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Inhibition of Host-Cell Protein and Ribonucleic Acid Synthesis by Newcastle Disease Virus

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The mechanisms of Newcastle disease virus-(NDV)induced inhibition of cell protein and ribonucleic acid (RNA) synthesis were investigated. It was observed that the ability of NDV to inhibit cell RNA synthesis is dependent on the virus strain. The inhibitors, azauridine and cycloheximide, were added to cell cultures at different times after infection to study the roles of protein and RNA synthesis in the viral inhibition process. Viral inhibition of cell RNA synthesis and viral inhibition of cell protein synthesis become resistant to cycloheximide at a different time after infection than that in which they become resistant to azauridine. The results indicate that the inhibition of cell RNA synthesis by the Texas strain involves the synthesis of inhibitory proteins which are coded by the viral genome. The Texas and Beaudette strains of NDV appear to employ different mechanisms for the inhibition of host-cell protein synthesis. Viral inhibition of cell protein synthesis does not appear to cause, or be the result of, viral inhibition of cell RNA synthesis.

Many viruses are capable of inhibiting protein and ribonucleic acid (RNA) synthesis in their host cells (6, 8, 10). The mechanisms of Newcastle disease virus (NDV)-induced inhibition of cell protein synthesis and cell RNA synthesis were investigated by use of the metabolic inhibitors actinomycin, azauridine, and cycloheximide. It has been shown that, in some virus systems, virus-specific "early proteins" are responsible for the virus inhibition of cell protein and cell RNA synthesis (6, 8). To learn more about the roles of RNA and protein synthesis in the NDV-induced inhibition process, inhibitors were added to infected cultures at different times after infection. The conclusions which follow are based on the observation that viral RNA synthesis and the viral inhibition processes do not become resistant to protein synthesis inhibitors at the same time after infection that they become resistant to RNA synthesis inhibitors.

It is recognized that the time when a viral synthetic process becomes resistant to a given drug depends on the chosen experimental conditions, such as temperature, virus strain, and multiplicity of infection. In an earlier publication (11), it was shown that viral RNA synthesis at 37 C is inhibited by the addition of puromycin as late as 3 hr after infection. In the present report, viral RNA synthesis at 39 C became resistant to cycloheximide 2 hr after infection. Thus, the observed times of events in the virus growth cycle should be considered relative and applicable only to the present work.

The results indicate that NDV inhibition of cell protein and RNA synthesis depends on the synthesis of viral RNA and inhibitory proteins. Throughout this paper, the term viral RNA refers to the RNA which is synthesized in response to the infecting virus in actinomycin D-treated cells. It has been demonstrated that this RNA consists of a population of (+) parental strands and (−) complementary copies of the parental RNA (2, 4). The presence of other molecular species in this RNA population, such as a replicative form, has not been excluded. In the experiments reported here, no attempt was made to resolve the function of the individual RNA species in the viral RNA population.

MATERIALS AND METHODS

Chemicals. Uridine-5-^3H (31 c/mmole) was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Uniformly labeled ^{14}C-L-leucine (250 mc/mmole) was obtained from New England Nuclear Corp., Boston, Mass. Cycloheximide and 6-azauridine were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Actinomycin D was a gift from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J.

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Cells and virus. Virus and chick embryo fibroblast culture methods have been described previously (1). The Texas (GB) strain and a heat-stable mutant "C" of the Beaudette strain (3) of NDV were used. Virus was centrifuged from allantoic fluid for 30 min at 30,000 × *g* and resuspended in phosphate-buffered saline (PBS). Chick embryo fibroblast monolayers were washed with PBS, and virus was added to give a multiplicity of 100 plaque-forming units per cell. The period of virus adsorption was 30 min. After adsorption, cells were washed with PBS to remove unadsorbed virus. This point in the experiment was taken as time zero or the time of infection. In all experiments, the culture medium was Hank's balanced salt solution containing 2% calf serum. The incubation temperature in all experiments was 39 C.

Analytical procedures. The techniques used to measure viral RNA synthesis were described previously (11). The rates of cellular protein and RNA synthesis were measured by exposing cell cultures to medium containing uridine-5-^3H (0.5 μc/ml) or ^{14}C-L-leucine (0.05 μc/ml) for 30 min. In all cases, the data show points plotted at the time a drug was added or the time when a 30-min pulse was terminated. Samples were prepared for radioactivity analysis as described previously (1) and counted in a liquid scintillation counter.

