthesized viral product.

We have recently reported (6) that two plaque size variants of VSV rapidly inhibit both RNA and protein synthesis in mouse L cells. The small-plaque (S_2) VSV virus inhibited 90% of L-cell protein synthesis within two hr after infection at a multiplicity of 10. In L cells infected with large-plaque (L_1) VSV at a multiplicity of 10, inhibition was efficient but not as rapid as with the S_2 mutant. A 90% inhibition of protein synthesis did not occur until 8 hr after infection. This paper reports our findings concerning the mechanism of inhibition of protein synthesis in L cells infected with L_1 or S_2 VSV.

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MATERIALS AND METHODS

Virus. The Indiana strain of VSV was propagated in primary chick embryo cell monolayers inoculated at low multiplicities of infection (MOI) to avoid formation of incomplete, autointerfering particles. Virus was concentrated, purified, and stored as described previously (6).

Cell cultures. A line of L cells (clone 929) was propagated and maintained in Eagle's minimal essential medium containing 4% calf serum as described in detail (9).

Media and solutions. Hanks balanced salt solution (BSS) was used as a diluent for tritiated compounds. Phosphate-buffered saline (PBS), 0.01 M, *p*H 7.4, was used to wash cells. Cycloheximide (Acti-Dione) was purchased from the Upjohn Co., Kalamazoo, Mich. It was dissolved in 7% ethanol and diluted in Eagle's medium to be used at a concentration of 100 μg/ml. L-[*4,5,-³H*] leucine, specific activity 40 Ci/mmole, was purchased from Schwarz BioResearch, Inc., Orangeburg, N.Y.

Measurements of protein synthesis. Growth medium was removed from monolayer petri dish cultures (2×10^6 cells) and replaced with 1 μCi of ^{3}H-leucine in 1 ml of BSS. The cultures were incubated at 37 C in a CO_2-gassed incubator for appropriate intervals. Immediately after the labeling period, the fluids containing the label were removed and the cells were washed twice with cold BSS. The cells were removed from the glass by scraping, resuspended in cold BSS to a concentration of 10^6 cells per ml, and disrupted by sonic oscillation for 5 min. The samples were prepared for assay of isotope incorporation by the following procedure. Duplicate 1-ml samples were

treated with 1 ml of 10% trichloroacetic acid containing filter aid (4 g/100 ml). The acid-precipitable protein fractions were collected by suction filtration on no. 1 Whatman filter paper. The precipitates were washed to remove unincorporated label: twice with cold 5% trichloroacetic acid, once with 95% ethanol, and once with ethyl ether. The dry samples were removed from the filters by scraping, placed in glass scintillation vials, and solubilized with 0.5 ml of 10× hydroxide of Hyamine for 20 hr at room temperature. To each solubilized sample was added 10 ml of a toluene-based scintillation mixture. The samples were counted in a Packard model 3375 liquid scintillation spectrometer, and radioactivity was expressed as counts per minute per 10^6 cells.

RESULTS

Inhibition of protein synthesis in L cells infected with S_2 VSV. A modification of the method of Moss (4) was used to determine whether inhibition of protein synthesis in L cells infected with VSV required new protein synthesis subsequent to infection. A general outline of the experiment follows. Cells were infected with virus in the presence of cycloheximide. This inhibitor does not affect adsorption and uncoating of VSV (Wertz, *unpublished data*). After a suitable interval, the drug was washed out, and the ability of the cells to synthesize protein was measured. The resumption of protein synthesis after removal of cycloheximide would indicate that new protein is required for subsequent inhibition of protein synthesis. Failure of the cells to resume protein synthesis after the cycloheximide is removed would indicate that the inhibition of host protein synthesis is effected by a component of the infecting virion.

