Studies of the Induction of Interferon System by Nonreplicating Newcastle Disease Virus[1] (34467)

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Viral infection frequently leads to induction of the interferon (IF) system. Indirect evidence indicates that the IF system is composed of two cellular proteins, IF, itself, and the proposed antiviral protein (AVP) which determines the antiviral resistance (AVR) (1–6). It is known that AVP can be directly induced by IF without the newly induced synthesis of intermediary proteins (5–7). However, it is not known whether AVP can be directly induced by viral infection independently of induction of IF or whether the induction of AVP by virus requires previous production of IF.

Another unresolved question is whether viral replicative events are required for induction of the IF system or whether input virus is capable of induction (8, 9). A study of these two unanswered questions was undertaken under conditions of alternate inhibition of protein and RNA synthesis as previously applied to related problems (5–7, 10).

Materials and Methods. Cycloheximide (Sigma Chemical Co.) was used at a final concentration of 10 μg/ml. DL-*p*-fluorophenylalanine (Mann Research Lab.) was used at a final concentration of 600 μg/ml. Actinomycin D (Merck, Sharp and Dohme) was used at a final concentration of 1 or 2 μg/ ml. The activity and/or the reversibility of the drugs was controlled biologically (effect on virus growth) and biochemically (effect on incorporation of labeled precursors) as described below.

Vaccine F strain of Newcastle disease virus (NDV), at a multiplicity of 100 egg infectious doses per cell, was used as the inducer of IF and AVR. For production or assay of IF and AVR, a strain of mouse L cells derived from clone CCL-1 was used in a single-cycle virus yield reduction test as previously described (11). Semliki Forest virus (SFV) was used as the challenge virus. IF samples were acidified at pH 2 and maintained at 4° for 7 days before neutralization and assay. SFV was assayed by viral plaque formation.

The effect of metabolic inhibitors on protein and RNA synthesis was studied by determining the rate of incorporation of ^{14}C-isoleucine and ^{3}H-uridine (0.5 μCi/ml; 30-min pulse in isoleucine-free or plain Eagle's medium). The amount of acid-precipitable incorporated label [phenol extracted RNA was used for evaluation of ^{3}H-uridine incorporation (12)] was determined in a Nuclear Chicago MK-1 scintillation counter.

Results. Effect of treatment of NDV-induced CCL-1 cells with inhibitors of protein synthesis followed by replacement with actinomycin D. Experiments were carried out to help determine whether the mRNA's for IF and AVP are produced in NDV-infected L cells during inhibition of protein synthesis. NDV does not replicate in these cells but is a strong inducer of IF and AVR (presumably determined by AVP). L cells were treated with NDV and cycloheximide for 4 hr at 37° (cycloheximide was added to the cells 15 min earlier than NDV). To insure further that no residual protein synthesis could take place during the first 5 hr an additional series of cultures was treated with fluorophenylalanine (FPA), another inhibitor of protein synthesis, together with cycloheximide at the start of the experiment. After 4 hr actinomycin D was added and 1 hr later the cultures

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TABLE I. Effect of Treatment of L Cells with NDV and Protein-Synthesis Inhibitors Followed by Replacement with Actinomycin D.

Treatment for 4 hr before addition of actinomycin D	Log_{10} yield of SFV and reduction of yield ( ) as compared with untreated control cultures at the:		IF yield (units/ml) at the:	
	Fifth hr	Eighth hr	Fifth hr[a]	Eighth hr
Experiment 1				
Control	6.0	6.0	<3	<3
NDV	3.7 (2.8)	3.7 (2.3)	330	330
NDV + cyclo	6.1 (0)	6.0 (0.1)	<3	330
NDV + cyclo + FPA	6.0 (0)	6.2 (0)	ND	330
Cyclo	6.1	6.1	<3	<3
Cyclo + FPA	6.0	6.1	ND	<3
Experiment 2				
Control	6.3	6.4	<3	<3
NDV	4.9 (1.4)	<3.7 (>2.7)	10	>30
NDV + cyclo	6.6 (0)	6.7 (0)	<3	>33
NDV + cyclo + FPA	6.4 (0.1)	6.8 (0.1)	ND	10
Cyclo	6.5	6.7	<3	<3
Cyclo + FPA	6.5	6.7	<3	<3
Experiment 3				
Control	6.2	6.4	<3	<3
NDV	3.9 (2.2)	3.7 (2.7)	33	330
NDV + cyclo	6.1 (0.2)	6.4 (0.5)	<3	33
NDV + cyclo + FPA	6.2 (0.1)	6.7 (0.5)	ND	100
Cyclo	6.3	6.9	<3	<3
Cyclo + FPA	6.3	7.2	ND	<3

[a] ND = not determined.

were washed four times, refed with fresh Eagle's medium not containing inhibitors and incubated for 3 more hr. IF and AVR were assayed at the fifth and at the eighth hr. During inhibition of protein synthesis by cycloheximide (first 5 hr) it has been shown that messenger RNA (mRNA) for newly induced proteins, but not the proteins themselves, can be synthesized (6, 7, 10, 13). When both actinomycin D and cycloheximide were present between the fourth and fifth hours neither proteins nor RNA could be synthesized. Finally, after washing at the fifth hour, which reverses the action of cycloheximide but not that of actinomycin D, translation of previously transcribed mRNA's can occur but no newly formed mRNA's can be transcribed.

