Action of Interferon: Kinetics and Differential Effects on Viral Functions

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A highly purified rabbit interferon was tested for its capacity to inhibit various manifestations of infection of primary rabbit kidney (RK) cells with vesicular stomatitis (VS) virus. A kinetic analysis of the actinomycin-sensitive phase of interferon-induced cellular resistance revealed that RK cells could transcribe virtually all of the hypothetical antiviral messenger ribonucleic acid (mRNA) within 3 hr. Similar exposure to interferon reduced virus yield by 95 to 99% and markedly inhibited cytopathic effect on RK cells infected at a multiplicity of 10 or less. Interferon was less effective in blocking cytopathic effects when RK cells were infected at a multiplicity of 100. However, RK cells pretreated with the same amount of interferon and infected at a multiplicity of 100 failed to incorporate ³H-amino acids into structural or nonstructural proteins of VS virus identified by polyacrylamide gel electrophoresis. Despite this inhibition of viral protein synthesis, interferon did not prevent the switch off by VS virus of cellular protein synthesis. The rapidity with which a high multiplicity of VS virus switched off cellular protein synthesis, even in cells rendered resistant to viral infection by interferon, is further evidence that this reaction is caused by an infecting virion component rather than by a newly synthesized viral product.

These experiments were designed to compare the capacity of interferon to induce cellular resistance to the following four consequences of viral infection: (i) viral replication, (ii) cytopathogenicity, (iii) viral protein synthesis, and (iv) viral switch off of cellular protein synthesis.

The system we selected for these studies consisted of primary rabbit kidney (RK) cells infected with vesicular stomatitis (VS) virus (19). Stewart et al. (21) have recently shown that VS virus is more sensitive to rabbit interferon than to interferons prepared and tested in cells of other animal species. In addition, we had available from other experiments stocks of purified RK interferon of extremely high specific activity, as previously reported (28). This system also seemed ideal for analyzing the kinetics of interferon action by timing its dependence on the capacity of RK cells to transcribe the hypothetical antiviral messenger ribonucleic acid (mRNA).

MATERIALS AND METHODS

Cell cultures and virus. Primary RK cells obtained from minced, trypsinized kidney tissue of 3-week-old

rabbits were cultivated in Falcon plastic flasks (75 cm² surface) or plastic plates (50 mm in diameter). Confluent monolayers formed in 4 to 5 days when cells were incubated at 37 C in 0.5% lactalbumin hydrolysate in Earle's buffered salt solution (BSS) containing 5% calf serum. Maintenance medium consisted of Eagle's basal medium (BME) with or without calf serum, as described in the text.

Monolayer cultures of mouse L cells grown in plastic plates with medium 199 containing 5% calf serum were used for plaque assays (10).

The Indiana strain of VS virus was grown in chick embryo cell cultures, as previously described (24). Stock virus was prepared from culture media of 18-hr infected RK cell cultures and stored at -60 C until used. A single lot of this stock virus was used in all experiments; the titer was 1.2×10^6 plaque-forming units (PFU)/ml.

Interferon. The procedures for production and purification of rabbit interferon have been described in detail (28). In brief, primary RK cells were infected with the CG strain of Newcastle disease virus at a multiplicity of about 200 PFU/cell. The culture media collected at 8 hr after infection were clarified by centrifugation at 2,500 rev/min for 3 min; interferon titers of this crude interferon were about 2,000 PDD₅₀/ml (50% plaque-depressing doses) as measured by plaque inhibition of VS virus on RK cells (28). Initial 10-fold purification and concentration were achieved by precipitation of inert cellular and viral protein

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with 0.15 *N* perchloric acid (PCA) followed by interferon precipitation with 0.02 *M* zinc acetate. After exhaustive dialysis against 0.1 *M* sodium phosphate at pH 6.0, the interferon was further purified by adsorption on a column of carboxymethyl Sephadex C50 equilibrated and washed with 0.1 *M* phosphate, pH 6.0. The interferon was then eluted by means of a rising pH gradient of 0.1 *M* phosphate. Specific activity of interferon eluted at pH 6.4 to 6.6 was greater than 10^6 PDD₅₀/mg of protein. This purified interferon appropriately diluted in BME was used in all experiments.

Chemicals. ³H-leucine (40.0 Ci/mmole), ³H-tyrosine (43.0 Ci/mmole), and ³H-uridine (20.0 Ci/mmole) were purchased from Schwarz BioResearch, Inc., Orangeburg, N.Y. Actinomycin D was a gift from Merck Sharp and Dohme, West Point, Pa.

