Mechanism of Interferon Action: Inhibition of Vesicular Stomatitis Virus Replication in Human Amnion U Cells by Cloned Human γ-Interferon

I. EFFECT ON EARLY AND LATE STAGES OF THE VIRAL MULTIPLICATION CYCLE*

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The molecular basis of the inhibition of vesicular stomatitis virus (VSV) replication by pure recombinant γ-interferon (IFN-γ) in human amnion U cells was examined. A saturating concentration of IFN-γ induced, at maximum, about a two log10 reduction in infectious VSV yield. The kinetics of induction of the antiviral activity by IFN-γ were first order over the period of about 6–18 h, following a lag of about 3 h, after treatment with a saturating concentration of IFN-γ. The relationship of the inhibition in VSV infectivity to the early and late events of the VSV multiplication cycle was investigated. IFN-γ treatment had no detectable effect on the adsorption and penetration of VSV virions or on their uncoating to yield viral nucleocapsids. The polypeptides of adsorbed or uncoated VSV particles were neither preferentially degraded nor detectably altered in IFN-γ-treated U cells, as compared to untreated U cells. Progeny virions isolated from IFN-γ-treated U cells, although greatly reduced in number, were found to be equally as infectious as those isolated from untreated U cells. Progeny virions from IFN-γ-treated cells also possessed the same composition of viral proteins as was observed for virions from untreated cells. These results suggest that conditions of IFN-γ treatment sufficient to reduce the yield of infectious VSV progeny 100-fold do not detectably affect either the early or the late stages of the VSV multiplication cycle.

A wide range of different RNA and DNA animal viruses are sensitive to the antiviral actions of type I (α, β) and type II (γ) interferons. Among these, the replication of vesicular stomatitis virus is acutely sensitive to IFNγ in almost every kind of animal cell which responds to this class of regulatory proteins (1).

The human α-IFNs constitute a multigene family (2). IFN-αA, equivalent to IFN-α2, is one of the two IFN-α subspecies which appears to be preferentially expressed in Sendai virus-induced human cells (3). By contrast, available evidence suggests that there is only one human IFN-γ mRNA and gene (4, 5). It has recently become possible, by molecular cloning and expression, to obtain pure samples of single species of human IFN proteins absolutely free of any other eukaryotic proteins. Using IFN-αA expressed in Escherichia coli, we have recently shown that this IFN-α subspecies inhibits VSV replication in human amnion U cells primarily, and probably solely, at the level of translation of viral mRNA into viral polyproteins (6, 7). No significant effect of IFN-αA is observed in U cells on the adsorption, penetration, and uncoating of infecting VSV virions, nor is there an inhibition of primary transcription in vivo or a reduction in the in vitro translational activity of viral mRNA synthesized in vivo (6–8).

Progeny VSV from IFN-αA-treated U cells, although greatly reduced in number, are equally as infectious as progeny VSV from untreated U cells (8).

Very little is known concerning the nature of the antiviral activities and biochemical changes induced by IFN-γ as compared to those responses induced by IFN-α subspecies. At least two different classes of high-affinity IFN receptors have recently been shown to exist on human lymphoblastoid and fibroblast cells, one to which IFN-α and IFN-β bind and another to which IFN-γ binds (9–11). Recombinant IFN-γ, like IFN-αA, induces an antiviral activity against VSV in human amnion U cells (12). However, the antiviral activities of IFN-γ and IFN-αA in U cells are synergistic for the inhibition of VSV, suggesting that the molecular mechanism of the antiviral action of IFN-γ may be different from that of IFN-αA (12). Synergistic effects of highly purified recombinant IFN-α or IFN-β in antiproliferative as well as antiviral systems have also recently been reported (13).

Using recombinant human IFN-γ, we have undertaken a systematic biochemical study of the effect of IFN-γ treatment of human amnion U cells on each stage of the multiplication cycle of VSV. In this paper, we report the effect of IFN-γ on the early and the late steps of VSV multiplication; in the following paper (14), the effect of IFN-γ on VSV macromolecular synthesis is examined.

EXPERIMENTAL PROCEDURES AND RESULTS†

DISCUSSION

We have examined in detail the molecular basis of the antiviral state induced against VSV in human amnion U cells.
by molecularly cloned human IFN-γ. In this paper, we have addressed the question of whether inhibition of VSV replication is manifested to any significant extent at the earliest or the latest steps of the virus multiplication cycle. The results obtained demonstrate that treatment of human U cells with IFN-γ has no detectable effect on the ability of VSV to initiate an infection or on the specific infectivity of the progeny virions produced even though the yield of progeny virions is greatly decreased by IFN-γ treatment.

