Inhibition of Protein Synthesis in Newcastle Disease Virus Infected L Cells[1] (35799)

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The infection of L cells by Newcastle disease virus (NDV) has been shown to be an inefficient cell–virus interaction which results in the production of relatively large quantities of interferon (1). In addition, the production of interferon has been found to occur relatively late in the cycle of viral replication, when a considerable decrease in the rate of cellular protein synthesis is observed (2). However, the relationship between the cycle of NDV replication and the inhibition of L cell protein synthesis has not been previously elucidated. This communication reports a characterization of the nature of the mechanism responsible for the inhibition of cellular protein synthesis, resulting from NDV infection of L cells.

Materials and Methods. The propagation and assay of NDV (Roakin strain), the cultivation of L cells, and the composition of media have been described (3).

Cellular protein synthesis. L cell monolayers were infected or treated as described for the different experiments and exposed to medium containing radioactive amino acid(s) for 30 min. The medium was then removed and the cells were thoroughly washed three times with cold saline. The cells were solubilized in 1 ml of alkaline saline (0.05 *N* NaOH, 0.15 *M* NaCl); and the protein was extracted and precipitated three times with hot trichloroacetic acid (TCA, 5%). An aliquot of the final protein precipitate, solubilized in alkaline saline was assayed for radioactivity in the Beckman LS 100 scintillation counter. The level of amino acids in the cellular amino acid pool was estimated by assaying an aliquot of the supernatant fraction for radioactivity following the first hot TCA precipitation.

Protein determinations. Protein content was determined colorimetrically by the method of Oyama and Eagle (4) using bovine serum albumin as the protein standard.

Nuclear RNA synthesis. L cell monolayers were treated as described (Fig. 1) and then incubated with medium containing ^{3}H uridine for 60 min. The monolayers were then thoroughly washed with cold saline and the cells were trypsinized from the culture vessels. The cells were washed to remove the trypsin, resuspended in a hypotonic buffer and broken with 12 strokes of a tight fitting Dounce homogenizer (5). The nuclei were pelleted by centrifugation (1000*g* for 10 min) extracted with phenol and sodium dodecyl sulfate (5) and precipitated three times with ethanol. The precipitable radioactivity was assayed as described above.

Ultraviolet (uv) inactivation of NDV. A 5-ml portion of an NDV pool [2.8×10^{9} plaque-forming units (pfu)/ml], was placed in a sterile petri dish and irradiated for 2 min employing a General Electric germicidal uv lamp at a distance of 10.0 cm. During the irradiation period, the dish was continuously rotated. Following the 2-min period, the irradiated sample was placed in a darkened cabinet for 30 min to prevent photoreactivation and then assayed for residual viable virus content (4.0×10^{3} pfu/ml) as previously described (3).

Chemicals. ^{14}C arginine (250 mCi/m-

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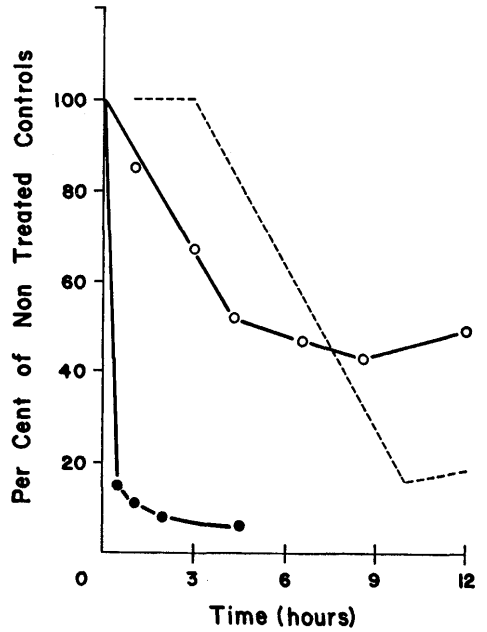

FIG. 1. Effect of actinomycin D on L cell protein and nuclear RNA synthesis: L cell monolayers were incubated with medium containing actinomycin D (1 μg/ml) for varying periods of time and then labeled with either ^{14}C arginine (0.5 μCi), a ^{3}H amino acid mixture (1.5 μCi) for 30 min or with ^{3}H uridine (2.0 μCi) for 60 min. Protein and nuclear RNA were extracted as described, and the rates of synthesis were determined [cpm/mg of total protein, or cpm/mg of cytoplasmic protein (RNA] and expressed as percentage of nontreated controls: (●—) nuclear RNA; (○—) protein; (- - -) rate of protein synthesis in NDV infected L cells (2).