Protein and RNA synthesis. Protein synthesis was measured by incorporation of a mixture of ³H-leucine and ³H-tyrosine into a trichloroacetic acid-precipitable fraction of disrupted cells. The following procedures were used in most experiments. Infected or uninfected confluent monolayer cultures were covered with 5 ml of special BME containing both ³H-leucine and ³H-tyrosine (1 or 10 μ Ci/ml each) but without unlabeled leucine, unlabeled tyrosine, or serum. After incubation at 37 C for the period indicated in each experiment, incorporation of labeled precursors was stopped by washing the monolayers or scraped cells with ice-cold BSS containing excess unlabeled leucine and tyrosine. Washed cells suspended in 1 ml of BSS were disrupted by freezing and thawing and by sonic treatment for 10 min at the 1-amp setting of a Raytheon sonic oscillator. The cell contents were precipitated and washed with 5% trichloroacetic acid and the precipitates were collected on HA filters (Millipore Corp., Bedford, Mass.). Dried filters were submerged in toluene-based fluors, and the radioactivity was counted by scintillation spectrometry.

RNA synthesis was measured by incorporation of ³H-uridine (1 or 10 μ Ci/ml) into the 0.5 *M* PCA-precipitable fraction of disrupted cells by the method previously described (9). The resulting precipitate was centrifuged at 2,500 rev/min for 15 min and washed twice with 0.5 *M* PCA and once with ethanol-ether (1:1). After drying, the precipitate was dissolved in 0.5 ml of hyamine and diluted in toluene-based fluors for counting of radioactivity.

Analysis of proteins by polyacrylamide gel electrophoresis. This procedure has been described in detail (25, 26).

RESULTS

Baseline studies on the effects of actinomycin and interferon. These experiments were done as controls for the studies, described later, to determine the effect of actinomycin on interferon-induced cellular resistance. Although it had been reported previously (2, 10) that growth of VS virus is resistant to actinomycin, as is true for many other but not all RNA viruses, it has been demonstrated that actinomycin can inhibit the

growth of an ordinarily resistant RNA virus in certain cells (27) and under various cultural conditions (3, 18). Therefore, actinomycin was tested for its effect on multiplication of VS virus in RK cells and compared with its capacity to suppress cellular RNA synthesis in uninfected RK cells.

FIG. 1. Comparative effects of actinomycin on cellular RNA synthesis and multiplication of VS virus. Monolayer cultures of 2×10^6 RK cells were incubated for 2 hr at 37 C in BME containing various concentrations of actinomycin or no actinomycin. Two cultures in each actinomycin-treated and control group were washed and reincubated for 2 hr in 5 ml of BME containing ³H-uridine, 10 μ Ci/ml. Incorporation of ³H-uridine into the PCA-precipitable fraction of scraped, disrupted cells was measured as described in Materials and Methods. Two additional washed cultures in each actinomycin-treated and control group were infected with VS virus (30-min adsorption) at a multiplicity of 10 and incubated in BME for 8 hr, at which time the virus titer in the medium of each culture was determined by plaque assay on L cells. The results are expressed as per cent of control ³H-uridine incorporation (11,382 counts per min per culture) and of control virus yield (9.0×10^6 PFU/ml) at each concentration of actinomycin.

Figure 1 shows that actinomycin at a concentration of 1 $\mu\text{g}/\text{ml}$ inhibited RNA synthesis in RK cells by $> 95\%$, but the same amount of actinomycin also reduced the yield of VS virus to $\sim 30\%$ of that from infected cells not exposed to actinomycin. Higher concentrations of actinomycin caused only slightly greater reductions in cellular RNA synthesis, but at these concentrations virus yields were significantly lower. Therefore, an actinomycin concentration of 1 $\mu\text{g}/\text{ml}$ was chosen to study its effect on interferon action. This dose of actinomycin administered at the appropriate time completely reversed the antiviral action of interferon.

To determine the dose of interferon required to induce maximum resistance of RK cells to VS viral infection, duplicate RK cell monolayers were exposed to serial dilutions of purified interferon at 4 C for 4 hr, washed three times, incubated in BME at 37 C for 2 hr, and then infected with VS virus at a multiplicity of 10. Virus yields at 8 hr were determined by plaque assay on triplicate cultures of L cells and compared with yields from control RK cells not exposed to interferon.

As shown in Table 1, interferon at a concentration of 100 PDD₅₀/ml reduced the yield of VS virus by 92%; concentrations as much as 40 times greater did not significantly enhance cellular resistance to viral infection under the conditions tested. Similar dose-dependent reduction of virus yields by interferon was noted when cells were infected with VS virus at a multiplicity of 100. These experiments formed the basis for the design of the studies described below.

Kinetics of interferon-induced cellular resistance.

