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Inhibitory Proteins in the Newcastle Disease Virus-Induced Suppression of Cell Protein Synthesis

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ABSTRACT

BOLOGNESI, D. P. (Rensselaer Polytechnic Institute, Troy, N.Y.), AND D. E. WILSON. Inhibitory proteins in the Newcastle disease virus-induced suppression of cell protein synthesis. J. Bacteriol. **91**:1896–1901. 1966.—Infection by Newcastle disease virus brings about a rapid and marked inhibition of cell protein synthesis (CPS) in chick embryo fibroblast monolayers. The block to CPS is initiated about 5 hr after infection, and by 9 hr about 85% of the host protein synthesis is shut off. Azauridine (3 mg/ml), a ribonucleic acid (RNA) synthesis inhibitor, prevents the virus-induced inhibition of CPS when added at the time of infection; but it does not prevent the inhibition when added at 3 hr after infection. When puromycin (60 μg/ml), a protein synthesis inhibitor, was added at 3.5 hr after infection, viral RNA was synthesized in normal amounts, but the virus-induced inhibition of CPS was prevented. Actinomycin D added at the time of infection does not, however, prevent the virus-induced inhibition of CPS. The results of these experiments indicate that proteins synthesized during Newcastle disease virus replication are responsible for the inhibition of host-cell protein synthesis. The synthesis of these inhibitory proteins depends on the prior synthesis of viral RNA.

A number of workers have shown that various ribonucleic acid (RNA) animal viruses strongly suppress host-cell protein synthesis during their growth cycle. Wheelock and Tamm (13) and Wheelock (12) reported a rapid inhibition of protein synthesis in HeLa cells infected with Newcastle disease virus (NDV). A rapid depression of protein synthesis was also demonstrated in HeLa cells infected with poliovirus (2, 9, 15) and in L cells infected by members of the Columbia SK group of small RNA viruses (3).

As yet, little information has been obtained regarding the mechanism of this virus-induced inhibition of host protein synthesis. Holland and Peterson (6) demonstrated that HeLa cell protein synthesis is inhibited by poliovirus infection more rapidly than by actinomycin D. This indicated that the inhibition of protein synthesis is not an indirect result of suppression of cell messenger RNA by poliovirus. Hausen and Verwoerd (4) obtained a similar result using the ME-virus L-cell system. Furthermore, Rich et al. (8) demonstrated that infection of HeLa cells with poliovirus results in a gradual decrease in the number of cell polysomes.

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Wheelock (12) demonstrated that puromycin, a protein synthesis inhibitor, delayed the inhibition of host-cell protein synthesis by NDV. Verwoerd and Hausen (11) have suggested that the inhibition of host-cell protein synthesis by ME virus is the result of the synthesis of virus-specific early proteins.

Our experiments were undertaken to study the mechanism of NDV-induced inhibition of host-cell protein synthesis by puromycin and 6-azauridine. Wilson and LoGerfo (14), using specific inhibitors for RNA and protein synthesis, were able to fractionate the growth cycle of NDV in chick embryo fibroblast (CEF) monolayers. They showed that, shortly after infection, virus-specific proteins are synthesized which are necessary for viral RNA replication. The synthesis of these early proteins is not affected by addition of 6-azauridine, indicating that RNA synthesis is not required for their formation. Addition of puromycin immediately after infection, however, does prevent the synthesis of virus-specific proteins. This sensitivity to puromycin is lost by 4.5 hr after infection, indicating that by this time virus-specific protein synthesis has been completed.

From then on, it can be assumed that these

virus-specific proteins remain stable, since no further protein synthesis is required for viral RNA synthesis.

In the present investigation, the use of higher temperatures during experiments considerably shortened the growth cycle of the virus, so that the phase dependent on virus-specific protein synthesis terminated at about 3 hr after infection. Thereafter, viral RNA synthesis begins and reaches a peak at 9 hr after infection.

The results of these experiments indicated that proteins synthesized during virus growth are responsible for the inhibition of host-cell protein synthesis.

MATERIALS AND METHODS

Cells and virus. Primary monolayer cultures of CEF were prepared by trypsinization of 9-day-old embryos. Cells were grown in Hank's balanced salt solution (BSS), supplemented with 10% calf serum and 0.5% lactalbumin hydrolysate, for 48 hr before use. This was the standard medium. In some experiments, isotopes were used in a medium of Hank's BSS supplemented with 2% calf serum. The Texas (GB) strain of NDV was centrifuged and resuspended in phosphate-buffered saline (PBS). CEF 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 at 39 C. After adsorption, cells were washed with PBS to remove unadsorbed virus. The cultures were then incubated in medium to which the various inhibitors were added at the desired times.