mole), ^{3}H valine (2.97 Ci/mmole) and a ^{3}H amino acid mixture were purchased from New England Nuclear Corp., Boston, Mass. ^{3}H uridine (9.53 Ci/mmole) was purchased from Schwarz Bioresearch Inc., Orangeburg, New York. Actinomycin D was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey. Puromycin was supplied by Dr. James Duncan, Northwestern University and DL-*p*-fluorophenylalanine (FPA) was purchased from Sigma Chemical Company, St. Louis, Missouri.

Results. Effect of transcriptional inhibition on protein synthesis. The cycle of NDV replication in L cells has been shown to result in an inhibition of both cellular transcriptional and translational processes (2, 6). These observations raised the question of whether the observed inhibition of transcription was sufficient to account for the inhibition of L cell protein synthesis. This was investigated by measuring the magnitude of the inhibition of protein synthesis resulting from a total block of cellular transcriptional events. Noninfected L cell monolayers were exposed to actinomycin D for varying periods of time, and the relative rates of nuclear RNA and total protein synthesis were then determined. The data indicated that a 30-min exposure to the antibiotic resulted in a 80 to 90% decrease in nuclear RNA synthesis (Fig. 1). The inhibition of cellular protein synthesis resulting from actinomycin treatment reached a maximum level of 55% through a 12-hr period. Despite an initial lag of 3 hr in NDV infected L cells, protein synthesis was inhibited by 85% after 12 hr. These results indicated the magnitude of the translational inhibition resulting from NDV infection was greater than the level of inhibition resulting solely from a block of transcriptional events.

Ultraviolet inactivation of DNV. Recent investigations have demonstrated that the exposure of cells to capsid components of some viruses resulted in pronounced inhibitions of cellular macromolecular biosynthesis (7, 8). These reports suggested the possibility that the inhibition of L cell protein synthesis by NDV may be the result of a toxic substance associated with the infecting virion. This hypothesis was tested by inactivating infectious NDV with uv light and then determining the relative rate of protein synthesis in L cells exposed to these uv inactivated virions. The results showed that uv inactivation of the infecting virion effectively prevented the inhibition of cellular protein synthesis which is observed in cells exposed to non-uv treated NDV (Table I).

Uptake of amino acids. The infection of cells by myxoviruses have also been shown to result in alterations in the chemical composition of the plasma membrane (9). These observations suggested the possibility that the inhibition of L cell protein synthesis may be due to an alteration in cell membrane permeability resulting in a decreased ability

TABLE I. The Effect of Ultraviolet Light Inactivation of NDV on the Inhibition of L Cell Protein Synthesis.[a]

Time after infection (hr)	Percentage of noninfected control Sp act	
	NDV treated	NDV uv treated
1	95.2	97.5
9	42.2	87.4
4	90.0	97.7

[a] L cell monolayers were exposed to equal numbers of NDV (multiplicity of infection, $m = 30$ pfu/cell) or ultraviolet light inactivated NDV or neither (controls). At different times after infection the monolayers were labeled with a ^3H amino acid mixture (1.0 μCi) for 30 min. The protein was then extracted as described and the specific activities were expressed as a percentage of the noninfected controls.

of the infected cell to take up amino acids. This possibility was examined by determining the quantity of labeled valine in the acid soluble fraction of L cells during the course of infection. The results showed that the amounts of acid soluble radioactivity from the cellular pool material of infected L cells did not decrease during the course of infection (Table II). In contrast, the concentration of ^3H valine was found to be elevated over control values at those time intervals when cellular protein synthesis had previously been found to be inhibited (Fig. 1). The relationship between the rate of cellular protein synthesis and the concentration of amino acids in the soluble pool was further examined by inhibiting protein synthesis with puromycin. These results indicated that the concentration of acid-soluble radioactive valine increased in noninfected L cells in which translational events were being inhibited (Table II).