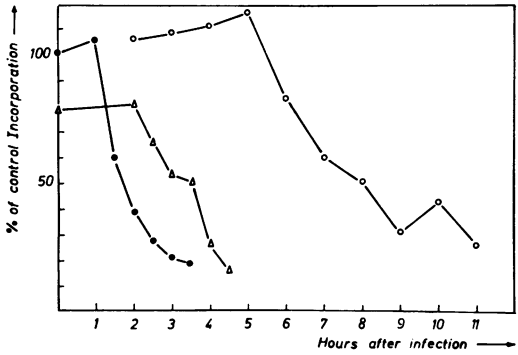

FIG. 1. *Cell protein synthesis inhibition by the Texas strain of NDV. Infected and uninfected chick embryo fibroblast cultures were overlaid with medium and incubated. The rate of cell protein synthesis was measured at different times after infection* (○) *by replacing the medium on some cultures with pulse medium containing* ^{14}C*-leucine. Data are plotted at times corresponding to the time when a 30-min pulse terminated. Beginning immediately after infection, and at times thereafter, the medium on a pair of infected and uninfected cultures was replaced with medium containing azauridine (3 mg/ml). At 8.5 hr after infection, the medium on all azauridine-treated cultures was discarded, the cells were washed with phosphate-buffered saline, and overlaid with* ^{14}C*-leucine pulse medium. The data* (●) *are plotted at times corresponding to the time when drug-containing medium was added. The same experiment was repeated with the use of medium containing cycloheximide (20 μg/ml) instead of azauridine to measure the effect of cycloheximide on the virus-induced inhibition of cell protein synthesis* (○). *The ratio per cent of control activity-counts per min per mg in infected cultures/counts per min per mg in uninfected cultures* × *100.*

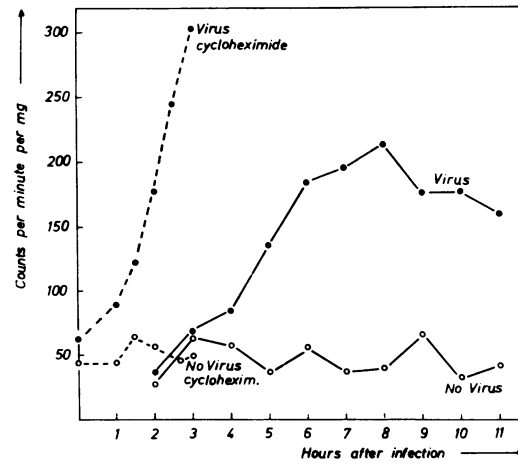

FIG. 2. *Viral RNA synthesis in cells infected with the Texas strain of NDV. Infected and uninfected cultures were overlaid with medium containing uridine-5-*^3H *and actinomycin D (10 μg/ml). At 2 hr after infection, and at times thereafter, a pair of infected and uninfected cultures was analyzed for* ^3H *incorporation. The data (solid lines) are plotted at times when a pair of cultures were taken for analyses. The effect of cycloheximide on viral RNA synthesis is shown by dashed lines. Immediately after infection, and at times thereafter, the medium on a pair of infected and uninfected cultures was replaced with medium containing uridine and actinomycin as above, and in addition containing cycloheximide (20 μg/ml). At 8 hr after infection, the incorporation in all cultures was measured.*

To correct for variations in cell numbers in different cultures, total protein was determined by the method of Lowry et al. (5). The results obtained from the radioactivity analysis were expressed as counts per minute per milligram of total protein.

RESULTS

Inhibition of cell protein synthesis by the Texas strain of NDV. The rate of protein synthesis in cells infected with the Texas strain of NDV began to decline approximately 6 hr after infection, and was 15 to 30% of the rate in control cells at 11 hr after infection (Fig. 1).

The role of viral RNA synthesis in the virus-induced inhibition of cell protein synthesis was investigated with the RNA synthesis inhibitor 6-azauridine. Azauridine was used at a concentration of 3 mg/ml, which acted within 15 min to cause an inhibition of over 90% of cell and viral RNA synthesis, but did not prevent the synthesis of virus specific proteins (1, 11). At different times after infection, the medium on a pair of control and infected cultures was replaced with drug-containing medium. At 8.5 hr the media on all

cultures were discarded, the cultures were washed with PBS, and the rates of protein synthesis were measured. In uninfected cultures which had been exposed to azauridine for 5 hr, the rate of cell protein synthesis in the first 30 min after removal of azauridine was 61% of the rate in control cells.

The addition of azauridine at the time of infection (Fig. 1) prevented the virus-induced inhibition of cell protein synthesis, but, when added at 3 hr after infection, azauridine did not prevent this process. Since actinomycin D, which prevents cellular but not viral RNA synthesis, does not prevent the virus-induced inhibition of cell protein synthesis (1), the action of azauridine must be directed against viral RNA synthesis. This result indicates that viral RNA synthesis is necessary for the virus-induced inhibition of host-cell protein synthesis.

