Formation of Non-infective Herpesvirus Particles in Cultured Cells Treated with Human Interferon

By ALBERTO MUÑOZ AND LUIS CARRASCO*

Departamento de Microbiología, Centro de Biología Molecular, Facultad de Ciencias, Universidad Autónoma, Canto Blanco, Madrid-34, Spain

(Accepted 27 February 1984)

SUMMARY

Treatment of HeLa cells with human lymphoblastoid interferon [HuIFN-α(Ly)] induced an antiviral state that rendered the cells refractory to infection by herpes simplex virus type 1 (HSV-1), and the yield of infectious HSV-1 was reduced by 98%. Analysis of the mechanism of the anti-herpes action of IFN indicated that no gross inhibition of viral protein synthesis took place and late proteins appeared, although the synthesis of some of them was partially inhibited. No differences were apparent between the glycoproteins or phosphoproteins synthesized in control and IFN-treated HSV-1-infected HeLa cells. No inhibition of the formation of new virus particles from IFN-treated cells was evident as assessed by electron microscopy and biochemical analyses, although their infectivity was drastically reduced. These virions exhibited some differences in their content of a few proteins as determined by PAGE. Studies on the second infection cycle with virions obtained from IFN-treated cells suggested that they attached and penetrated HeLa cells but that their development was impaired, since no viral protein synthesis was observed during the second infection. Altogether these results do not support the idea that the molecular mechanism of action of human IFN against HSV-1 is mediated via the 2'-5' A system, or by the inactivation of initiation factors. Rather, they focus the problem of the anti-herpes action of IFN at the level of formation of defective virions.

INTRODUCTION

Human interferon (IFN) is being used increasingly as an antiviral agent for clinical purposes. Positive results have been reported for a number of herpesvirus-associated diseases in several clinical trials in man. However, little is known about the molecular basis of the anti-herpes activity of human IFN.

The IFNs are a family of proteins secreted by mammalian cells in response to a varied array of stimuli. Cells exposed to IFN are resistant to infection by a number of animal viruses (Stewart, 1981). The step in the virus life-cycle that is inhibited in a cell where the antiviral state has been established varies, depending upon the particular virus–cell system being studied (Stewart & Lin, 1979; Sen & Herz, 1983; Muñoz & Carrasco, 1984).

For most viruses the step blocked by IFN is the transcription or the translation of the viral mRNA (Friedman, 1977). When DNA-containing viruses such as simian virus 40, a papovavirus, are used, either transcription or translation are inhibited depending on the time of IFN treatment (Yakobson et al., 1977) although this finding seems to be the result of a specific effect of IFN on the onset of viral RNA transcription (Brennan & Stark, 1983). The replication of poxviruses is inhibited by IFN acting by a different mechanism. When IFN-treated L cells grown in suspension are infected with vaccinia virus a drastic inhibition of cellular protein synthesis takes place and neither viral nor host protein synthesis is detected, although viral RNA is synthesized at higher levels than in control (IFN-untreated) cells (Joklik & Merigan, 1966; Metz & Esteban, 1972). Adenoviruses are thought to be very resistant to IFN action (Oxman et al., 1967). Although herpesviruses are sensitive to IFN and a number of reports indicate than IFN is a
potent antiviral substance against herpesvirus infection in man (Dunnick & Galasso, 1979; Merigan, 1981), the molecular mechanism of action of human IFN against herpesvirus infection is unknown. According to a recent report, treatment of cells with human IFN before cytomegalovirus infection led to the inhibition of immediate-early protein synthesis and, perhaps as a consequence, to the suppression of early viral RNA synthesis (Stinski et al., 1982).

According to current concepts of the antiviral state induced by IFN (Baglioni, 1979) it is thought that a 2′-5′A synthetase and a protein kinase are specifically synthesized de novo upon interaction of IFN with the cell. After virus infection, with the generation of dsRNA, the activity of both enzymes is stimulated. The synthetase makes the 2′-5′A oligonucleotide which activates a constitutive cellular nuclease that degrades RNA. The dsRNA also stimulates the action of a protein kinase enzyme that inactivates several proteins, one of which could be a subunit of the initiation factor eIF2. Although there is now overwhelming evidence that these two mechanisms are operative in cell-free systems obtained from IFN-treated cells (Williams & Kerr, 1980), and perhaps in intact cells (Williams et al., 1979; Wreschner et al., 1981; Epstein et al., 1981) it is not yet known whether they represent general mechanisms involved in the antiviral action of IFN. It is conceivable that these mechanisms apply only to a few species of animal viruses (Vaquero et al., 1981; De Ferra & Baglioni, 1981; Miyamoto & Samuel, 1981; Daher & Samuel, 1982; Muñoz et al., 1983b).