The kinetics of induction of the antiviral state against VSV in U cells by IFN-γ differ from those previously observed for IFN-αA. IFN-γ displayed a single kinetic phase following an initial lag. A maximum of about 2 log₁₀ reduction of VSV yield developed in cells treated for 24 h with saturating concentrations of IFN-γ, with the major extent of the reduction occurring after 6–18 h of IFN-γ treatment. By contrast, the antiviral state against VSV induced by IFN-αA in U cells is biphasic with the inflection between the first and second phases occurring after 6–8 h of treatment with saturating concentrations of IFN-αA (8). The first kinetic phase induced by IFN-αA in U cells accounts for the major extent of the total reduction of infectious VSV, typically about 3 log₁₀ (8, 15), and displays first-order kinetics at all IFN-αA concentrations examined (8). Our observation that, at saturating IFN concentrations, purified molecularly cloned IFN-γ induces an antiviral state more slowly than molecularly cloned IFN-αA against VSV in human U cells confirms the original observations reported for the antiviral action of natural IFN preparations in diploid human fibroblast cells measured with Sindbis virus (16).

Although it is generally accepted that IFN treatment has no effect on the adsorption, penetration, or uncoating of parental infecting virions, this notion is generalized from studies with type I (α, β) IFNs and a very small number of viruses. Recent studies indicating that the replication of SV40 and Sindbis virus (16) is inhibited by IFN-γ treatment are not essential for the initiation of budding of progeny virions which are otherwise normal.

The results described herein that were obtained with IFN-γ-treated U cell are similar to those recently reported for IFN-αA-treated U cells (8). The single molecularly cloned subspecies of α, IFN-αA, also did not detectably affect either the early or the late steps of VSV replication in human amnion U cells. Although neither IFN-γ nor IFN-αA affects either the early or the late steps of VSV replication, biologic and biochemical studies indicate that the molecular basis of their antiviral activity may be different (9–13). The fact that the antiviral actions of molecularly cloned human IFN-γ and IFN-αA are synergistic with each other for the inhibition of VSV in U cells strongly suggests that the two types of IFNs inhibit VSV replication by different mechanisms in the human amnion cell line (12). Recent biochemical studies have established that the major, and possibly only, effect of IFN-αA on VSV replication is translation inhibition (6, 7). By contrast, IFN-γ does not significantly affect the synthesis of either viral mRNA or viral polypeptides in U cells infected with either VSV (14) or revirus.³

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³ C. E. Samuel and G. S. Knutsom, unpublished observations.
**SUPPLEMENTAL MATERIAL**

**MECHANISM OF INTERFERON ACTION**

**YIELD OF VESICULAR STOMATITIS VIRUS REPLICATION IN HUMAN AMNION CELLS BY ELONGATED INFECTIOUS NUCLEOCAPSIDS**

by

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**EXPERIMENTAL PROCEDURES**

**Materials.** Rabbit antisera to the Indiana serotype of vesicular stomatitis virus was prepared by standard procedures (7). The sources of all other materials and reagents used for the experiments reported herein were as described previously (8).

**Cells and Virus.** The growth of human amnion cells in stable monolayer culture, and the growth and preparation of vesicular stomatitis virus (Indiana Strain) were as described previously (1,7).

**Interferon.** Highly purified (193-185) preparations of recombinant human interferon-1 alpha-synthesized in Escherichia coli were generously provided by Dr. E. T. Lowry; radioactivity associated with interferon was determined by the method of Lowry (9). Protein synthesis in the cell cultures was assessed by determining [35S]methionine incorporation into [35S]methionine-labeled progeny virus. The reduction of [35S]methionine incorporation in interferon-treated cultures was measured by autoradiography. The reduction of IFN-stimulated protein synthesis associated with single-cycle yield reduction was expressed as micrograms of protein/million cells.

**Cell Cultivation.** Madin-Darby canine kidney cells (MDCK) were maintained at 37°C in 5% CO2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All solutions used after IFN treatment contained 0.5% NaCl and 0.01% gentamicin.

**Infected Cells.** Infected MDCK monolayers were treated with varying concentrations of interferon. The concentration of interferon used was expressed as units/million cells. Single-cycle yield reductions were measured in parallel cultures of infected and uninfected cells at the same time that biochemical measurements were performed. The concentration of IFN was expressed in nanograms of protein/million cells; single-cycle yield reductions are expressed in log of reduction.