FPA effects on inhibition of protein synthesis. Previous communications have demonstrated a close relationship between inhibitions of cellular macromolecular biosynthesis and the synthesis of viral directed proteins during infection (10–12). This relationship was studied with the aid of FPA, an amino acid analog which can be incorporated into the primary structures of proteins (13) and thereby, possibly alter their biologic activity. The procedure used to determine the effect of FPA on the NDV directed inhibition of cellular protein synthesis is described in footnote *a* of Table III. The data showed that exposure of noninfected L cells to FPA up to 11 hr resulted in a 20 to 30% inhibition of protein synthesis, while protein synthesis in NDV infected L cells was inhibited 85% in 11 hr. However, protein synthesis in cells exposed to both NDV and FPA was inhibited by only 20 to 30% at 11 hr after infection. These observations suggest a relationship exists between the synthesis of viral directed proteins and the observed inhibition of cellular protein synthesis.

The temporal relationship between the synthesis of NDV directed protein(s) during the cycle of infection and the inhibition of protein synthesis could be evaluated by adding FPA to NDV infected L cells at various times after infection. The L cell monolayers were infected and FPA was added to duplicate cultures at various times postinfection as

TABLE II. The Effect of NDV Infection and Puromycin Treatment on the Quantity of TCA-Soluble Tritiated Valine in the Amino Pool of L Cells.[a]

Time after infection (hr)	Controls; TCA-soluble total counts (cpm)	NDV infected; TCA-soluble total counts (cpm)
1	2310	2440
3	3350	3390
6	3740	5700
9	4690	6130

	TCA precipitable total counts (cpm)	TCA-soluble total counts (cpm)
Controls	29,500	5430
Puromycin treated	8600	11,110

[a] L cell monolayers were infected with NDV ($m = 30$) or exposed to puromycin (10^{-4} *M* for 15 min) and then exposed to ^3H valine (1.0 μCi) for 30 min. The acid-soluble and acid-precipitable radioactivity was determined as described. The values presented are the mean determinations of duplicate or triplicate samples for each pulse period. The range of the total protein for all monolayers was 0.50 to 0.57 mg.

TABLE III. The Effect of FPA on the Inhibition of L Cell Protein Synthesis.[a]

Time after infection (hr)	Monolayer groups; (cpm/mg of protein)			
	Control	FPA treated	NDV infected	NDV infected and FPA treated
1	13,400	15,500	13,600	14,300
3	16,500	12,900	17,500	14,600
6	17,900	12,200	8500	13,200
9	15,500	11,900	4300	12,800
11	12,500	10,000	2100	8900

[a] Two groups of L cell monoloyers were infected with NDV ($m = 30$) while the other two groups were maintained as noninfected controls. After a 60-min viral adsorption period, two groups of monolayers received 4 ml of medium and the other two groups received 4 ml of maintenance medium containing 200 μg/ml of L *p*-fluorophenylalanine (FPA) as indicated above. At different times after infection one monolayer from each group was labeled with ^{3}H valine (1.0 μCi) for 30 min and the protein was extracted as described.

described (Table IV). The relative rate of cellular protein synthesis was then determined at 11 hr after infection, when cellular protein synthesis was found to be inhibited by approximately 80% (2). These determinations indicated that FPA completely blocked the inhibition of cellular translation events when it was added through 5 hr after infection. However, the ability of FPA to reverse the inhibition of cellular protein synthesis continually decreased thereafter as the level of inhibition in monolayers treated with FPA at 9.5 hr was approximately the same as that observed in non-FPA treated cells.

Discussion. Previous reports have indicated the inhibition of cellular protein synthesis in NDV infected L cells is greater than the degree of inhibition of nuclear transcription (2, 6). The relationship between these observations was examined by comparing the level of inhibition of L cell protein synthesis resulting from NDV infection with the level of inhibition resulting from a block of cellular transcription using actinomycin D. The results indicated that the inhibition of protein synthesis was substantially greater in NDV infected L cells compared with the level of inhibition which resulted from a total inhibition of L cell transcription. These observations suggest that the inhibition of L cell polypeptide biosynthesis resulting from NDV infection is a consequency of inhibi-

TABLE IV. Effect of Sequential Addition of FPA on the Inhibition of Protein Synthesis.[a]