Prior to undertaking studies on the effect of interferon on various viral functions, experiments were designed to determine the rate at which inter-

feron induces the antiviral state. These experiments were based on the finding originally described by Taylor (22) and confirmed by others (4) that actinomycin blocks the antiviral action of interferon, suggesting that interferon action requires intact transcription of cellular mRNA. The rate at which cellular resistance induced by interferon becomes refractory to actinomycin can be taken as a first approximation of the rate of synthesis of the hypothetical mRNA, the translation of which presumably results in the antiviral state. Therefore, the degree of cellular resistance to VS virus which develops in a given period of time after exposure to interferon and before introduction of actinomycin should reflect the relative amount of antiviral mRNA that is transcribed. To time the events even more precisely, advantage was taken of the fact that cells can interact with interferon at low temperature but the antiviral state does not develop until the cultures are incubated at 37 C (13).

Monolayer cultures of RK cells were exposed to 1,000 PDD₅₀ of purified rabbit interferon or BME for 4 hr at 4 C. The cultures were then washed three times, and the medium was replaced with BME and then incubated at 37 C. At intervals thereafter, actinomycin was added to paired sets of interferon-treated and control cultures to a final concentration of 1 $\mu\text{g}/\text{ml}$. After incubation in the presence of actinomycin for 2 hr, each culture was infected with VS virus at a multiplicity of 10. Yields of progeny VS virus were determined by plaque assay 8 hr after infection.

Figure 2 shows the kinetics of reversal of interferon action by actinomycin. The data reveal that actinomycin completely blocks antiviral action for the first 30 min after interferon adsorption. Thereafter, cellular resistance to viral infection becomes progressively refractory to actinomycin and reaches a peak at 3 hr, after which actinomycin no longer affects the antiviral state. These results are interpreted as evidence for transcription of antiviral mRNA between 30 min and 3 hr after induction by interferon. This plot of developing refractoriness to actinomycin shown in Fig. 2 provides an approximation of the kinetics of synthesis of the hypothetical mRNA, which is then translated into antiviral protein. The messenger appears to be relatively stable.

Effect of interferon on VS virus cytopathogenesis. Protection afforded against cytopathic effects of VS virus was compared with other antiviral effects of rabbit interferon. It was necessary to consider two potential types of cell killing that could result from infection by VS virus: (i) rapid cell death after infection at high multiplicity that has some of the characteristics of a toxic reaction

TABLE 1. *Cellular resistance to VS virus replication induced by various concentrations of interferon^a*

Interferon concn (PDD ₅₀ /ml)	Virus yield (PFU/ml)	Per cent of control yield
0	785×10^4	100
10	260×10^4	33
50	97×10^4	12
100	60×10^4	8
500	48×10^4	6
1,000	50×10^4	6
2,000	47×10^4	6
4,000	49×10^4	6

^a Duplicate cultures of 2×10^6 RK cells were exposed to purified interferon for 4 hr at 4 C, washed, incubated for 2 hr at 37 C, and infected with 2×10^7 PFU of VS virus. Virus yields at 8 hr were measured by plaque assay.

FIG. 2. Rate at which the interferon-induced cellular resistance to VS virus becomes refractory to actinomycin. Twenty-seven monolayer cultures of 2×10^6 RK cells each were exposed at 4 C to 5 ml of BME containing 1,000 PDD₅₀/ml of purified rabbit interferon (specific activity = 1.6×10^6 PDD₅₀/mg of protein). After contact for 4 hr, the residual interferon was completely removed by washing each cell layer three times with 10-ml amounts of cold BME; the cultures were then replenished with BME containing 2% calf serum and transferred to 37 C. An equal number of control cultures were treated in exactly the same way, except that they were not exposed to interferon. At the end of the 4-hr period of interferon adsorption and at intervals thereafter, three control and three interferon-treated cultures were exposed for 2 hr to actinomycin, 1 μ g/ml. The cell layers were then washed and infected with VS virus at a multiplicity of 10 (30-min adsorption). Virus yields from groups of three control and three interferon-treated cultures were determined by plaque assay at 8 hr after infection. The data are plotted as the per cent yield of VS virus from interferon-treated cultures based on the yield from control cultures at each time of actinomycin exposure.

(9) and (ii) the somewhat less rapid cytopathic effect caused by virus replication in cells infected at low multiplicity. Therefore, cell protection assays were performed at three different concentrations of purified interferon and tested against three multiplicities of infection.

Monolayer cultures of 2×10^6 RK cells were incubated with 5 ml of BME containing 2,000, 1,000, 200, or 0 PDD₅₀/ml of interferon for 6 hr at 37 C. The media were removed and cell layers exposed to each interferon concentration were infected with VS virus at multiplicities of 100, 10, or 1. Uninfected cultures exposed or unexposed to interferon served as controls. The cultures were then reincubated at 37 C in BME without serum and examined periodically by light microscopy for cytopathic effects scored on a scale of 0 to 4+.

Table 2 shows that 1,000 units of interferon, which inhibited viral multiplication in RK cells by 95% (Table 1), afforded only slight protection against infection at a multiplicity of 100. On the other hand, as little as 200 units of interferon

induced greater and longer-lasting resistance to infection of cells challenged at a multiplicity of 1. Intermediate degrees of resistance to increasing doses of interferon were noted when cells were infected at a multiplicity of 10.