Monolayers of 2×10^6 L cells were treated for 30 min with medium which contained 100 μg of cycloheximide per ml; this concentration of cycloheximide inhibited 98% of cellular protein synthesis within 20 min. The cells were infected with S_2 VSV at an MOI of 10 in the presence of cycloheximide. Virus used for these experiments was purified by differential centrifugation alone or by differential centrifugation followed by sucrose gradient centrifugation; identical results were obtained with virus purified by either method. After a 30-min adsorption period at 37 C, the virus inoculum was removed, and the cells were washed twice with PBS and incubated at 37 C in medium containing cycloheximide. Control cultures, treated with either cycloheximide or virus, were processed in the manner described above. After 2 hr at 37 C, the cycloheximide was washed out of half of the cultures, ^{3}H-leucine (1 μCi in 1 ml of BSS) was added to all cultures, and incubation was continued. When necessary, the effective concentration of cycloheximide was maintained throughout the experiment. At 20, 50, and 70 min after the addition of the labeled precursor, two cultures from each experimental group were processed as described above to determine the amount of radioactivity incorporated into trichloroacetic acid-precipitable material. Table 1 shows the results of this experiment. In untreated, uninfected control cultures, there was a rapid incorporation of ^{3}H-leucine into protein which continued through 70 min (group 1). The presence of cycloheximide throughout the experiment markedly inhibited the incorporation of ^{3}H-leucine into protein (group 2); however, when cycloheximide was removed from the cultures after 2 hr, there was a rapid renewal of protein synthesis which emphasized the ready reversibility of the effects of this antibiotic (group 3). A marked inhibition of protein synthesis was seen in infected cultures without antibiotic treatment (group 6). As expected from the findings with group 2, the ability of infected cells treated with cycloheximide throughout the experiment to synthesize proteins was inhibited even more severely (group 4). However, when cycloheximide was removed from the infected cultures 2 hr after infection (group 5), there was a rapid resumption of protein synthesis. The recovery of infected cells from the action of cycloheximide paralleled the resumption of protein synthesis in uninfected cells freed from the action of this antibiotic (group 3). These results show that infection with S_2 VSV did not shut off

TABLE 1. *Inhibition of L-cell protein synthesis by S_2 VSV: requirement for protein synthesis*[a]

Exptl group	Virus (MOI)	Cycloheximide (μg/ml)	Cycloheximide (μg/ml in wash)	Counts/min in trichloroacetic acid-precipitable material at		
				20 min	50 min	70 min
1	0	0	0	32,681	55,756	68,247
2	0	100	100	756	791	1,683
3	0	100	0	10,735	28,705	33,769
4	10	100	100	898	746	1,623
5	10	100	0	13,259	27,303	30,987
6	10	0	0	6,573	11,115	13,220

[a] Monolayer cultures of 2×10^6 L cells were pretreated with 100 μg of cycloheximide per ml as shown. After 30 min, the cultures were infected with S_2 VSV. After an adsorption period of 30 min, the unadsorbed virus was removed and cells were washed twice with PBS. The cultures were then reincubated in medium with or without 100 μg of cycloheximide per ml. After 2 hr at 37 C, the cycloheximide was washed out as indicated and 1 μCi of ^{3}H-leucine per ml was added. At indicated times, cultures were processed to determine the radioactivity of trichloroacetic acid-precipitable protein.

host protein synthesis in the presence of cycloheximide and indicate that new protein must be synthesized after infection with S_2 VSV for inhibition of host cell protein synthesis to occur.

An alternate explanation of the data obtained with group 5 would be that virion polymerase transcribed viral RNA during cycloheximide inhibition of protein synthesis and then this RNA was translated to viral protein after the reversal of the cycloheximide inhibition. This explanation is unlikely since experiments have been done which show that, in L cells infected with S_2 VSV, virion polymerase does not transcribe any detectable RNA in the presence of 100 μg of cycloheximide per ml (G. W. Wertz and M. Levine, *unpublished data*).