Chemicals. DL-Leucine-*1*-C^{14} (2 mc/mmole), uniformly labeled L-leucine-C^{14} (250 mc/mmole), uridine-H^3 (7.7 c/mmole), and guanosine monophosphate-H^3 (GMP-H^3; 1.23 c/mmole) were obtained from New England Nuclear Corp., Boston, Mass., and Schwarz Bioresearch, Inc., Orangeburg, N.Y. Puromycin dihydrochloride (PU) and 6-azauridine (AU) were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Actinomycin D was a gift from Karl Pfister, Merck, Sharpe and Dohme Research Laboratories, Rahway, N.J.

Analytical procedures. RNA synthesis was measured by exposing cell cultures to medium containing uridine-H^3. Protein synthesis rates were measured by exposing cells to C^{14}-leucine medium for 30 min. The method of Schmidt and Thannhauser (10) was used to extract the RNA and protein fractions from cells. Alkaline hydrolysis was carried out overnight at room temperature in 0.3 N KOH. The hydrolysates from two cultures were pooled for each point. Protein synthesis was measured by counting the radioactivity in the alkaline hydrolysate. RNA synthesis was measured by counting the supernatant fluid derived from acidification of the alkaline hydrolysate with perchloric acid. Radioactivity in 0.2-ml samples was measured with a windowless flow counter (C^{14}) or a liquid scintillation counter (C^{14}, H^3).

To correct for variations in cell numbers in different cultures, total protein was determined by the method of Lowry et al. (7). The counts obtained are expressed as counts per minute per milligram of total protein.

RESULTS

Effect of 6-azauridine on the virus-induced inhibition of cell protein synthesis Figure 1 shows the time course of the cellular protein synthesis (CPS) inhibition by NDV. The block to CPS is initiated about 5 hr after infection, and by 9 hr the rate of host protein synthesis has declined by 85%.

The effect of actinomycin D on the virus-induced inhibition of CPS was investigated by exposing infected and control cultures to actinomycin D immediately after infection. The rate of CPS was measured 8.5 hr after infection. The results in Table 1 show that, although actinomycin has an inhibitory effect on CPS, it does not prevent the virus-induced inhibition of CPS. This result also indicates that the virus-induced inhibition of CPS does not depend on viral inhibition of cell RNA synthesis.

The role of viral RNA synthesis in the virus-

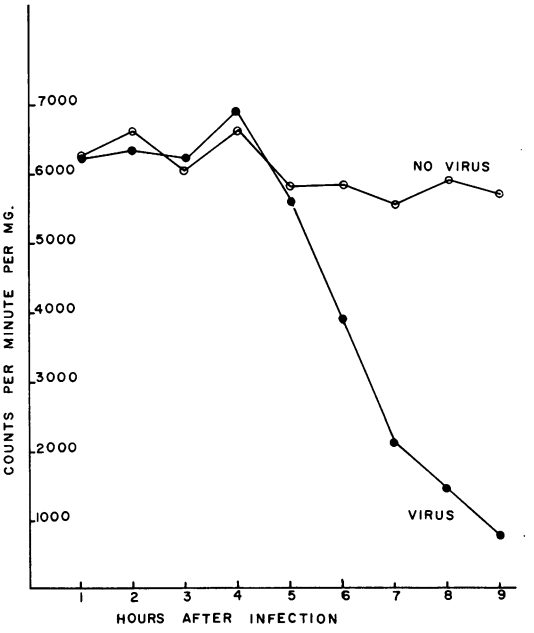

FIG. 1. *Cell protein synthesis inhibition by NDV. Infected and uninfected chick fibroblast cultures were overlaid with standard medium and incubated at 39 C. Beginning 1 hr after infection, and at successive 1-hr intervals thereafter, the medium on a pair of infected and uninfected cultures was discarded and replaced with Hank's BSS, 2% calf serum, and 0.066 μc of uniformly labeled C^{14}-leucine per ml. Duration of the leucine pulse was 30 min.*

TABLE 1. *Effect of actinomycin D on the inhibition of cell protein synthesis induced by NDV**

Culture	Actinomycin D	Counts per min per mg of protein
Virus	+	17
No virus	+	98
Virus	−	44
No virus	−	350

* Infected and uninfected fibroblast monolayers were overlaid with medium containing actinomycin D (10 μg/ml) immediately after infection. The controls were untreated. Protein synthesis was measured 9 hr after infection by pulsing with leucine-*1*-C^{14} medium (0.250 μc/ml) for 30 min.

induced inhibition of CPS was investigated by use of the RNA synthesis inhibitor 6-azauridine. This drug was used at a concentration of 3 mg/ml, which causes an inhibition of over 90% of cell and viral RNA synthesis but does not prevent virus-specific protein synthesis (14).