The results are reported in Table I. It can be seen that neither AVR nor IF production occurred in NDV-infected cultures when cycloheximide or cycloheximide plus FPA were present (5-hr samples). After their removal and in the presence of actinomycin D action, amounts of IF often comparable with those produced by the cultures treated with NDV alone were produced, but no AVR developed. The production of interferon during the 3-hr period after reversal of inhibition of protein synthesis indicates that the mRNA for IF had been produced by the NDV-infected cells during the 5-hr period of inhibition of protein synthesis. Analogously the failure of AVR to develop under the same conditions indicates that the mRNA for the AVP was not produced by the NDV-infected cells during inhibition of protein synthesis. This finding will be discussed below.

Control experiments showed that: (1) the same concentrations of cycloheximide and FPA were able to inhibit more than 99% of SFV yield when left in contact with the cells

TABLE II. Effect of Cycloheximide[a] and FPA[b] on ^{14}C Isoleucine Incorporation[c] in L Cells.

Treatment	Time of treatment (min)	^{14}C counts/min (×100)	% Inhibition
None	30	139.4	
Cyclo	30	9.3	93.6
Cyclo + FPA	30	4.6	97.0
None	60	120.4	
Cyclo	60	6.4	95.1
Cyclo + FPA	60	3.1	97.9
None	120	106.0	
Cyclo	120	7.6	93.2
Cyclo + FPA	120	2.8	97.8

[a] 10 μg/ml.
[b] 600 μg/ml.
[c] 0.5 μCi/ml; 30-min pulse.

during the viral infection; (2) cells treated with the metabolic inhibitors for the times indicated below gave full virus yield after the removal of the drugs; (3) the dose of actinomycin D used in the experiments was able to prevent completely the development of IF and AVR when applied at the same time as NDV.

As the presence of actinomycin D in the 5-hr harvests could affect the IF assay (1–4), some of the cultures were kept in contact with cycloheximide for 5 hr without addition of actinomycin D, then harvested, dialized, and assayed for IF. In this way it was shown that IF was not produced during the 5 hr of cycloheximide's presence.

Effect of metabolic inhibitors on protein and RNA synthesis. Table II reports the results of a representative experiment carried out to test the effect of cycloheximide and cycloheximide plus FPA on protein-synthesis inhibition in L cells. It can be seen that in both conditions ^{14}C-isoleucine incorporation was almost completely inhibited after 30 min of treatment with the drugs.

In addition further experiments (Table III) demonstrated that: (1) inhibition of protein synthesis by cycloheximide was effective through the entire period of the experiments; (2) 4 hr of cycloheximide treatment resulted in at most a 17% inhibitory effect on RNA synthesis; (3) the inhibitory activity of cycloheximide was fully reversible; (4) actinomycin D, when applied to L cells for 1 hr, was capable of inhibiting RNA but not protein synthesis and its action was irreversible after washing; (5) when combined, the metabolic inhibitors affected RNA and protein synthesis independently, thereby indicating absence of interaction.

Determination of replication activity of NDV in L cells. As the ability of the virus inducer of IF to undergo partial or complete replication would influence the interpretation of the results, replication of NDV in L cells was studied. No production of infectious virus or hemagglutinin was detected by biological assay. To evaluate the possibility that some abortive synthesis of viral RNA might take place, L cells that were treated with

TABLE III. Effect of Cycloheximide and Actinomycin D on ^{3}H-uridine and ^{14}C-iso-Leucine Incorporation in L Cells.[a]

Expt. no.	Hr after addition of the drugs: Cycloheximide	Hr after addition of the drugs: Actinomycin	Min after reversal of cycloheximide	^{3}H counts /min (× 100)	% Inhibition	^{14}C counts /min (× 100)	% Inhibition
1	0	0	0	155.7		76.7	
	4	0	0	129.0	17.2	7.5	90.1
2	0	0	0	127.8		66.0	
	5	1	0	12.2	90.4	5.4	91.8
	0	0	30	89.0		58.4	
	5	1	30	6.5	92.7	69.8	0

[a] Cycloheximide = 10 μg/ml; actinomycin D = 1 μg/ml; ^{3}H-uridine and ^{14}C-iso-leucine = 0.5 μCi/ml; 30-min pulse; timing of metabolic inhibitors as in Table I and accompanying text.

TABLE IV. Effect of RNase Treatment on ^{32}P RNA Extracted from Cells Infected with NDV in Presence or Absence of Protein-Synthesis Inhibitors.