These data indicate that interferon affords more effective protection of cells infected at low multiplicity than it does at high multiplicity. The extremely early cytopathic effects at high multiplicity suggest a toxic effect of this virus which is not readily inhibited by interferon even at relatively high concentrations. In contrast, small amounts of interferon are effective against low multiplicity infection, the cytopathic effects of which are undoubtedly the result of viral replication. These somewhat different effects of interferon on viral cytopathogenicity may be related to the divergent action of interferon on viral protein synthesis and on viral effect on cellular protein synthesis reported below.

Interferon inhibition of viral protein synthesis. Although the inhibition of viral RNA synthesis by interferon has been adequately described (22) and confirmed, the evidence for inhibition by interferon of viral protein synthesis is not as convincing because cellular protein synthesis cannot be selectively inhibited as can cellular RNA synthesis by actinomycin. A clear demonstration of the suppression by semipurified interferon of Semliki Forest virus protein synthesis was provided by Friedman (5). We performed similar studies with our purified preparation of rabbit interferon by examining its effect on the distinctive electrophoretic profile of VS viral proteins. When isotopically labeled proteins extracted from L cells or chick embryo cells infected with VS virus are analyzed on neutral sodium dodecyl sulfate (SDS)-urea polyacrylamide gels, five major protein peaks are readily identified (26). Three of these proteins are assembled into mature virions (25, 26) and are designated G (for glycoprotein), N (for nucleoprotein), and S (for surface protein); in addition, two nonstructural proteins (designated NS₁ and NS₂) are present in infected cells. It was of interest to determine whether synthesis of any or all of these VS viral proteins is inhibited by rabbit interferon.

Replicate cultures of RK cells were incubated at 37 C for 6 hr in the presence of 1,000 PDD₅₀ of interferon per ml in BME or in the presence of BME alone to serve as controls. One set of controls was not infected; the other set was infected with VS virus at a multiplicity of 100 as were the interferon-treated cultures. All cultures were then incubated for 5 hr at 37 C in special BME containing ³H-leucine and ³H-tyrosine. The cells from each set of cultures were harvested, and the pro-

TABLE 2. *Inhibitory effect of interferon on cytopathic effects (CPE) induced by VS virus infection of RK cells at various multiplicities*

Virus multiplicity	Interferon concn (PDD ₅₀ /ml)	Time (hr) after infection ^a							
		1	2	3	4	5	6	7	20
100	2,000	0	0	0	±	+	+	++	++
	1,000	0	0	0	+	+	++	++	+++
	200	0	0	±	+	++	++	++	+++
	No interferon	0	±	+	++	++	+++	++++	++++
10	2,000	0	0	0	0	0	±	+	+
	1,000	0	0	0	0	±	±	+	+
	200	0	0	0	0	+	+	+	++
	No interferon	0	0	±	+	++	++	+++	++++
1	2,000	0	0	0	0	0	0	0	0
	1,000	0	0	0	0	0	0	0	±
	200	0	0	0	0	0	0	0	+
	No interferon	0	0	0	0	±	+	+	++++
0	2,000	0	0	0	0	0	0	0	0
	No interferon	0	0	0	0	0	0	0	0

^a Symbols: 0 = no CPE; ± = slight CPE; + = CPE area less than 50%; ++ = CPE area about 50%; +++ = CPE area more than 50% and some cells detached; ++++ = CPE of the whole monolayer and all cells detached.

teins were extracted for analysis by electrophoresis in neutral SDS-urea-polyacrylamide gels along with marker ¹⁴C-proteins extracted from purified VS virions.

Figure 3 shows representative electropherograms of the proteins synthesized under each experimental condition. At least 16 ³H-protein peaks and a rather amorphous pattern are seen in the gel profile of the uninfected cells (Fig. 3A). The electropherogram of the proteins extracted from infected cells not exposed to interferon shown in Fig. 3B reveals a considerably lower base line of radioactivity and three distinct ³H-protein peaks that migrated to the same gel positions as the three coelectrophoresed virion marker ¹⁴C-proteins G, N, and S; the two nonstructural proteins NS₁ and NS₂ are also evident in infected cells. In striking contrast, none of the five viral proteins can be detected in infected cells pretreated with interferon. It would appear, therefore, that under these conditions interferon suppresses the synthesis of all the structural and nonstructural proteins of VS virus.