Azauridine was added at successive 1-hr intervals to infected and control cultures. At 9.5 hr after infection, the rate of protein synthesis in all cultures was measured. Since AU inhibits all messenger RNA synthesis, the rate of protein synthesis decreases with time in the AU medium. This effect can be seen in Fig. 2 where the control cultures show an increase in the rate of protein synthesis as the time in the AU medium decreases.

The results shown in Fig. 2 indicate that the inhibition of RNA synthesis at 2 hr after infection or earlier prevents virus-induced inhibition of CPS. The relative inhibition of CPS in virus-infected cultures remains almost constant when AU is added at 3 to 6 hr after infection. Since actinomycin D, which prevents cellular but not viral RNA synthesis, does not prevent virus-induced CPS inhibition, the action of azauridine must be directed against viral RNA.

These results indicate two things. (i) Viral RNA synthesis is necessary to bring about the maximal inhibition of CPS. (ii) The virus-specific proteins which have been shown by Wilson and LoGerfo (14) to be synthesized after infection and in the presence of AU are not responsible for this process. This then points to some process which is dependent on viral RNA as the cause of virus-induced inhibition of CPS.

Since actinomycin D does not prevent the virus-induced inhibition of CPS (Table 1), we may conclude that the only cell messengers coding for proteins are those synthesized prior to actinomycin treatment and virus infection.

Role of viral RNA in the inhibition of CPS. It was then of interest to determine how much viral

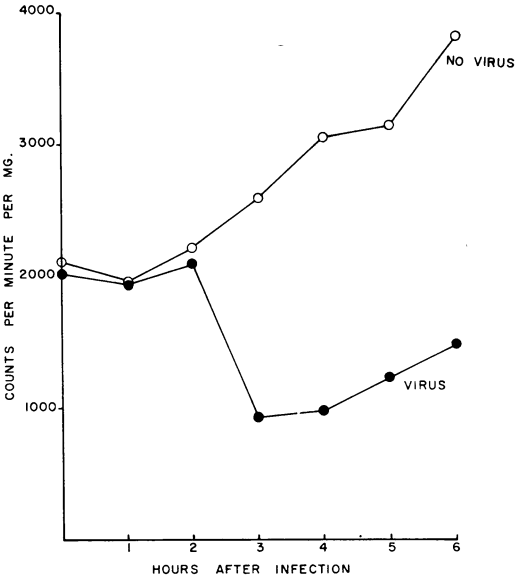

FIG. 2. *Cell protein synthesis inhibition by NDV in the presence of azauridine. Infected and uninfected CEF cultures were overlaid with Hank's BSS and 2% calf serum, and were incubated at 39 C. Beginning immediately after infection, and at successive 1-hr intervals thereafter until the 6th hr, the medium on a pair of infected and uninfected cultures was discarded and replaced with Hank's BSS, 2% calf serum, and azauridine (3 mg/ml). At 9.5 hr after infection, the medium on all cultures was discarded and replaced with Hank's BSS, 2% calf serum, azauridine (3 mg/ml), and 0.075 μc of uniformly labeled C^{14}-leucine per ml. Duration of the pulse was 30 min.*

RNA was necessary to bring about the inhibition of CPS.

Azauridine was added to infected and control cultures at successive 0.5-hr intervals beginning 3.5 hr after infection. Azauridine remained in each culture for 3 hr and then C^{14} pulse medium with AU was added for 30 min to measure the rate of CPS. Cultures which received AU 3.5 hr after infection showed some virus-induced inhibition of CPS at 6.5 hr, and cultures which received AU at 4.5 hr after infection showed essentially full virus-induced inhibition of CPS at 7.5 hr after infection (Fig. 3).

To be sure that additional RNA synthesis does not occur after the addition of AU, the rapidity with which this drug is able to inhibit cell RNA synthesis was tested. The results in Table 2 show that AU inhibits over 90% of the cell RNA synthesis within 15 min. The action of AU on cellular and viral RNA synthesis is quantitatively similar (14).