The same experiments were also performed with the protein synthesis inhibitor cycloheximide, in place of azauridine. Cycloheximide was used at a concentration of 20 μg/ml, which caused a 94% inhibition of cell protein synthesis within 15 min after contact with cells. In uninfected cultures which had been exposed to cycloheximide for 5 hr, the rate of protein synthesis during the first 30 min after removal of the drug was 45% of the rate in control cells.

The results shown in Fig. 1 indicate that the virus-induced inhibition of cell protein synthesis was prevented by the addition of cycloheximide at the time of infection. This result was to be expected, since this process requires viral RNA synthesis, and cycloheximide, added at the time of infection, blocked viral RNA synthesis (Fig. 2). It is interesting to note, however, that in cultures which received cycloheximide at 2 hr after infection, the yield of viral RNA at 8 hr was 85% of the normal 8-hr yield of viral RNA in drug-free controls. In these cultures treated with cycloheximide at 2 hr, the virus-induced inhibition of cell protein synthesis was prevented (Fig. 1). These results indicate that viral RNA alone did not inhibit cell protein synthesis, and that virus-induced protein synthesis was also necessary for this process. These results are in agreement with the previously reported observation that the inhibition of cell protein synthesis by the Texas strain of NDV depends on the synthesis of virus-induced inhibitory proteins (1).

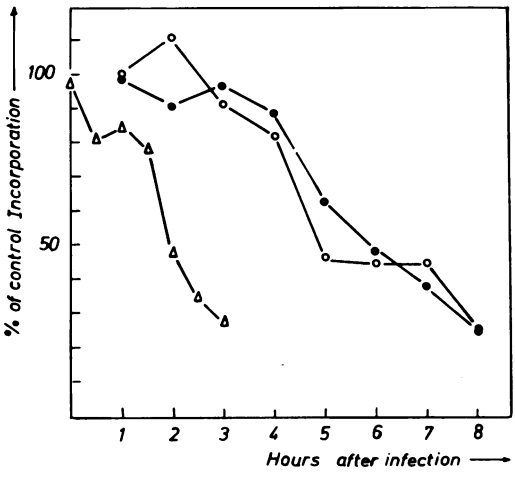

FIG. 3. *Cell protein synthesis inhibition by the Beaudette strain of NDV. See Fig. 1 for the experimental details. Rates of cell protein synthesis at different times after infection (●). Rates of cell protein synthesis at 8 hr after infection in cultures which received azauridine at different times after infection (○). Rates of cell protein synthesis at 8 hr after infection in cultures which received cycloheximide at different times after infection (△).*

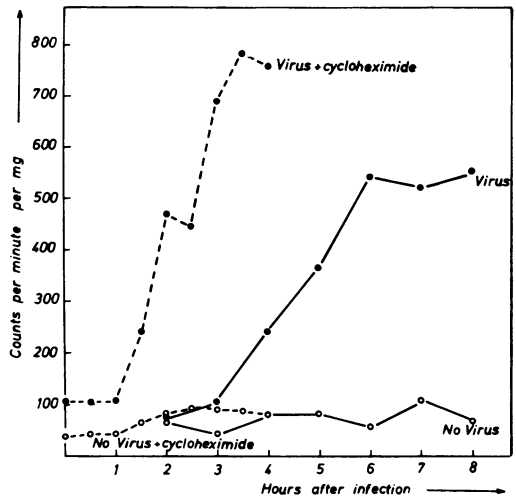

FIG. 4. *Viral RNA synthesis in cells infected with the Beaudette strain of NDV. See Fig. 2 for experimental details.*

Inhibition of cell protein synthesis by the Beaudette strain of NDV. Experiments similar to those shown in Fig. 1 were also performed with the Beaudette strain of NDV (Fig. 3). From 0 to 4 hr after infection, no significant virus-induced inhibition of cell protein synthesis was observed, and the addition of azauridine at any time during this period prevented the later development of protein synthesis inhibition. When azauridine was added at 5 hr after infection, or later, i.e., at times when some viral inhibition of cell protein synthesis had already taken place, the rate of protein synthesis at 8 hr postinfection was about the

same as the rate at the time of azauridine addition. Thus, addition of azauridine at any time after infection prevented the further development of protein synthesis inhibition by the Beaudette strain. As a control for this experiment, infected and uninfected cells were exposed to actinomycin D (2 μg/ml) from 0 to 8 hr after infection. At 8 hr after infection, the rate of protein synthesis in infected cells was 5% of the rate in uninfected cells. These results indicate that inhibition of cell protein synthesis by the Beaudette strain depends on viral RNA synthesis but not on host-cell RNA synthesis.