**Cell Electrophoretography.** MDCK cell electrophoretograms were obtained by autoradiography and were expressed as previously described (13). Infected MDCK monolayers were treated with varying concentrations of molecularly cloned IFN-1 ranging from 0.3 to 100 units per million cells. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were visualized by autoradiography. The autoradiographs were quantitated by densitometry and quantitated by densitometry and radioactivity incorporation calculated.

**RESULTS**

**Infectivity of the Antiviral State.** Human amniotic fluid cells were treated for 24 h with varying concentrations of interferon. Infections were then allowed to proceed for 48 h. The maximum antiviral response induced by IFN was observed between 100 and 1,000 units of IFN. A saturating concentration of 300 units of IFN was selected for use in the experiments described in this report.

**Figure 1. Concentration Dependence of the Inhibition of Vesicular Stomatitis Virus Replication by Interferon a.** U cells were treated with varying concentrations of IFN for 48 h and then infected with VSV at a multiplicity of 10 PFU/cell. After a 2-h incubation, the cultures were washed three times with PBS and then reincubated in fresh medium. Control cultures were treated with varying concentrations of IFN for 24 h and then infected with VSV at a multiplicity of 10 PFU/cell. A saturating concentration of 300 units of IFN was selected for use in the experiments described in this report.
cells and uncoating within cells constitutes the earliest stage of VSV infection. To study this, monolayers were treated with trypsin prior to measurement of cell-associated radioactivity, rather than at 4°C, in order to duplicate the standard conditions under which reduction of viral yield and degradation or alteration of input virion polypeptides was observed. Furthermore, the yield of uncoated nucleocapsids described above. Therefore, the yield and structure of uncoated nucleocapsids derived from 35S-labeled parental viral particles was examined. Nucleocapsids were separated from parent viral input and 35S-labeled virions by immunoprecipitation of cytoplasmic extracts prepared at 2 h post-infection from untreated and IFN-treated U cells. The separated nucleocapsids were then collected by ultracentrifugation and analyzed by nondenaturing gel electrophoresis. As shown in Fig. 3, the polypeptide profiles of nucleocapsids recovered from IFN-treated and untreated U cells were indistinguishable. The recovered nucleocapsids were composed predominantly of L protein with smaller amounts of M protein. In longer autoradiographic exposures, trace amounts of NS and M polypeptides appear, along with a polypeptide designated S which is believed to derive from M protein (8). No enhanced degradation or alteration of input nucleocapsids as a result of IFN-γ treatment was detected. Furthermore, the yield of uncoated nucleocapsids recovered from IFN-treated U cells was comparable to that of untreated U cells as was previously observed in IFN-α treated U cells (6).

To determine the fate of input cell-associated parental virions, the 35S-labeled virions were recovered by immune precipitation from cell lysates prepared at 2 h post-infection. When analyzed by nondenaturing gel electrophoresis, no difference was detected between the profiles of viral polypeptides recovered from untreated cells as compared to cells treated with IFN-γ for 24 h at concentrations ranging from 0.3 to 300 ng/ml (data not shown). However, IFN-γ treatment did not enhance the proteolytic degradation or alteration of input viral polypeptides. In fact, the 35S-labeled polypeptides composing input parental virions were surprisingly stable in IFN-γ treated cells as was previously observed in IFN-α treated U cells (6).

### Table 1

| Treatment | Total Radioactivity (cpm) | % of Input |
|-----------|--------------------------|------------|
| No IFN    | 1,230                    | 8.1        |
| IFN-γ     | 510                      | 7.3        |
| 0.3 ng/ml | 1,120                    | 7.4        |
| 3 ng/ml   | 1,330                    | 8.1        |
| 30 ng/ml  | 1,300                    | 8.0        |
| 300 ng/ml | 1,280                    | 7.8        |

In this table, the total radioactivity of input nucleocapsids was measured as % of input viral radioactivity. The effect of IFN-γ treatment on penetration of 35S-labeled virions into human amnion cells is shown in Table 2.