The effect of UV irradiation on the infectivity of S_2 VSV and on the ability of the virus to inhibit protein synthesis was investigated. L cells were infected with S_2 VSV at an MOI of 5 or with the same concentration of virus which had been exposed to a 15-w G.E. germicidal lamp for 10, 20, 60, or 180 sec at a distance of 17.5 cm. Table 2 shows the effect of these doses of irradiation on the infectivity of the virus. The inoculum was removed after a 30-min adsorption period at 37 C, the cells were washed, fresh medium was added, and the cells were incubated at 37 C. At intervals starting at the end of the adsorption period, the medium was removed and replaced with 1 μCi of ^{3}H-leucine in 1 ml of BSS. At the end of a 20-min labeling period, the cells were processed as described above and assayed for radioactivity incorporated into trichloroacetic acid-precipitable material.

The results presented in Fig. 1 show that the inhibition of protein synthesis in infected cells was less severe when the infecting virus was exposed to larger doses of UV irradiation. These results also indicate that there may be two types of inhibition of protein synthesis in L cells infected with S_2 VSV. The initial inhibition (i.e., the inhibition seen at the end of the adsorption period)

TABLE 2. *Effect of UV irradiation on infectivity of VSV*

Time of exposure to UV (sec)[a]	Infectivity (PFU/ml)[b]	
	S_2 VSV	L_1 VSV
0	1.4×10^8	2.7×10^8
10	3.5×10^4	2.7×10^5
20	9.0×10^3	1.4×10^3
60	10	10
180	0	0

[a] Conditions of irradiation as described.
[b] PFU, plaque-forming units.

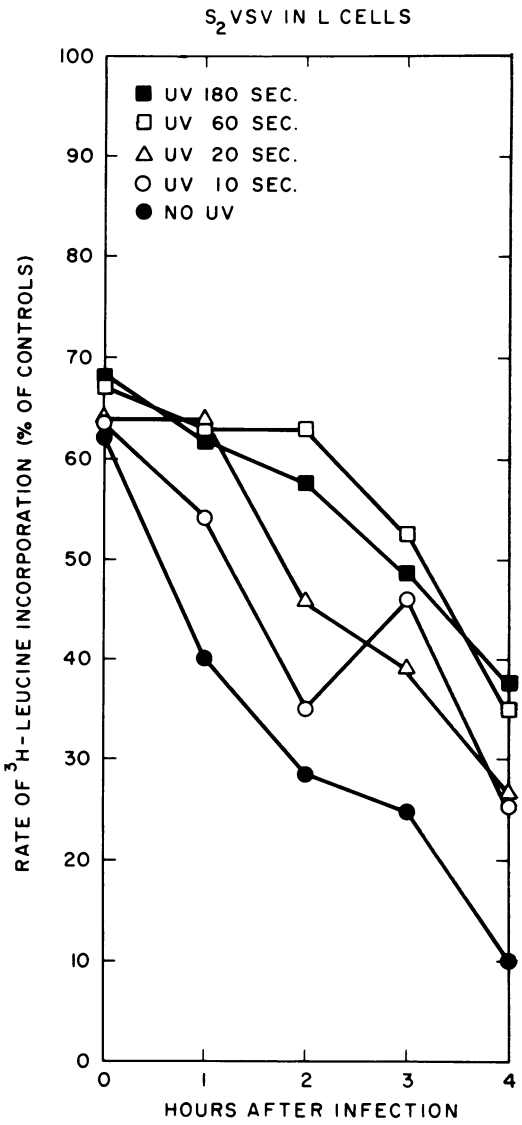

FIG. 1. *Protein synthesis in L cells infected with S_2 VSV: effect of UV-irradiated virus. L-cell monolayers were infected with S_2 VSV at an MOI of 5 and with the same concentration of virus which had been exposed to different doses of UV irradiation. At intervals from time 0, infected cultures and uninfected control cultures were exposed for 20 min to 1 μCi of ^{3}H-leucine. The label was removed, the cultures were washed twice with BSS, and the cells were suspended in BSS (10^6 cells/ml). Trichloroacetic acid-precipitable radioactivity per 10^6 cells in infected cultures was expressed as per cent of the counts incorporated into uninfected controls. Control values (counts per minute per 10^6 cells) for 0 hr = 4,544, 1 hr = 4,040, 2 hr = 4,239, 3 hr = 4,466, and 4 hr = 4,589.*

did not seem to be affected by a dose of UV irradiation which significantly altered progressive inhibition (i.e., the inhibition seen between adsorption and 4 hr) of protein synthesis. As shown in Fig. 2, the ability of S_2 VSV to produce progressive inhibition of protein synthesis was lost as the infectivity of the virus was destroyed by UV irradiation.