Viral RNA synthesis was measured at different times after infection by following the incorpora-

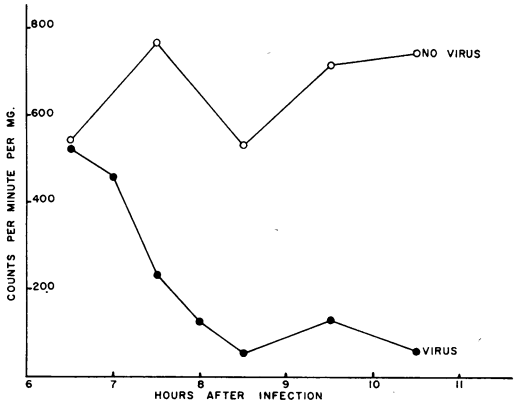

FIG. 3. *Cell protein synthesis inhibition by NDV after short exposures to azauridine. Infected and uninfected cultures were overlaid with standard medium and incubated at 39 C. At 3.5 hr after infection, and at 0.5-hr intervals thereafter until 7.5 hr after infection, the medium on a pair of infected and uninfected cultures was replaced with Hank's BSS, 2% calf serum, and AU (3 mg/ml). After a pair of cultures had been in the AU medium for 3 hr, this medium was replaced with leucine-1-C^{14} medium (0.225 μc/ml) containing AU. Pulse duration was 30 min.*

TABLE 2. *Inhibition of RNA synthesis by azauridine**

AU	Time	Counts per min per mg of protein
	min	
+	0	5
+	15	8
+	30	25
+	45	14
+	60	12
−	60	614

* Uninfected chick embryo fibroblast monolayers were overlaid with medium containing AU (3 mg/ml). The control was untreated. RNA synthesis was measured by incorporation of H^3-uridine (0.67 μc/ml) at the indicated times and read 15 min later.

tion of GMP-H^3 (Table 3). For comparison, the effect of virus on CPS (data from Fig. 1) and the effect of virus and AU on CPS (data from Fig. 3) are also shown in Table 3. One can see from the table that at 4.5 hr after infection 35% of the maximal amount of viral RNA has been synthesized and that no inhibition of CPS has occurred. When AU was added at 4.5 hr after infection, the rate of protein synthesis 3 hr later was depressed by the virus to the same level as observed in infected cultures which were not exposed to AU. Thus, as little as 35% of the viral RNA, if

TABLE 3. *Role of viral RNA in the inhibition of CPS induced by NDV**

Time after infection	Viral RNA relative to RNA synthesized 9 hr after infection	Virus-induced CPS inhibition	Virus-induced CPS inhibition with 3-hr AU treatment
hr	%	%	%
3.5	20	0	—
4	30	0	—
4.5	35	0	—
5	41	3	—
5.5	48	17	—
6.5	60	45	3
7	—	62	30
7.5	72	68	70
9	100	85	85

* Viral RNA synthesis was measured by addition of standard medium with GMP-H^3 (0.655 μc/ml) and actinomycin D (10 μg/ml) to infected and uninfected CEF monolayers. Pairs of cultures were withdrawn after incubation (39 C) at the indicated times. The effect of virus on CPS is calculated from Fig. 1. The effect of virus and 3-hr AU treatment on CPS is calculated from Fig. 3.

given enough time, can bring about a 70% inhibition of CPS, and this is accomplished in the presence of AU, under which conditions viral RNA cannot replicate further. Even smaller amounts of viral RNA may bring about an inhibition of CPS. The results shown in Fig. 2 indicate that the viral RNA synthesized at 3 hr after infection causes over 60% reduction in CPS at 9.5 hr after infection.

This result suggests two possibilities: (i) that viral RNA can act as a direct inhibitor of protein synthesis (if given enough time), or (ii) that during the period of AU treatment viral RNA is directing the synthesis of proteins which themselves act as the inhibitory agents.

Effect of puromycin on the inhibition of CPS. To determine whether protein synthesis is necessary for the virus-induced inhibition of the CPS, the effect of a protein synthesis inhibitor, puromycin, was tested.

AU was added to infected and uninfected cultures at 0.5-hr intervals beginning 3.5 hr after infection, as in the AU experiment shown in Fig. 3. In addition, puromycin was added to all cultures at 3.5 hr after infection and maintained until the rate of CPS was to be measured. PU was then removed by a quick wash with PBS containing AU, and C^{14} pulse medium was then added in the presence of AU. Cell protein synthesis was measured at times corresponding to the previous AU experiment. Viral RNA synthesis at 9 hr in cultures treated with puromycin at 3.5 hr was 26% greater than in untreated infected cultures.

