Inhibition of Arbovirus Protein Synthesis by Interferon

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Infection of cells treated with guanidine and actinomycin D and then washed to remove the guanidine inhibition of virus growth had no effect on antiviral activity already established by interferon. Protein synthesis in interferon-treated cells infected under these conditions was decreased as compared to control cells similarly treated but not exposed to interferon. In these control cells, analysis by polyacrylamide gel electrophoresis indicated that six proteins were produced during the first hour after guanidine reversal. Five of these proteins have been previously identified as probably being viral in origin. In interferon-treated cells, only a single major protein was produced. Ribonucleic acid (RNA) synthesis by Semliki Forest virus during the first hour after guanidine reversal was markedly depressed by incubation at 42°C, but no inhibition of total virus protein synthesis was seen; this finding suggested that much of the virus protein produced in the first hour after guanidine reversal was carried out by input virus RNA. Interferon was fully active in cells incubated at 42°C. The results suggested that interferon inhibits the production of Semliki Forest virus proteins ordinarily produced under the direction of the virus genome.

Recent studies have indicated that the mechanism of action of interferon involves a step between the transcription and the translation of virus genetic information (7). Additional studies have suggested that the step inhibited may be the binding of parental viral ribonucleic acid (RNA) to the ribosome or to a ribosomal subunit (2, 10) or the translation of viral RNA (10). Moreover, it has been shown that the production of viral polymerases relatively late in infection by menogovirus (12) or Semliki Forest virus (15) is decreased by interferon and that the entry of parental Semliki Forest virus into a ribonuclease-resistant form is also inhibited by interferon treatment (5). The latter result suggested that at least one virus protein, the viral RNA polymerase, is not produced in interferon-treated cells during SFV infection. In the present study, the effect of interferon treatment on the production of several viral proteins very early in infection was investigated. Interferon treatment was found to inhibit the production of SFV proteins.

MATERIALS AND METHODS

Details on the following methods are included in the accompanying paper (4) from this laboratory: preparation of chick embryo fibroblast (CEF) monolayer cultures, Semliki Forest virus (SFV) pools, and purified SFV; assay of viral infectivity and interferon;

estimation of cellular RNA and protein synthesis; and polyacrylamide gel electrophoresis.

Infection procedure. CEF in monolayers containing about 5×10^6 cells were treated for 1 hr with actinomycin D (2 µg/ml). They were then infected at a virus-cell multiplicity of 250 plaque-forming units (PFU) in the presence of guanidine (3 mg/ml). After 4 hr, the cells were washed five times and fresh medium was added.

Interferon. A partially purified pool of chick interferon prepared by the method of Fantes was employed (3). This pool contained 10,000 units of interferon per ml and 179 µg of protein per ml. It was previously characterized and found to have all of the usually described properties of interferon (5). The preparation was donated by Karl Fantes, Glaxo Laboratories, Ltd., Stoke Poges, England.

Reagents. Actinomycin D was donated by Merck Sharp and Dohme, Rahway, N.J. ³H-leucine (38 mc/mg) and ³H-uridine (2 c/mole) were purchased from New England Nuclear Corp., Boston, Mass., and from Schwarz BioResearch Inc., Orangeburg, N.Y., respectively. The purified ¹⁴C-amino acid mixture (54 mc/matam carbon) employed was prepared by the Radiochemical Centre, Amersham, England, from *Chlorella* protein hydrolysate.

RESULTS

Effect of guanidine treatment on SFV-infected cells. In a previous study (4), it was shown that SFV replication and RNA synthesis were mark-

edly inhibited by infection of CEF in the presence of actinomycin D (2 μ g/ml) and guanidine (3 mg/ml). Protein synthesis was depressed to less than 3% of controls under these conditions. When guanidine inhibition was reversed by washing the cells, virus replication and RNA and protein synthesis began immediately, but cell protein synthesis remained inhibited.

Lack of guanidine effect on interferon action. Since the object of this study was to determine the effect of interferon on early virus protein synthesis by the methods previously discussed, it was mandatory to show that this treatment had no effect on the antiviral activity of interferon. The data shown in Table 1 indicate that the guanidine-actinomycin D treatment employed was not inhibitory to the antiviral activity of interferon. As previously shown, virus growth was somewhat greater in cells treated with actinomycin D (17).