Of particular interest in these experiments was the fortuitous finding that most, if not all, of the protein peaks appearing in electropherograms of uninfected cells (Fig. 3A) were not present or greatly reduced in electropherograms of proteins synthesized in cells infected with VS virus regardless of whether the cells were pretreated with interferon (Fig. 3B). Moreover, total protein synthesis was evidently far greater in the uninfected RK cells than in either infected control cells or those pretreated with interferon. The elec-

tropherograms in Fig. 3, for example, revealed a total of 398,359 counts/min of ³H recovered from the gel loaded with a 100-μliter extract of uninfected cells compared with recovery of 96,441 counts/min (about 24%) from the gel loaded with the same amount of virus-infected cell extract. The total ³H-protein in a 100-μliter extract of interferon-pretreated infected cells was 66,759 counts/min (~ 17% of that from the uninfected control cells). These results suggested that inhibition of cellular protein synthesis by VS virus was not affected by interferon and led us to investigate this phenomenon in greater detail.

Inability of interferon to reverse the switch off by VS virus of cellular protein synthesis. The selective inhibition by interferon of synthesis of all VS viral proteins, as described above, provided another probe for determining whether VS viral protein synthesis is required for switch off of cellular macromolecular synthesis (9).

Base line, control data on the effects of interferon and VS virus on RK cells were required to set the stage for these experiments. First, purified rabbit interferon was found not to affect RNA or protein synthesis by uninfected RK cells; pretreatment with interferon for 6 hr neither inhibited nor enhanced incorporation of ³H-uridine or ¹⁴C-amino acids into acid-precipitable fractions of uninfected RK cells. Second, the effects of VS virus on RNA and protein synthesis by RK cells was measured by incorporation of ³H-uridine and ³H-amino acids into acid-precipitable cell fractions. Figure 4 shows the comparative amounts of RNA and protein synthesized in replicate cultures

FIG. 3. Comparative electropherograms of ^3H -proteins synthesized in uninfected cells, in VS virus-infected cells pretreated with interferon, and in VS virus-infected cells not pretreated with interferon. Five monolayer cultures of 2×10^6 RK cells each were first exposed to 1,000 PDD₅₀ of purified interferon per ml for 6 hr at 37 C and then exposed to 0.5 μg of actinomycin per ml for 1 hr. Two other groups of five cultures each were similarly treated but not exposed to interferon. The interferon-treated cultures and one group of control cultures were then infected with VS virus at a multiplicity of 100. After virus adsorption for 30 min at 37 C, all cultures were washed and covered with 5 ml of special BME containing ^3H -leucine and ^3H -tyrosine (each 10 $\mu\text{Ci}/\text{ml}$) but without unlabeled leucine, tyrosine, or serum. After labeling for 5 hr, the cultures were washed three times and the cells were scraped into 1 ml of Earle's BSS. Proteins were extracted from whole cells by treatment at 37 C with one-tenth volume of glacial acetic acid, 0.5 M urea, and 1% SDS, and then by dialysis overnight against 0.01 M sodium phosphate (pH 7.2) containing 0.1% SDS, 0.5 M urea, and 0.1% 2-mercaptoethanol. Samples of 100 μl each were subjected to electrophoresis for 5.5 hr at 5 ma/gel in 7.5% neutral polyacrylamide gels containing 0.5 M urea and 0.1% SDS. Gels were sliced into lengths of 1.25 mm, dissolved in 0.5 ml of NCS (Nuclear Chicago solubilizer) at 60 C for 2 hr, diluted in toluene-based fluors, and counted by scintillation spectrometry corrected for double labeling. Migration of viral structural ^3H -proteins was determined by co-electrophoresis with ^{14}C -proteins extracted from purified virions. The peak

FIG. 4. Incorporation of ^3H -amino acids and ^3H -uridine into acid-precipitable fractions of RK cells infected at different multiplicities with VS virus. Six monolayer cultures of 2×10^6 RK cells each were infected with 0.5 ml of VS virus at each of the multiplicities shown. Six uninfected control cultures were exposed to 0.5 ml of BME. After adsorption for 30 min at 37 C, the cultures were washed and replenished with 5 ml of BME. After incubation for 1 hr at 37 C, the media were drained; three cultures at each multiplicity were labeled for 1 hr with ^3H -leucine plus ^3H -tyrosine (1 $\mu\text{Ci}/\text{ml}$ each) and three additional cultures at each multiplicity were labeled for 1 hr with ^3H -uridine (1 $\mu\text{Ci}/\text{ml}$). Incorporation of ^3H into acid-precipitable fractions of each triplicate group of control and infected cultures was measured as described in Materials and Methods. Uninfected control cells incorporated 126×10^3 counts per min per culture of ^3H -amino acids and 69×10^3 counts per min per culture of ^3H -uridine.

of RK cells during the first 2 hr after infection with VS virus at various multiplicities. Unlike the finding with Krebs-2 ascites carcinoma cells (9), infection at a multiplicity of 100 caused only moderate reduction in cellular RNA synthesis (47% of uninfected controls). Under comparable conditions, RK cells infected at a multiplicity of 100 synthesized only 20% of the control level of protein. These results determined the design of the following experiments.