In this paper we have undertaken a study of the mode of action of human IFN against herpes simplex virus type 1 (HSV-1).

**METHODS**

**Materials.** Human lymphoblastoid interferon [HuIFN-α(Ly); 1 × 10⁶ IU/mg protein] was a generous gift of Drs N. B. Finter, K. H. Fantase and M. D. Johnston, Wellcome Research Laboratories, Beckenham, U.K. Radioactive compounds were purchased from Amersham International. Dulbecco’s modified Eagle’s medium (DMEM), newborn calf serum and trypsin were purchased from Gibco Biocult. Petri dishes and multiwell plates were from Falcon; HEPES was from Serva; sucrose was AnaLaR grade; other chemicals were from Merck.

**Viruses and cells.** HeLa cells were grown in DMEM supplemented with 10% newborn calf serum.

HSV-1 (KOS strain) was grown in HeLa cells in DMEM containing 2% calf serum. Cells were scraped off and the internal virus was extracted by successive cycles of freezing and thawing; the fraction obtained after removal of cell debris by low-speed centrifugation was used as source of virus.

**Virus infection and measurement of protein synthesis.** HeLa cells were grown on 16 mm 24-well plates at a concentration of 4 × 10⁵ cells/well and infected with HSV-1 at the m.o.i. described in each experiment; after a 1-5 h incubation at 37 °C the medium was removed and 0-5 ml DMEM medium (2% calf serum) added. To radiolabel the proteins 0-5 ml of DMEM medium without methionine, but containing 1% calf serum and 0-1 to 0-2 μCi [³⁵S]methionine (110 Ci/mmol; 5-4 mCi/ml) was added to the cells in a 1 h pulse. The medium was removed, and the cells were washed with phosphate-buffered saline (PBS) solution and precipitated with 5% TCA; the precipitate was washed three times with ethanol. The precipitated cell monolayer was dried under an infrared lamp and solubilized with 225 μl 0-1 M-NaOH plus 1% SDS. A 125 μl amount was counted using Bray’s liquid as a scintillation cocktail in an Intertechnique Scintillation Spectrometer.

**Preparation of radiolabelled virions.** In both cases (DNA- and protein-labelled virions) cells infected for 22 h were scraped off the 100 mm plates on which they had been grown and given ten strokes with a Potter–Elvehjem homogenizer. After a low-speed centrifugation at 6000 r.p.m. for 15 min at 2 °C, the supernatants were centrifuged on a 10% sucrose cushion in PBS at 30000 r.p.m. for 90 min at 2 °C in a SW50 rotor. The pelleted virus was resuspended in PBS.

**Virus entry.** The entry of virus into cells was measured by estimating the TCA-precipitated radioactivity in the cell monolayer at different times after infection with the radiolabelled virions (see above) at 37 °C, taking values obtained at 4 °C as controls for the virus adsorption. At the indicated times the medium was removed and cell...
monolayers on 16 mm 24-well plates were washed with PBS, DMEM and 0.0025% trypsin-0.08% EDTA in PBS, precipitated with 5% TCA and processed as above.

RESULTS

Effect of IFN treatment on virus production, macromolecular synthesis and membrane integrity in HSV-1-infected HeLa cells

Human cells grown in culture and treated with IFN are resistant to infection by a number of animal viruses to different degrees (Stewart, 1981). We first tested the antiviral effect of HuIFN-α(Ly) against infection of HeLa cells by HSV-1 (Table 1). Treatment of HeLa cells with concentrations of IFN as low as 10 IU/ml decreased the virus yield in a single step by more than 90%. Treatment with 400 IU/ml led to a 98% reduction of infectious HSV-1 as measured by the standard plaque assay on Vero cells. The capacity of the cells to synthesize proteins under conditions of HSV-1 infection is shown in Fig. 1. Infection with HSV-1 at multiplicities above

![Graph showing the effect of IFN treatment on virus production in HeLa cells](image)

**Fig. 1.** Protection by HuIFN-α(Ly) against HSV-1 infection of HeLa cells. Cells were grown in 24-well dishes and treated with IFN 18 h before infection with HSV-1 at the indicated m.o.i. After 48 h the level of protein synthesis in these cells was measured as described in Methods. 100% of the control corresponds to 27967 c.p.m. (without IFN), 25843 c.p.m. (4 U/ml), 25446 c.p.m. (40 U/ml) and 23757 c.p.m. (400 U/ml).