Another way of studying the antiviral activity of interferon is the assay of protein synthesis in cells treated with interferon and then infected with SFV in the presence of guanidine and actinomycin D. After reversal of guanidine inhibition, almost all of the protein produced was viral in origin (4). Interferon treatment under these conditions gave rise to more than a fivefold inhibition of protein synthesis (Table 2). Since interferon treatment had no effect on cell protein synthesis in this system (5), the marked inhibition of protein synthesis observed must have been specific for virus-directed protein synthesis. These results indicate, therefore, that the antiviral

TABLE 1. *Lack of guanidine effect on interferon action*

Treatment ^a	Virus titer (PFU)	Virus growth as a percentage of control
None.....	67×10^{-7}	100
Interferon.....	9×10^{-7}	13
Actinomycin D, guanidine.....	95×10^{-7}	100
Interferon, actinomycin D, guanidine.....	14×10^{-7}	15

^a Monolayers of chick cells were treated with 5 units of partially purified chick interferon per ml for 14 hr. These cells and controls were then washed five times and were treated with guanidine (3 mg/ml) and actinomycin D (2 μ g/ml) or medium as described in Materials and Methods. After 4 hr, the cells were again washed and infected with Semliki Forest virus at a multiplicity of 20 PFU/cell. Cells were washed after 1 hr, and, after 6 additional hr of incubation, virus was harvested by freezing and thawing. The fluids were then assayed for virus titers.

TABLE 2. *Inhibition of protein synthesis in interferon-treated cells*

Treatment ^a	Specific activity (counts per min per μ g of protein)
None.....	46.4
Interferon.....	8.0

^a Chick cells were treated with 1,000 units of purified chick interferon per ml for 14 hr. After washing, these cells and untreated control were incubated with actinomycin D (2 μ g/ml) and were infected with Semliki Forest virus in the presence of guanidine (3 mg/ml). After 4 hr, the monolayers were washed and incubated for 1 hr with 10 μ C of a ¹⁴C-amino acid mixture per ml. Total protein and acid-precipitable radioactivity were then determined.

activity of interferon persists despite infection with virus in the presence of guanidine and actinomycin D.

Nature of proteins produced in interferon-treated cells. To study the nature and number of proteins produced in interferon-treated, virus-infected cells, extracts of the cytoplasm of virus-infected cells were analyzed by polyacrylamide gel electrophoresis (16). Microdensitometer tracings from autoradiograms of gels on which various cytoplasmic extracts had been analyzed are shown in Fig. 1. When ¹⁴C-amino acids were incubated with infected CEF for 1 hr immediately after reversal of guanidine inhibition, the autoradiogram showed six distinct lines (Fig. 1A). Five of these lines have previously been identified in SFV-infected CEF, 1 to 2 hr after guanidine reversal. These lines are identified in Fig. 1A, as they were previously, by the designations 1, 2, or 3 for proteins which are present in the virion and A and B for nonstructural proteins. The protein not previously identified is designated C' (Fig. 1A).

In cells treated with 1,000 units of partially purified interferon per ml for 14 hr before infection and then treated as the specimen shown in Fig. 1A, only a single protein could be clearly identified, even after prolonged exposure of films to the dehydrated gel slices (Fig. 1B).

In uninfected CEF treated with actinomycin D and guanidine and then washed (Fig. 1C), a pattern of lines qualitatively similar to that seen in untreated and uninfected CEF was present (4). The prominent lines in the autoradiogram corresponded to the major lines present in the stained gel.

These results suggested that interferon inhibited the accumulation of SFV proteins. Peak C' in Fig. 1A or the single peak present in inter-

FIG. 1. Polyacrylamide gel electrophoresis of interferon-treated and control cell cytoplasmic extracts. Chick cell monolayers were treated with 1,000 units of partially purified chick interferon per ml for 14 hr. These cells and untreated controls were treated with actinomycin D (2 μ g/ml) and were infected in the presence of guanidine (3 mg/ml) as described in Materials and Methods. After 4 hr, the cells were washed and 10

feron-treated cells (Fig. 1B) may not be a viral protein, since both corresponded in position to the major chick cell protein produced (Fig. 1C), and since protein C' is not present in autoradiograms made from cytoplasmic extracts of cells labeled from 1 to 2 hr (instead of 0 to 1 hr) after virus infection (4).

Effect of growth at 42 C on virus protein synthesis. The preceding results suggested that interferon treatment inhibited the synthesis of SFV proteins, but it is well known that interferon treatment inhibits virus RNA synthesis (11); thus, it is possible that the results observed might have been secondary to an inhibition of progeny virus RNA production. The bulk of the protein synthesized in the first hour of infection under the conditions employed, however, may be produced under the direction of input virus RNA. To indicate whether this was likely, advantage was taken of the fact that SFV is a temperature-sensitive virus which does not grow at 42 C (14).

Even though no virus multiplication took place in cultures incubated at 42 C after guanidine reversal, some actinomycin D-resistant RNA was produced (Table 3). This, however, amounted to only 12% of that produced in similar cultures incubated at 37 C. On the other hand, no inhibition of protein synthesis was seen in cultures incubated at 42 C. These results indicated that, under conditions which markedly reduced the production of viral RNA, no inhibition of protein synthesis took place. The bulk of the viral protein made in the first hour after guanidine reversal, therefore, may be produced by input RNA.