Cells infected with S_2 VSV at an MOI of 1 showed an effect of UV irradiation on infectivity and progressive inhibition of protein synthesis in L cells similar to that seen at an MOI of 5. These results indicate that multiplicity reactivation of UV-irradiated virus is not responsible for progressive inhibition in cells infected at MOI > 1.

Inhibition of protein synthesis in L cells infected with L_1 VSV. An experiment similar to the one described with S_2 VSV involving reversal of the action of cycloheximide was not feasible with L_1 VSV. In the case of this virus, host protein synthesis was reduced to only 30% of the control rate by 6 hr after infection (6). After this length of exposure to cycloheximide, the ability of even uninfected cells to synthesize protein is seriously compromised by the time the action of the antibiotic is reversed. In view of this limitation, the effect of UV irradiation on the infectivity of L_1 VSV and on the ability of the virus to inhibit protein synthesis was investigated. L cells were infected with L_1 VSV at an MOI of 10 or with the same concentration of virus which had been UV-irradiated as described above for S_2 VSV; Table 2 shows the effect of irradiation on the infectivity of the virus. After a 30-min adsorption period at 37 C, the inoculum was removed, the cells were washed, fresh medium was added, and the cells were reincubated at 37 C. At intervals starting at the end of the adsorption period, the medium was removed and replaced with 1 μCi of ^{3}H-leucine in 1 ml of BSS. After a 20-min labeling period, the cells were harvested and the radioactivity incorporated into trichloroacetic-precipitable material was determined.

The results presented in Fig. 3 show that the inhibition of protein synthesis in infected cells was less severe when the infecting virus was exposed to larger doses of UV irradiation. As seen with cells infected with S_2 VSV, these results also indicate that there may be two types of inhibition of protein synthesis in L cells infected with L_1 VSV. The initial inhibition did not seem to be affected by a dose of UV irradiation which significantly altered progressive inhibition of protein synthesis. When the data with L_1 VSV were plotted as for S_2 VSV in Fig. 2, almost identical curves were obtained, indicating that the ability of L_1 VSV to produce progressive inhibition of protein synthesis in L cells was lost as the infectivity of the virus was destroyed by UV irradiation.

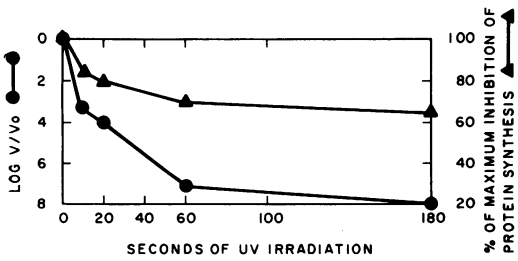

FIG. 2. *Comparison of effects of UV irradiation on S_2 VSV infectivity and ability to inhibit protein synthesis in L cells. Infectivity of irradiated virus is expressed as the ratio (V/V_0) of the residual plaque-forming units (PFU) after irradiation, divided by the PFU of the unirradiated virus. The effect of UV-irradiated virus on protein synthesis in L cells is expressed as the per cent of the maximal inhibition produced by unirradiated virus. See legend to Fig. 1 for experimental details.*