<table>
<thead>
<tr>
<th>Expt.</th>
<th>M.o.i.</th>
<th>HuIFN-α(Ly) (IU/ml)</th>
<th>Infectious virus production (p.f.u./ml x 10^-7)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>–</td>
<td>8.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>0.71</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>400</td>
<td>0.17</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1000</td>
<td>0.16</td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>0.1</td>
<td>–</td>
<td>3.1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>400</td>
<td>0.06</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>–</td>
<td>7.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>400</td>
<td>0.15</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>–</td>
<td>7.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>400</td>
<td>0.60</td>
<td>8</td>
</tr>
</tbody>
</table>

* IFN was added to cells 18 h before infection. The virus was collected 48 h later as indicated in Methods and titrated by standard plaque assay on Vero cells.
Fig. 2. Effect of HuIFN-α(Ly) on protein synthesis in HSV-1-infected HeLa cells. Cells were grown in 30 mm dishes. IFN (400 U/ml) was added 18 h before infection with 5 p.f.u./cell of HSV-1. The measurement of protein synthesis in the infected cells (○, IFN; ●, control) and analysis by PAGE were performed at the indicated times post-infection as described in Methods.

0.1 p.f.u./cell resulted in cell death even when the cells had been pretreated with IFN. When the cells were infected at lower multiplicities, the monolayer was protected, because the virus yield in the first step growth-cycle decreased and hence many cells remained uninfected.

When HeLa cells are treated with IFN and infected with picornaviruses (encephalomyocarditis, poliovirus), togavirus (Semliki Forest virus) or rhabdovirus (vesicular stomatitis virus, VSV) no viral protein synthesis occurs (Muñoz & Carrasco, 1981, 1983, 1984). The effect of IFN treatment on translation in HSV-1-infected HeLa cells is shown in Fig. 2. The decrease in total protein synthesis that occurred in HeLa cells infected with HSV-1 was also observed in cells...
Antiherpes action of interferon

1073

treated with 400 IU/ml HuIFN-α(Ly). Analysis by polyacrylamide gel electrophoresis of the proteins synthesized at different times after infection indicated that there was no major difference between control and IFN-treated cells. We conclude from these results that the effect of IFN treatment on HSV-1 development is quite different from that previously reported for SV40 (Yakobson et al., 1977), vaccinia virus (Metz et al., 1975) and cytomegalovirus (Stinski et al., 1982), since herpesvirus protein synthesis evidently takes place in cells treated with HuIFN-α(Ly).

In L cells treated with mouse IFN and infected with VSV there is a drastic reduction in infective virus production. Maheshwari et al. (1980) have reported that the step blocked in this system, when low doses of IFN are used, is the maturation of a viral protein. In particular, the viral G protein is not glycosylated, although it is synthesized and incorporated into virions. Those virions, however, showed a much reduced infectivity. Analyses of the glycoproteins in uninfected HeLa cells, in cells infected with HSV-1 and in IFN-treated cells infected with HSV-1 have been carried out, and no differences in glycosylation of viral proteins were observed after IFN treatment (results not shown). Since a higher level of phosphorylation occurs in IFN-treated cells, we analysed the level of phosphoproteins in HSV-1-infected HeLa cells, both in control and in IFN-treated cells at different times after infection. We did not detect a difference between the two samples that could account for the massive decrease in virus yield in IFN-treated cells (results not shown).

We also investigated the integrity of the cell membrane throughout the course of infection in HSV-1-infected HeLa cells with and without IFN treatment. An increased loss of $^{86}$Rb$^+$ from untreated HSV-1-infected HeLa cells started at 6 to 8 h after infection. This loss was higher after the 10th hour when the cells had been treated with IFN. The consequences of this greater decrease in ion content in IFN-treated cells infected with HSV-1 are not clear.