	Treatment of the cells before RNA extraction	Amount of radioactivity in the sample (counts/ml)	Acid-precipitable radioactivity after RNase treatment in:	
			1 *M* NaCl	0.01 *M* NaCl
1	NDV + cycloheximide + FPA	50,000	914	548
2	NDV	50,000	834	274
3	Cycloheximide + FPA	50,000	963	379

actinomycin D (1 μg for 1 hr) were infected with NDV and at preestablished times the rate of ^{3}H-uridine incorporation was determined. The results showed that no increase of ^{3}H-uridine incorporation occurred over 4 hr of infection, suggesting absence of synthesis of viral RNA. An additional experiment was carried out to test the ability by NDV to produce RNase-resistant double-stranded RNA in L cells in the presence or absence of cycloheximide and FPA. Three liters of spinner L cells containing 1.5×10^{10} cells were divided into three lots. One lot was infected with NDV (100 egg infectious doses per cell) in the presence of cycloheximide (10 μg/ml) and FPA (600 μg/ml); a second lot was infected with NDV without inhibitors, and the third lot received inhibitors but was not infected. Four hours later actinomycin D (1 μg/ml) was added to each lot and 1 hr later 18 mCi of carrier-free ^{32}P Na_3PO_4 were added. After a 30-min pulse the cells were washed three times with phosphate-buffered saline in the presence or absence of the inhibitors. RNA was phenol-extracted (12), and the amount of incorporated radioactivity was determined. Two portions of RNA from each lot containing 50,000 cpm were dissolved in 1 *M* and in 0.01 *M* NaCl respectively and then treated with RNase (5 μg/ml) at 37°. Twenty minutes later residual acid-precipitable radioactivity was measured. The results are reported in Table IV. It can be seen that there was no substantial difference in the amount of residual radioactivity among the three groups, indicating that no detectable amounts of RNase-resistant RNA were produced in cells infected with NDV in presence or absence of inhibitors of protein synthesis.

Discussion. An outline of sequential cellular events leading to the production of IF and AVP in NDV-treated L cells can be proposed on the basis of the experimental results reported above (Table I). It was shown that while protein synthesis was inhibited by cycloheximide, neither IF nor AVP was synthesized in NDV-induced cells. However, since IF but not AVR was produced during the 3-hr period after reversal of inhibition of protein synthesis, it can be concluded that the mRNA for IF but not the mRNA for the AVP was transcribed during the 5-hr period of inhibition of protein synthesis. The accumulation of mRNA during treatment of certain cells with cycloheximide has been reported (5–7, 10). Previous experiments on mouse embryo cells showed that mRNA for IF-induced AVP is stable under cycloheximide activity (6, 7) and similar experiments carried out in L cells confirmed this finding. The failure of AVR to develop under these conditions indicates that although NDV was able to induce the mRNA for IF, NDV could not induce the mRNA for AVP without the mediation of the IF protein. Thus, despite the observation that AVR is often detectable before IF in stimulated cells (14), including the present system (15), the production of AVP in NDV-infected cells requires the prior production of IF.

The failure of NDV to undergo replicate events was evidenced by the inability of NDV-infected L cells to produce virus-specific RNA, hemagglutinin, and infectious virus. Under the experimental conditions viral replication was further prevented by cycloheximide. Even if very small amounts of viral-specified proteins could be produced in the presence of cycloheximide, they would be

nonfunctional due to the additional presence of FPA (16). The finding that the mRNA for IF may be produced in the absence of even partial replication by NDV indicates that the stimulus for induction of IF in this system is provided by a component of the input virus or an associated physical event.

Studies employing temperature-sensitive arboviruses (8, 9) suggest a requirement of partial replication for induction of IF by these viruses. This may indicate either the need for the formation of an inducing substance not present in the input virus or of larger quantities of an inducing substance which is present in the input virus but in insufficient quantity. The difference between their and our results may also be due to differing experimental conditions. For example, a very high multiplicity of virus infection and a different cell system were used in the present study.

If the interferon-inducing component of NDV is RNA, and since the input RNA of NDV is single-stranded, then it may be concluded that at least certain single-stranded viral RNA's are capable of inducing IF. This view would be consistant with the findings that natural and synthetic single-stranded RNA's can induce IF (17–19).

Summary. Mouse L cells infected with nonreplicating Newcastle disease virus first developed antiviral resistance and then produced the interferon protein. Such infected cells, during inhibition of protein synthesis by cycloheximide, did not produce viral components or interferon and did not develop antiviral resistance. However, after reversal of protein inhibition and the addition of actinomycin D interferon was produced but antiviral resistance failed to develop. The production of interferon but not antiviral resistance after reversal of inhibition of protein synthesis was interpreted to indicate that the mRNA for interferon, but not the mRNA for antiviral resistance, was transcribed during infection in the presence of inhibitors of protein synthesis. The failure of development of the proposed mRNA for antiviral resistance in the absence of production of interferon suggests that induction of resistance by this virus is mediated by interferon. Finally, the production of the mRNA for interferon in the absence of even partial replication of Newcastle disease virus indicates that the stimulus for induction of interferon in this system is provided by a component of the input virus or an associated physical event.

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