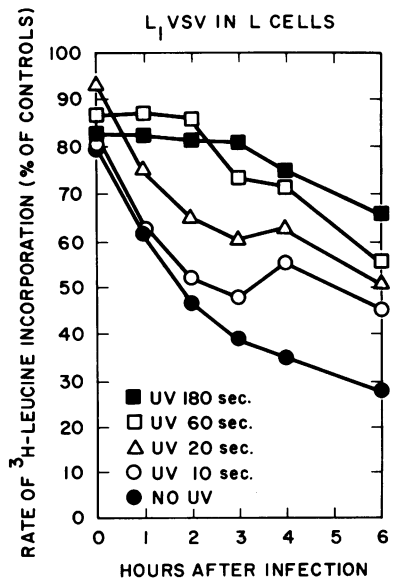

FIG. 3. *Protein synthesis in L cells infected with L_1 VSV: effect of UV-irradiated virus. L-cell monolayers were infected with L_1 VSV at an MOI of 10 and with the same concentration of virus which had been exposed to different doses of UV irradiation. At intervals from time 0, infected cultures and uninfected control cultures were exposed for 20 min to 1 μCi of ^{3}H-leucine. The label was removed, the cultures were washed twice with BSS, and the cells were suspended in BSS (10^6 cells/ml). Trichloroacetic acid-precipitable radioactivity per 10^6 cells in infected cultures was expressed as per cent of the counts incorporated into uninfected controls. Control values (counts per minute per 10^6 cells) for 0 hr = 7,452, 1 hr = 7,841, 2 hr = 8,922, 3 hr = 8,902, 4 hr = 8,709, and 6 hr = 8,872.*

DISCUSSION

The data presented indicate that the progressive inhibition of protein synthesis in L cells infected with VSV is due to new protein synthesized after infection. The results also suggest that a functional viral genome may be required for progressive inhibition of protein synthesis by S_2 and L_1 VSV. However, two mechanisms appear to be involved in the inhibition of cell protein synthesis by VSV: an initial multiplicity-dependent, UV-insensitive inhibition as well as a progressive, cycloheximide- and UV-sensitive inhibition. It is possible that the initial UV-resistant inhibition of protein synthesis in L cells infected with high multiplicities of S_2 or L_1 VSV may result from changes in the cell membrane caused by the infection process. Morphological evidence for membrane changes in infected cells was provided by Simpson et al. (5) and by Heine and Schnaitman (2), who examined VSV-infected L cells by electron microscopy. These workers found that the regions of the L-cell membrane associated with attached VSV particles were markedly thickened and that fusion of viral and cell membranes occurred. The initial inhibition seen at the end of the adsorption period may depend on the degree to which the cell membrane is affected by high or low MOI; initial inhibition of L-cell protein synthesis by L_1 or S_2 VSV is always greatest at high MOI (6).

Evidence for an analogous mechanism of inhibition was provided by Yamazaki (7), who investigated early inhibition of protein and RNA synthesis in *Escherichia coli* cells infected with the RNA phage R17. He found that the inhibition of both protein and RNA synthesis was stoichiometrically dependent on MOI, that the intracellular functioning of the phage genome was not required for these inhibitions, and that the phage infection caused a rapid and marked inhibition of cellular amino acid transport. It is possible that the initial, multiplicity-dependent, UV-resistant inhibition of protein synthesis in L cells infected with L_1 VSV may represent a mechanism of inhibition of host macromolecular synthesis by animal viruses similar to that described by Yamazaki for R17.

More recently, Yamazaki and Wagner (8) concluded that the rapidity with which a high multiplicity of VSV switches off cellular protein synthesis in the presence of a concentration of interferon which inhibits measurable viral protein synthesis provides evidence that this reaction is caused by an infecting virion component rather than by a newly synthesized viral product.

In regard to RNA synthesis, an initial multiplicity-dependent inhibition of RNA synthesis was also observed in L cells infected with S_2 and L_1 VSV (6). This rapid inhibition of cellular RNA and protein synthesis may be the result of alterations in host cell membranes after infection by high multiplicities of VSV. These suggestions are in accord with those of Huang and Wagner (3) concerning the inhibition of RNA synthesis by Krebs-2 cells infected with VSV. On the basis of experiments with UV-irradiated virus, they concluded that a component of the infecting virion most likely was the factor responsible for the inhibition of cellular RNA synthesis.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI-06264 and training grant AI-0080 from the National Institute of Allergy and Infectious Diseases.

Gail Wertz was a predoctoral fellow of the National Institutes of Health under fellowship 1 F01-GM-43,186-01 from the National Institute of General Medical Sciences.

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