Table 3 compares incorporation of a mixture of ^3H -leucine plus ^3H -tyrosine into the acid-precipit-

position of each marker protein G, N, and S is shown by the arrows, as are the positions of nonstructural proteins NS₁ and NS₂.

TABLE 3. Inhibition of cellular protein synthesis by VS virus infection of RK cells pretreated or not pretreated with interferon^a

Treatment of cells	³ H counts/min	Per cent standard deviation	Per cent of uninfected control
No interferon, uninfected.....	206,700	0.1	100
No interferon, infected.....	54,665	0.1	26
Interferon-treated, infected.....	37,917	0.2	18

^a Triplicate RK cell cultures exposed to 1,000 PDD₅₀/ml of interferon or BME for 6 hr at 37 C were treated with actinomycin for 1 hr and infected with VS virus at a multiplicity of 100. Each culture was labeled with ³H-leucine plus ³H-tyrosine for 5 hr, and the radioactivity was determined in trichloroacetic acid-precipitable fractions of disrupted cells.

able fraction of triplicate cultures of uninfected RK cells, RK cells infected with VS virus, and RK cells pretreated with interferon for 6 hr and then infected with VS virus. All cultures were exposed to actinomycin (0.5 µg/ml) for 1 hr after treatment with interferon and before infection with VS virus at a multiplicity of 100. The infected cells were incubated for 5 hr post-infection with ³H-leucine plus ³H-tyrosine (1 µCi/ml each), disrupted, and analyzed for acid-precipitable radioactivity. Infection with VS virus resulted in a 74% reduction in cellular protein synthesis over a period of 5 hr. Pretreatment with interferon not only failed to reverse this effect on cellular protein synthesis but accentuated it somewhat, presumably owing to inhibition of specific viral protein synthesis (Fig. 2). These data suggest that synthesis of viral proteins is not essential for switch off by VS virus of cellular protein synthesis. Moreover, the fact that this switch off is not blocked by actinomycin suggests that induction of a host protein by the virus is not essential for the reaction.

Figure 5 illustrates the kinetics of the switch off of cellular protein synthesis caused by infection with VS virus at high multiplicity. In this experiment, a series of replicate cultures was pretreated with interferon or BME, infected with VS virus at a multiplicity of 100, and pulse-labeled with ³H-leucine plus ³H-tyrosine for 15 min at intervals after infection. Identical uninfected pulse-labeled cultures served as controls for each time point. About 80% of cellular protein synthesis was inhibited within 30 min after virus adsorption, regardless of whether the cells were pretreated with interferon. Unlike the preceding experiments

FIG. 5. Failure of interferon to reverse the inhibition by VS virus of protein synthesis in RK cells. One group of 18 monolayer cultures of RK cells was exposed to 1,000 PDD₅₀ of interferon per ml for 8 hr at 37 C, whereas another group of 18 cultures was exposed to BME for the same period. Sixteen cultures of each group were infected with VS virus at a multiplicity of 100; the other two cultures of each group served as uninfected controls. After adsorption of virus for 30 min at 37 C, duplicate cultures were washed and pulse-labeled for 15 min with a mixture of ³H-leucine plus ³H-tyrosine (1 µCi/ml each) at intervals after virus adsorption. Radioactivity was measured in the 5% trichloroacetic acid precipitable fraction of 4×10^6 disrupted RK cells (two cultures). Percentages of ³H incorporation are based on counts of uninfected control cells in each group pulsed for 15 min with ³H-amino acids. Time zero is the end of the 30-min period of virus adsorption, and the data are plotted at the onset of each 15-min pulse. Per cent incorporation of ³H-amino acids in infected cultures was based on radioactivity of control uninfected cultures of 14,951 counts per min per culture for interferon-treated cells and 15,594 counts per min per culture for cells not treated with interferon.

on cumulative 5-hr effects of viral infection shown in Table 3, pulse labeling revealed considerable recovery of the capacity of the infected cells to synthesize protein. Repeated experiments showed similar rapid decline and recovery of cellular protein synthetic capacity. This rapid decline in cellular protein synthesis after infection at high multiplicity of interferon-pretreated cells suggests a toxic effect of the input virus that is partially reversible.

DISCUSSION

Although most investigators agree that interferons act by selectively inhibiting translation of viral RNA, the precise mechanism and site of action are unknown. Kerr et al. (12) could not confirm the reports (15, 17) that ribosomes from interferon-treated cells had impaired capacity to

bind virion RNA and to translate it. Part of the problem relates to the incontrovertible evidence that interferon itself is not a direct antiviral agent but requires intact capacity of the target cell to synthesize RNA (22) and protein (6). The kinetics of induction by interferon of the antiviral state have not been worked out in as great detail as has the synthesis of interferon (23), but approximately 7 hr appear to be required for mouse embryo cells to reach maximal levels of resistance after exposure to crude interferon (4). Development of resistance to viral infection is temperature-dependent and does not occur at 4 C, although interferon appears to be absorbed at this temperature by cells which gradually become resistant to viral infection after incubation at 37 C (13). The rate at which resistance develops is partially dependent on interferon concentration (4) and undoubtedly varies for each virus-cell system. The present experiments, as well as those reported previously (19), reveal that rabbit kidney cells become resistant to viral infection at the most rapid rate yet described. The interferon-induced hypothetical mRNA is completely transcribed in RK cells by 3 hr, as judged by failure of actinomycin to arrest the progression of cellular resistance to infection with VS virus.