The addition of cycloheximide at the time of infection prevented the inhibition of cell protein synthesis by the Beaudette strain (Fig. 3). Addition of cycloheximide at 3 hr after infection, however, failed to prevent this process. This was probably due to the fact that viral RNA synthesis in cells infected with the Beaudette strain depends on protein synthesis in the period immediately after infection (Fig. 4). There was no direct evidence that inhibition of cell protein synthesis by the Beaudette strain depends on protein synthesis after the proteins necessary for viral RNA synthesis have been formed.

Inhibition of cell RNA synthesis by the Texas strain of NDV. Previous studies showed that NDV inhibits host-cell protein and deoxyribonucleic acid synthesis, but NDV was reported to have only a slight depressing or enhancing effect on cell RNA synthesis (7, 10). In the present study, it was found that the Texas strain of NDV inhibits cell RNA synthesis (Fig. 5). Azauridine and cycloheximide were used to study this process. Cultures were treated with these drugs as described above. In uninfected cultures which had been exposed to azauridine for 5 hr, the rate of cell RNA synthesis in the first 30 min after removal of azauridine was 46% of the rate in control cells, and the corresponding figure for cycloheximide was 58%.

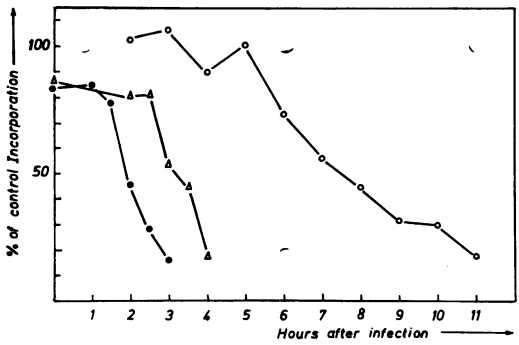

FIG. 5. *Cellular RNA synthesis inhibition by the Texas strain of NDV. The rates of cell RNA synthesis at different times after infection* (○), *and the effect of azauridine* (●) *and cycloheximide* (△) *on the virus-induced RNA synthesis inhibition was measured as in Fig. 1, with pulse media containing uridine-5-³H.*

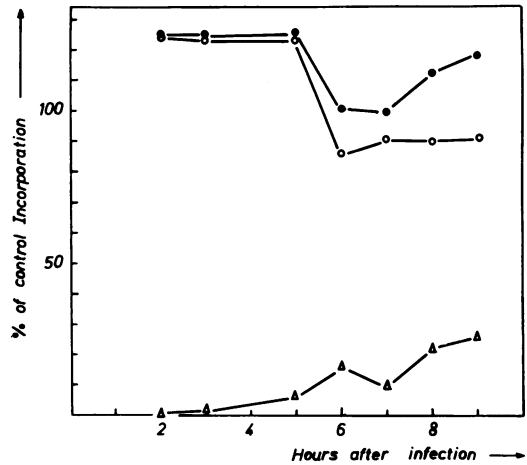

FIG. 6. *RNA synthesis in cells infected with the Beaudette strain of NDV. The rate of RNA synthesis in uninfected and infected cells was measured at different times after infection with a 30-min pulse of uridine-5-³H,* (●). *To measure the viral component of the total RNA synthesis, infected and uninfected cultures were overlaid with medium containing actinomycin D (2 μg/ml). At different times after infection, pairs of control and virus-infected cultures were pulsed for 30 min with medium containing actinomycin and uridine-5-³H. The data* (△) *show the incorporation in infected cells minus the incorporation in uninfected cells, plotted as the percentage of the rate of RNA synthesis in the untreated control cells. The rate of host-cell RNA synthesis* (○) *was calculated by subtracting the rate of viral RNA synthesis from the total rate of cellular RNA synthesis.*

The inhibition of cell RNA synthesis by the Texas strain was prevented by azauridine when added at the time of infection, but not when added at 3 hr after infection (Fig. 5). This result suggests that the RNA synthesis inhibition process depends on viral RNA synthesis.

The role of protein synthesis in this process may be deduced from the cycloheximide experiments also shown in Fig. 5. The addition of cycloheximide to cells at 2 hr after infection prevented the inhibition of cell RNA synthesis, although, as shown in Fig. 2, viral RNA synthesis was not prevented. These results indicate that viral RNA alone cannot inhibit cell RNA synthesis and that virus-induced protein synthesis is also necessary for this process.