Although approximately the same amounts of viral RNA were synthesized at each time in both experiments, protein synthesis was prevented in the latter experiment. Thus, if viral RNA is directly responsible for the inhibition of CPS, one would expect that PU would have no effect on this inhibition. If, however, protein synthesis is necessary for this process, CPS inhibition should then be prevented by PU. The results of this experiment are shown in Fig. 4. It can be seen that the virus-induced inhibition of CPS is significantly prevented by PU, whereas the normal degree of inhibition occurs in the presence of AU alone. One can therefore conclude that protein synthesis is necessary for the inhibition of CPS. These results do not show, however, whether these are virus-specific proteins or cellular proteins; but only that their synthesis is stimulated by the viral RNA.

DISCUSSION

The results of these experiments indicate that several steps in virus replication must take place before the virus can inhibit CPS. The synthesis of virus-specific proteins, which are necessary for the synthesis of viral RNA, must first occur. These virus-specific proteins are synthesized in the presence of azauridine (14), but since azauridine prevents the virus-induced inhibition of CPS (Fig. 2), we may conclude that these proteins are not able to inhibit CPS directly.

The virus-induced inhibition of CPS is also dependent on the synthesis of viral RNA. Wheelock (12) observed that puromycin delayed the NDV-induced inhibition of CPS in HeLa cells when added 1 hr after infection. The observed delay was equal to duration of the puromycin treatment (2 hr). The effect of puromycin in this case can be attributed to the inhibition of virus-specific proteins which are essential for viral RNA synthesis.

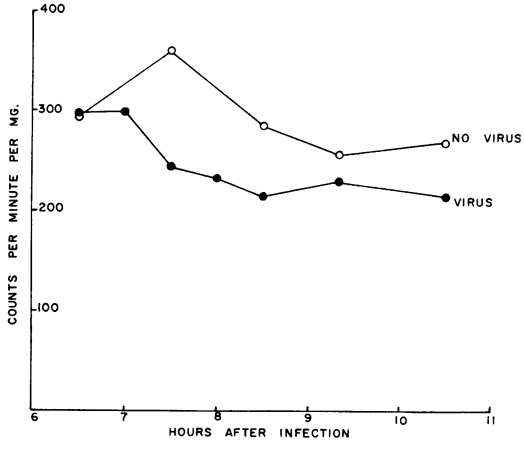

FIG. 4. *Cell protein synthesis inhibition by NDV in the presence of azauridine and puromycin. The same procedure was followed as in the experiment shown in Fig. 3 and, in addition, puromycin (60 μg/ml) was added to all cultures at 3.5 hr after infection.*

It appears that as little as 35% of the peak value of viral RNA is sufficient to initiate the last process leading to the inhibition of CPS. This phase of viral replication, immediately preceding the inhibition of CPS, depends on the synthesis of protein. Although it is clear that these inhibitory proteins are synthesized in response to the viral RNA, it is not known whether they have cell messenger RNA or viral RNA templates.

Since actinomycin D added at the time of infection does not prevent the virus-induced inhibition of CPS, we may conclude that this process does not depend on the synthesis of new messenger RNA molecules which have host genome templates. A similar effect of actinomycin D was observed by Hausen and Verwoerd (4) with ME virus in L cells. It seems most likely that in the case of NDV the inhibitory proteins have viral RNA templates.

If, as we suspect, viral messenger RNA is the template for inhibitory proteins, it then appears that, in the presence of azauridine, this viral messenger RNA of the entering particles is not able to direct the synthesis of sufficient quantities of these proteins to bring about the block to CPS. This may result from a viral regulatory mechanism which prevents the synthesis of enough inhibitory protein to block CPS.

Evidence for inhibitory protein was also obtained by Verwoerd and Hausen (11). They observed that *p*-fluorophenylalanine inhibits the suppression of host CPS by ME virus when added at the time of infection. These authors suggested that the action of *p*-fluorophenylalanine was directed against virus-induced "early proteins" which inhibit cellular protein and RNA synthesis. Evidence was obtained which indicated that a protein which blocks cell RNA synthesis was formed before viral RNA. It is interesting to note that Holland (5) obtained evidence that the suppression of host CPS by high multiplicities of poliovirus is caused by the viral RNA alone.

That the NDV-induced inhibition of CPS may be the result of degradation of cell polysomes (8) has not been determined, but it is an attractive hypothesis. This mechanism would enable the virus to "capture" the ribosomes actively for its own use to complete its growth cycle. If this is so, this inhibitory agent must be able to distinguish between the cell messenger polysome and the viral RNA-polysome complexes.

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