### Table 2

| Treatment | Trypsin penetration (cpm) | % of Input |
|-----------|---------------------------|------------|
| No IFN    | 1,230                     | 8.1        |
| IFN-γ     | 510                       | 7.3        |
| 0.3 ng/ml | 1,120                     | 7.4        |
| 3 ng/ml   | 1,330                     | 8.1        |
| 30 ng/ml  | 1,300                     | 8.0        |
| 300 ng/ml | 1,280                     | 7.8        |

In this table, the total radioactivity of input nucleocapsids was measured as % of input viral radioactivity. The effect of IFN-γ treatment on penetration of 35S-labeled virions into human amnion cells is shown in Table 2.
Incorporation of de novo Synthesized Polypeptides into Progeny Nucleocapsids and Virions. The observed two bag reduction in infectious virus yield (Figs. 1 and 2) does not appear to be the result of an IFN-γ-induced alteration in the early steps of viral multiplication, attachment, penetration or uncoating of parental virions (Tables 1 and 2, Fig. 3). Likewise, IFN-γ does not cause a major reduction in viral protein polypeptide synthesis. It could be that the major site IFN-γ-induced action is a late step in viral multiplication such as the post-translational modification of viral polypeptides or the assembly of newly synthesized viral nucleocapsids into mature viral particles. To begin to test these possibilities, the physical yield and specific infectivity of virions and the polypeptide composition of both virions and nucleocapsids produced by IFN-γ-treated U cells were compared to those of untreated cells.

U cell monolayers were left untreated or treated for 24 h with varying concentrations of IFN-γ ranging from 0.3 ng/ml to 10 ng/ml. Monolayers were labeled with [35S]methionine from 4-5 h post-infection and then incubated in maintenance medium for the balance of the standard 12 h single-step viral growth period. Cell lysates were assayed for infectious titer and then fractionated on sucrose gradients to resolve progeny nucleocapsids and virions. It was observed that treatment of U cells with 300 ng/ml of IFN-γ caused 95% reduction in yield of infectious progeny based on plaque assay and 90% reduction in physical viral particles based on radioactivity.

Figure 4. Sucrose Gradient Profiles of [35S]Methionine-Labeled VSV Fractions Pelleted and Assessed Nucleocapsid and Virion Proteins. U cell monolayers, either untreated or treated with IFN-γ at the indicated concentration for 24 h, were then left uninfected (a) or were infected with VSV at a multiplicity of 10 PFU/cell (b). Cells were labeled with [35S]methionine from 4-5 h post-infection and then incubated in maintenance medium until the standard 12 h harvest time. A small aliquot of each sample was assayed for infectious titer, and the remainder of each cell lysate was fractionated on 5 to 40% sucrose gradients as described in "Experimental Procedures." The radioactivity in a 50-μl aliquot of each 1-ml fraction was counted. The direction or sedimentation is from left to right. The shaded areas indicate the fractions pooled for further analysis.

Aliquots containing approximately equal amounts of radioactivity from pooled virion fractions of the untreated and IFN-γ-treated samples were pelleted by ultracentrifugation and analyzed by NaDODSO4 polyacrylamide gel electrophoresis (Fig. 5). There was no selective reduction of 5 or 6 or any of the other three VSV polypeptides in assembled virions obtained from IFN-γ-treated cells as compared to untreated U cells at any of the IFN-γ concentrations tested (Fig. 5). Functional protein bands appeared more prominent in the samples isolated from U cells treated with the higher concentrations of IFN-γ (Fig. 5, lanes b-f). However, this was due to the larger volume of sample which was required to obtain an amount of labeled protein similar to that of the other samples isolated from untreated cells or cells treated with lower concentrations of IFN-γ. This undoubtedly reflects the significant reduction in physical particle yield from IFN-γ-treated VSV as compared to untreated cells. The same autoradiograms were observed upon gel electrophoresis of the pooled uninfected U cell sample (Fig. 5, lane a), suggesting that these bands derive from co-sedimenting cellular material.

Figure 5. Polypeptide Composition of Progeny Virions Released from IFN-γ-Treated and Untreated Human U Cells. Equal amounts of radioactivity from each of the pooled viral peaks from Fig. 4 were pelleted by ultracentrifugation and analyzed by NaDODSO4 polyacrylamide gel electrophoresis. In the case of the untreated, uninfected sample (lane a), the corresponding gradient fractions were pooled and analyzed by autoradiography. The radioactivity in a 50-μl aliquot of each fraction was counted. The direction of sedimentation is from left to right. The shaded areas indicate the fractions pooled for further analysis.

Similar analysis of the pooled sucrose gradient fractions containing nucleocapsids isolated from untreated and IFN-γ-treated U cells likewise indicated that there was no detectable effect of IFN treatment on the polypeptide composition of nucleocapsids obtained from cells treated with the highest concentration of IFN, only nucleocapsids released from cells treated with representative lower concentrations of IFN were analyzed. As can be seen in Fig. 6, the labeled nucleocapsids were composed predominantly of N and M proteins, with smaller amounts of G, V, and L proteins, and the polypeptide designated 9 (8) was also detectable.