The results of our studies on the capacity of rabbit interferon to prevent cytopathic effects caused by VS virus are similar to those reported by Haase et al. (7) who showed that only large doses of crude mouse interferon delayed cytopathic effects in mouse L cells infected with mengovirus at a multiplicity of 20. Our experiments also show that much more interferon is needed to inhibit viral cytopathic effects than to inhibit viral replication, but, in addition, this effect is also multiplicity-dependent. Large doses of interferon only caused slight delay in cytopathic effect on RK cells infected with VS virus at a multiplicity of 100. It is apparent that interferon is more effective in rendering cells resistant to viral replication than to viral cytopathogenicity.

Purified rabbit interferon was also more effective in suppressing synthesis of viral proteins in RK cells infected with VS virus at a multiplicity of 100. There is general agreement among investigators in this field that the primary effect of interferon must be on viral protein synthesis (11, 12); inhibition of viral RNA synthesis of RNA viruses appears to be secondary to inhibition of viral RNA polymerase (20). Friedman (5) has shown that five out of six of the individual proteins synthesized in cells infected with Semliki Forest virus are inhibited by pretreatment with chick interferon. Our data indicate that synthesis of all five identifiable peptides coded by the VS viral genome

is suppressed by interferon. It seems likely, therefore, that interferons do not selectively inhibit synthesis of specific viral proteins but interfere with viral RNA translation in general.

The VS viral function found to be most resistant to interferon action was the capacity to switch off cellular protein synthesis. Levy (14) also found that relatively small doses of mouse interferon that lowered virus yield, and that partially reversed switch off of cellular RNA synthesis, had no effect on mengovirus inhibition of cellular protein synthesis in L cells. Haase et al. (7) showed that large doses of mouse serum interferon could partially block mengovirus inhibition of L-cell protein synthesis, but their data also show either cellular recovery or some late viral protein synthesis which was not monitored. On the other hand, Long and Burke (16) reported that partially purified chick interferon could completely prevent the inhibition of CE cell protein synthesis caused by fowl plague virus, but their measurements were made only 7 to 8 hr after infection. These somewhat contradictory data are difficult to reconcile with the known effects of interferon. It seems likely that these conflicting results are attributable to some extent to variability in experimental conditions, such as dose of interferon, multiplicity of infection, and failure to account for viral protein synthesis. However, it is even more likely that there are fundamental differences among viruses in respect to the mechanisms by which they affect cellular macromolecular synthesis. Picornavirus, for example, may code for early switch off proteins (1), whereas VS viral protein synthesis does not seem to be essential for switch off of cellular RNA (9) or protein synthesis. As shown in this report, the same amount of purified interferon that blocked viral protein synthesis in RK cells infected at a multiplicity of 100 had no effect on inhibition of cellular protein synthesis in RK cells infected at the same multiplicity. Moreover, the greatest reduction in cellular protein synthesis caused by VS virus occurs earlier than any viral protein synthesis can be detected (26).

These data appear to provide additional evidence for a "toxic" component of the infecting VS virion that rapidly inhibits cellular RNA (9) and protein synthesis. This hypothesis is supported by inability to prevent these cellular effects by prior ultraviolet irradiation of VS virions or by puromycin (9) or by pretreatment of cells with interferon sufficient to eliminate viral protein synthesis. Conceivably, these effects on cellular macromolecular synthesis caused by the infecting VS virion could be due to altered plasma membrane function resulting from fusion with VS

virions (8) or by the product of the virion transcriptase (1a), neither of which functions is likely to be susceptible to the action of interferon.