Assembly and integrity of herpes virions in IFN-treated cells

Since the analysis of the synthesis of viral proteins, glycoproteins and phosphoproteins in cells infected with HSV-1 showed no major differences between cells treated or not treated with IFN, we analysed whether treated cells produced virions and whether those virions were similar to normal ones. For this purpose, the infected cells were labelled with $[^3]$Hthymidine from 3 to 22 h post-infection; the virions were extracted as indicated in Methods and analysed on a Sepharose 6B column. Particles of comparable size were indeed obtained from control and IFN-treated cells. Moreover, the content of $[^3]$Hthymidine in particles of each origin are also comparable, suggesting that viral DNA synthesis was not affected by IFN treatment (results not shown).

To analyse further whether there might be some specific defect in the assembly of virions in cells treated with IFN, such as lack of membrane envelope, the virions obtained from control and IFN-treated cells were analysed by electron microscopy. According to others (Watson et al., 1963) four different types of HSV-1 particles are distinguishable by electron microscopy after negative staining: enveloped full particles, naked full particles, enveloped empty particles and naked empty particles. No differences were observed either in the morphology or in the percentage of each class of virion formed within IFN-treated or untreated cells.

The percentage of virions observed to contain DNA is about the same in both control and IFN-treated cells, suggesting once again that no significant inhibition of viral DNA synthesis takes place in IFN-treated cells. Analysis by electron microscopy of sections from infected cells also did not show any differences in the virion particle formation, cytopathic effect or virus cycle (results not shown).

Next we examined the nature and content of proteins incorporated into purified virions. Fig. 3 indicates a number of differences in the protein content of virions from control or IFN-treated cells. Some viral bands were considerably reduced in the IFN-treated samples: the absence of a protein $M_r$ 18,000 from these is particularly apparent. The relevance of these differences to the lack of infectivity of these virions is presently being investigated.

Finally, we designed experiments to test the infectivity of these virions. First, we examined the effect on cellular protein synthesis induced by virions grown in control or in IFN-treated
Fig. 3. Effect of HuIFN-α(Ly) on HSV-1 proteins incorporated into virions. Cells were grown, IFN-treated (400 U/ml) and infected as in the legend to Fig. 2. [35S]Methionine (5 μCi/ml) was added in DMEM without methionine plus 1% calf serum at the end of virus incubation and every 2 h post-infection (2, 4, 6, 8). Extraction of virions was carried out 22 h after infection as described in Methods, and proteins present in the virions were analysed by PAGE. (a) Virions from untreated cells; (b) virions from IFN-treated cells.

cells both in control HeLa cells (Fig. 4a) or in cells pretreated with IFN (Fig. 4b) using the same number of virus particles. Their ability to induce c.p.e. was also analysed microscopically and by dye exclusion. A reduced infectivity of IFN-treated cell-derived virions which paralleled their capacity to depress cellular protein synthesis was observed, the reduction being 90% (Fig. 4a), in agreement with the results obtained from analysis of yields in terms of p.f.u. The difference between virions from control and IFN-treated cells was also found when the cells had been pretreated with IFN. This result is important since it indicates that the replication of those few infective virus particles produced from IFN-treated cells was inhibited when they infected a second cell treated with IFN. In other words, the infective virions produced in the first round of
Antiherpes action of interferon

Fig. 4. Inhibition of cellular protein synthesis induced by HSV-1 grown in cells with or without HuIFN-α(Ly) treatment. Cells grown in 24-well dishes containing 4 × 10⁵ cells/well were infected with HSV-1 grown in cells treated, where appropriate, with IFN (400 U/ml) and the level of protein synthesis was measured 24 h later in a 1 h pulse. (a) Control cells; (b) cells treated with IFN 18 h before infection. ●, Virus from IFN-treated cells; ○, virus from untreated cells.

replication in IFN-treated cells were not intrinsically resistant to IFN and hence selected out, since their replication was blocked when a second round of infection of an IFN-treated cell took place.

The possibility that the virions formed from IFN-treated cells might be defective in recognition, attachment and entry into HeLa cells was also tested. For this purpose, virions were labelled in their DNA with [³H]thymidine or in their protein with [³⁵S]methionine. The internalization of the label into HeLa cells was followed using similar numbers of virus particles from control or IFN-treated cells (Fig. 5). A slight reduction of around 10 to 30% was observed in the entry of label from virions obtained from IFN-treated cells. These results suggest that the virion particles formed in IFN-treated cells are almost as effective as control HSV-1 virions as regards attachment and entry into a second cell to initiate a second cycle of infection.