The nature of the protein produced in the infected cells incubated at 42 C after guanidine reversal is shown in the tracing from an autoradiogram of a gel pattern produced by extracts of these cells (Fig. 2A). Virus proteins were present. Therefore, most of the protein produced

μ C of a 14 C-amino acid mixture per ml was added in otherwise amino acid-free medium. After 1 hr, cytoplasmic extracts were prepared by the method of Summer et al. (16). Polyacrylamide gel electrophoresis was performed on 50- to 150- μ l samples of these extracts containing about 20,000 counts/min. After the run, the gels were sliced and stained; autoradiography was performed with dehydrated gel slices. Microdensitometer scans were made on the autoradiograms: (A) infected cells, no interferon treatment; (B) infected cells, interferon treatment; (C) extract of cytoplasm of uninfected chick cells treated with actinomycin D and guanidine. Film was exposed to the dehydrated gels for 7 days (A, C) or 14 days (B). Longer exposure did not reveal additional lines. Arrows in this and other densitometer tracings indicate the cathodal end of the gel. All densitometer tracings shown in this study are direct photographs of the original tracings.

FIG. 2. Polyacrylamide gel electrophoresis of interferon-treated and control cell cytoplasmic extracts. Chick cells were treated with interferon and were then

TABLE 3. Effect of incubation at 42 C on viral RNA and protein synthesis^a

Incubation temp (C)	Specific activity (counts per min per μ g of protein)	
	RNA	Protein
37	15.9	12.1
42	2.0	18.8

^a Actinomycin D-treated (2 μ g/ml) cells were infected with virus in the presence of guanidine (3 mg/ml) as previously described. After washing, the cells were incubated for 30 min at 37 or 42 C in Eagle's medium. The cells were then pulse labeled for 10 min with 10 μ c of ³H-uridine or ³H-leucine per ml. Total protein and acid-precipitable radioactivity were then determined.

in these cells was viral in origin. Interferon was active in virus-infected cells incubated at 42 C after guanidine reversal, since the electrophoretogram of such interferon-treated cells incubated at 42 C contained only one distinct band (Fig. 2B).

An additional observation may be made on the tracing shown in Fig. 2A. Proteins A and B appear to be reduced in amount when compared to the distinct peaks of these proteins seen in Fig. 1A. Current studies suggest that the rate of destruction of proteins A and B at 42 C probably exceeds their rate of synthesis at this temperature.

DISCUSSION

The results of this investigation indicate that interferon inhibits SFV growth by inhibiting the production of SFV proteins. Experiments performed at 42 C suggest that much of the protein ordinarily produced during the first hour of infection after guanidine reversal may be formed under the direction of parental (input) virus RNA.

The conclusion that interferon may inhibit the synthesis of viral proteins formed under the direction of parental virus RNA is consistent with previous findings. Interferon treatment did not inhibit the formation of vaccinia virus messenger RNA, but polysomes containing this RNA failed to form in interferon-treated cells (7). Interferon treatment has also been shown to inhibit the entry of parental mengovirus 37S RNA into a 50S cytoplasmic form (9). It was suggested that

infected as described in the legend to Fig. 1. The cells were labeled as described but incubation was at 42 C for 1 hr. Extracts were made and run on polyacrylamide gels; the radioautograms prepared from the gels were scanned as previously described. (A) Incubation for 1 hr at 42 C, no interferon treatment. (B) Treated with 1,000 units of interferon per ml before infection and incubated for 1 hr at 42 C after guanidine reversal.

this form normally represents an attachment of the 37S RNA to a 40S ribosomal subunit as a prelude to viral polysome formation. Finally, interferon treatment has been shown to inhibit the formation of a ribonuclease-resistant RNA form from parental (input) Semliki Forest virus RNA (5). Since the polymerase responsible for this process was fully active in the presence of purified interferon (15), the results suggested that the enzyme was not formed in interferon-treated cells rather than that it was inactivated by interferon.

Previous studies have indicated that cellular RNA and protein synthesis are necessary for the development of antiviral activity in interferon-treated cells (6, 8, 17). Results in cell-free systems have suggested that interferon alters the ability of viral RNA to bind to cell ribosomes. These results have led to the notion that a cell protein induced by interferon treatment binds to ribosomes or to ribosomal subunits and inhibits the attachment (2, 9, 10) or translation (10) of viral RNA. However, these *in vitro* studies (2, 10) have not been confirmed by investigations carried out in two laboratories (J. A. Sonabend, *personal communication*; R. Z. Lockart, *personal communication*), and it is therefore possible that the site of antiviral activity is elsewhere than directly on the ribosome. Therefore, on the basis of available evidence, the primary site of interferon action could just as well be on viral RNA, transfer RNA, ribosome-messenger RNA binding proteins (13), or another factor, as directly on the ribosome. Recent findings indicating that interferon treatment inhibits the growth of *Toxoplasma gondii* (J. S. Remington and T. C. Merigan, *personal communication*) or *Malaria berghei* (R. I. Jahiel et al., *personal communication*) also make it unlikely that the site of action of interferon is directly on host ribosomes.

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