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LITERATURE CITED

1. Baltimore, D., R. M. Franklin, and J. Callender. 1963. Mengo-virus-induced inhibition of host ribonucleic acid and protein synthesis. *Biochim. Biophys. Acta* 76:425-430.
- 1a. Baltimore, D., A. S. Huang, and M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. *Proc. Nat. Acad. Sci. U.S.A.* 66:572-576.
2. Black, D. N., and F. Brown. 1968. The influence of mitomycin C, actinomycin D and ultraviolet light on the replication of the viruses of foot-and-mouth disease and vesicular stomatitis. *J. Gen. Virol.* 3:453-457.
3. Cooper, P. D. 1966. The inhibition of poliovirus growth by actinomycin D and the prevention of the inhibition by pretreatment of the cells with serum or insulin. *Virology* 28:663-678.
4. Dianzani, F., C. E. Buckler, and S. Baron. 1968. Kinetics of the development of factors responsible for interferon-induced resistance. *Proc. Soc. Exp. Biol. Med.* 129:535-538.
5. Friedman, R. M. 1968. Inhibition of arbovirus protein synthesis by interferon. *J. Virol.* 2:1081-1085.
6. Friedman, R. M., and J. A. Sonabend. 1965. Inhibition of interferon action by puromycin. *J. Immunol.* 95:696-703.
7. Haase, A. T., S. Baron, H. Levy, and J. A. Kasel. 1969. Mengovirus-induced cytopathic effect in L-cells: protective effect of interferon. *J. Virol.* 4:490-495.
8. Heine, J. W., and C. A. Schnaitman. 1969. Fusion of vesicular stomatitis virus with the cytoplasmic membrane of L cells. *J. Virol.* 3:619-622.
9. Huang, A. S., and R. R. Wagner. 1965. Inhibition of cellular RNA synthesis by nonreplicating vesicular stomatitis virus. *Proc. Nat. Acad. Sci. U.S.A.* 54:1579-1584.
10. Huang, A. S., and R. R. Wagner. 1966. Defective T particles of vesicular stomatitis virus. II. Biologic role in homologous interference. *Virology* 30:173-181.
11. Joklik, W. K., and T. C. Merigan. 1966. Concerning the mechanism of action of interferon. *Proc. Nat. Acad. Sci. U.S.A.* 56:558-565.
12. Kerr, I. M., J. A. Sonabend, and E. M. Martin. 1970. Protein-synthetic activity of ribosomes from interferon-treated cells. *J. Virol.* 5:132-144.
13. Levine, S. 1966. Persistence of active interferon in cells washed after treatment with interferon. *Proc. Soc. Exp. Biol. Med.* 121:1041-1045.
14. Levy, H. B. 1964. Studies on the mechanism of interferon action. II. The effect of interferon on some early events in mengo virus infection in L cells. *Virology* 22:575-579.
15. Levy, H. B., and W. A. Carter. 1968. Molecular basis of the action of interferon. *J. Mol. Biol.* 31:561-577.
16. Long, W. F., and D. C. Burke. 1970. The effect of infection with fowl plague virus on protein synthesis in chick embryo cells. *J. Gen. Virol.* 6:1-14.
17. Marcus, P. L., and J. M. Salb. 1966. Molecular basis of interferon action. Inhibition of viral RNA translation. *Virology* 30:502-516.
18. Schaffer, F. L., and M. Gordon. 1966. Differential inhibitory effects of actinomycin D among strains of poliovirus. *J. Bacteriol.* 91:2309-2316.
19. Smith, T. J., and R. R. Wagner. 1967. Rabbit macrophage interferons. I. Conditions for biosynthesis by virus-infected and uninfected cells. *J. Exp. Med.* 125:559-577.
20. Sonabend, J. A., E. M. Martin, E. Mécs, and K. H. Fantes. 1967. The effect of interferon on the synthesis and activity of an RNA polymerase isolated from chick cells infected with Semliki Forest virus. *J. Gen. Virol.* 1:41-48.
21. Stewart, W. E., II, W. D. Scott, and S. E. Sulkin. 1969. Relative sensitivities of viruses to different species of interferon. *J. Virol.* 4:147-153.
22. Taylor, J. 1965. Studies on the mechanism of action of interferon. I. Interferon action and RNA synthesis in chick embryo fibroblasts infected with Semliki Forest virus. *Virology* 25:340-349.
23. Wagner, R. R., and A. S. Huang. 1965. Reversible inhibition of interferon synthesis by puromycin: evidence for an interferon-specific messenger RNA. *Proc. Nat. Acad. Sci. U.S.A.* 54:1112-1118.
24. Wagner, R. R., T. C. Schnaitman, and R. M. Snyder. 1969. Structural proteins of vesicular stomatitis viruses. *J. Virol.* 3:395-403.
25. Wagner, R. R., T. C. Schnaitman, R. M. Snyder, and C. A. Schnaitman. 1969. Protein composition of the structural components of vesicular stomatitis virus. *J. Virol.* 3:611-618.
26. Wagner, R. R., R. M. Snyder, and S. Yamazaki. 1970. Proteins of vesicular stomatitis virus: kinetics and cellular sites of synthesis. *J. Virol.* 5:548-558.
27. Yamazaki, S. 1968. Effect of endogenous interferon and actinomycin D on growth of Japanese encephalitis virus in chick embryo cells. *Jap. J. Microbiol.* 12:171-178.
28. Yamazaki, S., and R. R. Wagner. 1970. Purified rabbit interferon: attempts to demonstrate interferon-specific ³H-protein. *J. Virol.* 5:270-273.