Finally, since protection against induction of the c.p.e. was found when virions from IFN-treated cells were used (Fig. 6), we analysed protein synthesis throughout the course of infection both in control HeLa cells and IFN-treated cells using the same numbers of HSV-1 particles...
grown in HeLa cells or in IFN-treated cells. Fig. 6 shows that when the HSV-1 used had been grown in cells treated with IFN, no viral proteins could be detected at any stage in the normal period of infection and only cellular protein synthesis was apparent. This result agrees with the finding that there is protection against the c.p.e. as observed under the phase-contrast microscope, and indicates that although virions from IFN-treated cells gain entry into HeLa cells, the second round of replication is blocked at an early stage since no late viral proteins appear.

DISCUSSION

The action of IFN against herpesvirus infections is now well established (Merigan, 1981). However, we know very little about the molecular basis by which an IFN-treated human cell becomes resistant to infection by herpesviruses. According to current models for the molecular
mechanisms of IFN action, drawn mainly from studies on cell-free systems, protein synthesis is the major step blocked during viral infection (Baglioni, 1979). This inhibition of protein synthesis becomes effective when viral dsRNA is synthesized and activates a 2'-5'A synthetase which makes a 2'-5'-oligo(A) from ATP. The 2'-5'-oligo(A) in turn activates a constitutive nuclease that degrades RNA. The dsRNA also activates a protein kinase which phosphorylates a number of proteins, including a subunit of initiation factor eIF2. Although it is reasonable to assume that these mechanisms operate in IFN-treated virus-infected cells, since RNA and DNA viruses form dsRNA during their replication, there is as yet no direct evidence that they are responsible for the antiviral effect of IFN in intact cells. Infection of IFN-treated mouse L cells grown in suspension with vaccinia virus induces a drastic inhibition of both cellular and viral protein synthesis (Joklik & Mergan, 1966; Metz & Esteban, 1972) but whether this effect is mediated by the mechanisms discussed remains to be established.

Our results on the replication of HSV-1 in IFN-treated cells indicate that viral protein synthesis continues at almost the same rate as in control cells. We take this to mean that the viral mRNAs must also be present and that the components of the protein-synthesizing machinery remain active, indicating that the mechanisms suggested for the antiviral action of IFN are not applicable in herpesvirus-infected cells. On the other hand, the replication of the viral genome does not seem to be affected either, since the synthesis of late proteins would then have been impaired.

Studies on the antipherpetic activity of IFN have been interpreted on the assumption that this antiviral action is mediated via stimulation of the immune system. Indeed, IFN has been shown to enhance the lytic activity of natural killer cells against HSV-1-infected cells (Fitzgerald et al., 1982) and even to increase the susceptibility of HSV-1-infected HeLa cells to lysis by these immune cells (Muñoz et al., 1983a). Our present results show that besides this indirect effect mediated by the immune system, IFN exerts a direct antipherpetic effect inhibiting viral replication in infected cells.

We believe that this direct action of IFN against herpesviruses is very subtle. Our studies have already revealed that new viral particles are formed at levels comparable to those of controls. However, most of these virions are inactive. Comparison of control virions with these indicates that some differences exist in the amounts of a number of proteins. The role that those proteins might play during the replicative cycle of HSV-1 is not known.

Whatever the defects that exist in virions obtained from IFN-treated cells, they retain the ability to infect a new cell, although their replication in that cell is impaired. Bearing in mind these findings, we believe that future research on the molecular basis of the antipherpetic effects of IFN could profitably concentrate on two questions: (i) why are defective virions formed in IFN-treated cells and what exactly are the molecular differences between normal virions and those obtained from IFN-treated cells, and (ii) what step is impaired in the second replicative cycle of those virions. Attempts to answer these questions are in progress in our laboratory.

The expert technical assistance of Ms M. A. Ramos is acknowledged. We also thank M. Caballero for the development of computer programs for image processing and J. de la Torre for his help in the electron microscope experiments. This work has been supported by CAICYT, 'Fundación Científica de la Asociación Española Contra el Cáncer' and 'Plan Concertado de Investigación'.

REFERENCES


(Received 26 October 1983)