	Time after infection of FPA addition (hr)	Sp act (cpm/mg of protein)	Percentage of noninfected controls
Control	1	9900	100.0
Infected	1	10,300	104.5
	2	11,500	115.9
	3	11,600	116.7
	4	9700	97.6
	5	9200	92.7
	6	7500	75.7
	7.5	5300	53.6
	8.5	4000	40.4
	9.5	2800	28.2

[a] Duplicate L cell monolayers were differentially treated as indicated. At 11 hr after infection all monolayers were labeled with ^{3}H valine (1.0 μCi) for 30 min; and the protein was extracted as described.

tory effects at both the transcriptional and translational levels.

An examination of the relationship between NDV viability and the inhibition of protein synthesis was performed. The results indicated that the inhibition of protein synthesis was sensitive to uv inactivation of the infecting virion. These observations suggested that the inhibition of cellular protein synthesis was neither the result of NDV attachment to cellular receptors nor was it due to a toxic substance or enzyme associated with the infecting virion. In contrast, these data indicated the inhibition of L cell protein synthesis was the result of an active albeit inefficient cycle of NDV replication.

An examination of the levels of the acid-soluble amino acid pool material during the course of infection revealed increased concentrations of ^{3}H valine at times when cellular protein synthesis was inhibited. A similar phenomenon could be reproduced by simply treating cells with puromycin which inhibits protein synthesis at the ribosomal level (14). These observations suggested that the elevated concentrations of acid soluble ^{3}H valine were a reflection of the inhibition of protein synthesis. More importantly, these results indicated that the inhibition of L cell protein synthesis was not the result of a depletion of intracellular amino acids.

FPA has been shown to be incorporated into protein with the results that the functional nature of the protein could be impaired (13). An evaluation of the effectiveness of FPA in blocking the inhibition of cellular protein synthesis was performed by adding the analog immediately after infection. The data (Table III) indicated that FPA alone reduced the level of protein synthesis by only 20 to 30% in 11 hr in noninfected L cells. When FPA was added to NDV infected L cells, the analog completely blocked the inhibition of protein synthesis. Since the level of FPA employed would completely block the cycle of NDV replication (15) these results suggested a close relationship existed between the cycle of NDV infection and the inhibition of cellular protein synthesis.

The addition of FPA to NDV infected L cells at various times after infection enabled a temporal examination of the ability of this amino acid analog to reverse the viral mediated inhibition of cellular protein synthesis. If FPA were added early after NDV infection and prior to the synthesis of those molecules which were responsible for inhibiting cellular protein synthesis, then the analog could be incorporated into their structure and possibly impair their inhibitory effect. The data indicated that the protein molecules responsible for the inhibition of cellular protein synthesis were synthesized or activated from 5 to 9.5 hr after infection by the inability of FPA to prevent the translational inhibition when FPA was added during these time intervals. A comparison of the inhibition of cellular protein synthesis (2) and the time course for the synthesis of the inhibitors that are resistant to the effects of FPA indicates a close relationship between these two phenomena. In addition, a comparison of these results with the time of interferon synthesis (1, 2) indicates these proteins are produced just prior to and during the synthesis of interferon mRNA and that interferon production proceeds while these molecules are exerting their inhibitory activity on cellular protein synthesis. These observations, which may be related to the marked alteration of mitotic activity observed in NDV infected HeLa cells (16, 17), further indicate that proteins synthesized relatively late in the cycle of NDV replication are closely associated with or responsible for the observed inhibition of L cell protein synthesis.

Summary. The nature of the inhibition of cellular protein synthesis in NDV infected L cells has been examined and found to be a consequence of inhibitions at both transcriptional and translational levels. It was determined that the inhibition of protein synthesis was not a result of viral attachment, any detectable toxic substance associated with the infecting virion, the depletion of intracellular amino acids, or an inhibition of amino acid uptake. The inhibitory action of the virus could be completely reversed by the addition of FPA prior to 5 hr after infection. Thereafter, the effectiveness of the analog in reversing the inhibition of protein synthesis

continually diminished. Consequently, the inhibition of cellular protein synthesis was found to be closely associated with proteins synthesized relatively late in the cycle of NDV replication in L cells.

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