RNA synthesis in cells infected with the Beaudette strain of NDV. The effect of Beaudette virus on cell RNA synthesis (Fig. 6) resembled the previously reported observations on other NDV

strains (7, 10). The total rate of RNA synthesis shown in Fig. 6 represents the sum of cell RNA synthesis and viral RNA synthesis. The rate of viral RNA synthesis in the presence of actinomycin was measured and then subtracted from the total RNA synthesis to obtain the rate of cell RNA synthesis. Since actinomycin usually suppresses the yield of infectious NDV by about 50% (7), the rate of viral RNA synthesis in infected cultures without actinomycin might be somewhat higher than the values shown in Fig. 6.

Beaudette virus infection stimulates the synthesis of cellular RNA in the period 0 to 5 hr after infection, as previously reported for the Italian strain (7). Beaudette virus clearly lacks the capacity to induce the severe depression of cell RNA synthesis which is observed in Texas virus-infected cells.

DISCUSSION

It was previously reported that the inhibition of cell protein synthesis by the Italian strain of NDV is reversible, and that infected cells recover the capacity to synthesize proteins by 10 hr after infection (7). The present studies were extended to 11 and 12 hr after infection with the Texas and Beaudette strains, but no recovery of the protein synthetic capacity of infected cells was observed.

A comparison of the results obtained with the Texas and Beaudette strains, and the previously reported results with the Italian (10) and Hickman strains (14), indicates that the ability of NDV to inhibit cell RNA synthesis is dependent on the virus strain. This observation is taken to indicate that the information for cell RNA synthesis inhibition is coded by the virus genome. Although normal amounts of viral RNA are synthesized in Texas virus-infected cells treated with cycloheximide at 2 hr after infection, no RNA synthesis inhibition develops. This result is taken to indicate that viral RNA alone cannot block cell RNA synthesis and that cycloheximide prevents cell RNA synthesis inhibition by blocking the formation of inhibitory proteins coded by the viral genome.

Although the cycloheximide experiments indicate that inhibitory proteins play a role in the inhibition of cell protein and RNA synthesis by the Texas strain of NDV, the azauridine experiments indicate that viral RNA synthesis is also necessary for this process. The inhibition of cell protein and cell RNA synthesis is prevented when azauridine is added immediately after infection. These results are consistent with the hypothesis that inhibition of cell protein and RNA synthesis by the Texas strain involves: (i) synthesis of virus-specific proteins which are responsible for the synthesis of viral RNA, followed by (ii) synthesis of viral RNA, which is then followed by (iii) synthesis of inhibitory proteins using mainly the newly formed viral RNA templates.

One question which is not resolved is why the incoming messenger RNA from the infecting virus particle does not directly code for inhibitory proteins, as appears to be the case with poliovirus (6). This could result from a regulatory mechanism which prevents the reading of part of the viral messenger RNA template. This could also be due to a requirement for the synthesis of larger quantities of protein than can be made on the limited number of viral messenger RNA molecules present in the cell at the time of infection.

Cell protein synthesis inhibition by the Beaudette strain of NDV appears to involve a different mechanism than in the case of the Texas strain. In the former case, azauridine blocks cell protein synthesis inhibition at times in the growth cycle when cycloheximide does not prevent this process. Since azauridine can prevent Beaudette virus-induced inhibition when added late in the infectious cycle, it is possible that viral RNA is, in some way, directly inhibiting cell protein synthesis. It is also possible, on the other hand, that inhibitory proteins formed soon after infection act in cooperation with viral RNA formed at later times to inhibit cell protein synthesis.

One may ask if the viral inhibition of cell protein synthesis is a specific effect caused by the virus, or the result of viral inhibition of cell messenger RNA synthesis. Since both Texas (1) and Beaudette strains inhibit protein synthesis in cells treated with actinomycin at the time of infection, we may conclude that the viral inhibition process does not depend on the inhibition of host-cell RNA synthesis. There is also evidence that viral inhibition of cell RNA synthesis is a specific virus-induced process, and is not caused by the lower rate of protein synthesis in infected cells. First, there are the observations that Beaudette and some other NDV strains do not inhibit cell RNA synthesis, although they do inhibit cell protein synthesis. Second, there is the observation that inhibition of protein synthesis by cycloheximide does not produce a rapid inhibition of cell messenger RNA synthesis (9). Third, the results shown in Fig. 1 and 5 indicate that the development of RNA synthesis inhibition does not lag behind the development of protein synthesis inhibition. Although virus-induced cell protein synthesis inhibition and cell RNA synthesis inhibition are separate processes, the possibility remains that, in Texas virus-infected cells, a single virus-induced protein is responsible for both processes.

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