Inhibition by Interferon of Herpes Simplex Virus Thymidine Kinase and DNA Polymerase in Infected and Biochemically Transformed Cells

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SUMMARY

The induction of thymidine kinase (TK) and DNA polymerase was inhibited by interferon (IFN) in mouse L-cells infected with herpes simplex virus type 1 (HSV-1). The inhibitory activity of IFN at this early stage of HSV-1 replication was followed by a reduced virus yield and was dependent on the multiplicity of infection. The expression of a cloned thymidine kinase (tk) gene of HSV-1, in biochemically transformed L-cells (LTK⁺), was not affected by IFN. These same LTK⁺ cells, however, developed an antiviral state since, upon HSV-1 infection, the induction of TK and DNA polymerase of the replicating virus was inhibited by IFN. Furthermore, IFN inhibited the transactivation of the HSV-1 tk gene in the biochemically transformed LTK⁺ cells, which followed infection by a virus mutant defective in the tk gene (HSV-1 TK⁻). This transactivation is dependent on expression of immediate-early HSV-1 α-genes. These results indicate that IFN inhibits HSV-1 replication at an early step prior to DNA synthesis. In addition, IFN displays a differential effect on the HSV-1 thymidine kinase gene, either when part of the replicating virus or when expressed as a cellular gene in biochemically transformed cells.

INTRODUCTION

In studies on the inhibition of herpesviruses by interferon (IFN) three assays have been used. These include inhibition of cytopathic effect, plaque reduction and inhibition of virus yield (Glasgow et al., 1967; Lerner & Baily, 1976; Rasmussen & Farley, 1975). Although some of the early reports suggested that herpesviruses were not sensitive to IFN (Ho & Enders, 1959; Glasgow et al., 1967), more recent studies have indicated that virus yields are reduced in IFN-treated cells (Rasmussen & Farley, 1975). Nonetheless, relatively high concentrations of IFN were required for this effect as compared with other virus groups.

Little information is available to date on the mechanisms of herpes simplex type 1 (HSV-1) inhibition by IFN. The complexity of the virus genome and the cascade mechanism of virus protein synthesis (for review, see Spear & Roizman, 1980) make such an analysis difficult. In the present work we describe the effects of IFN on the replication of HSV-1, using as markers two of the virus β-proteins, namely, the thymidine kinase (TK) and DNA polymerase. Effects of IFN on these virus enzymes were studied following virus infection and when a cloned thymidine kinase (tk) gene was stably integrated in the cell DNA (Wigler et al., 1977).

METHODS

Cells and viruses. LTK⁺ cells were obtained by transformation of murine L-cells LTK⁻, APRT⁻ to the TK⁺ phenotype with a cloned tk gene of HSV-1 (BamHI fragment of HSV-1 DNA, cloned in pBR322; Wigler et al., 1977). A cell clone RALH2-grown in selective medium (HAT) was isolated and used in the present study (Stein et al., 1982). The LTK⁺ cell line was received from R. Stein and H. Cedar of the Hebrew University, Jerusalem. L₉ mouse cells have been previously described (Epstein et al., 1981). The HSV-1 wild-type, NIH strain and HSV-1 TK⁻ mutants, derived from the NIH strain (Becker et al., 1982), were obtained from Y. Shtram and Y. Becker of the Hebrew University, Jerusalem. HSV-1 virus stocks were grown in BSC-1 cells, and HSV-1 TK⁻ was grown in the presence of bromodeoxyuridine (10 µg/ml) (Becker et al., 1982). The HSV-1 TK⁻ was checked periodically; it was found to be unable to induce TK activity after infection of L-cells deficient in TK (LTK⁻ cells). HSV-1 titres
were determined by plaque assay on BSC-1 cells with an agar overlay. Encephalomyocarditis virus was propagated and titrated in L<sub>n</sub> cells.

For virus infection, 200 000 cells were seeded in 35 mm plates in RPMI 1640 medium (2 ml) containing 10% calf serum [for LTK<sup>+</sup> cells, hypoxanthine, aminopterin and thymidine (HAT) were added to the medium]. After 24 h the medium was changed and the cells were exposed to IFN for 18 to 24 h. For virus infection, medium was removed, the inoculum (0.3 ml) was added for 30 min and cultures were washed and fed with medium containing 10% calf serum (1 ml). Ten h after infection, monolayers were washed with phosphate-buffered saline (PBS, 2 ml) and with cold extraction buffer (2 ml) (10 mM-Tris-HCl pH 8.0, 2 mM-2-mercaptoethanol, 20% glycerol). Cells were removed in the extraction buffer (0.3 ml) with a rubber policeman, sonicated and suspensions were centrifuged for 15 min at 12000 g. The supernatants were kept at −20 °C before enzyme assays. All experiments were done with duplicate cell cultures.

Mouse IFN (1 × 10<sup>8</sup> units/mg) was induced in L-cells with Newcastle disease virus (Paucker <i>et al.</i>, 1970) and purified by adsorption to a column of Sepharose linked to antibodies specific for IFN.

**Enzyme assays.** HSV-1 DNA polymerase activity in cell extracts was determined as described by Purifoy & Benyesh-Melnick (1975). The incorporation of [3H]TTP (sp. act. 22000 ct/min/pmol) into trichloroacetic acid (TCA)-insoluble material was followed with activated DNA as template-primer. KCl (0.2 M) was included in the assay mixtures to suppress cellular DNA polymerases.

HSV-1 TK activity was assayed by the phosphorylation of [3H]thymidine (sp. act. 880 ct/min/pmol) (Jamieson <i>et al.</i>, 1974); [3H]TMP was determined after binding to DEAE-cellulose filters (DE-81, Whatman). Both the DNA polymerase and TK reactions were linear with respect to incubation time (30 min at 37 °C). All enzyme assays were carried out in duplicate (20 μl and 40 μl of cell extract in 100 μl reaction mixture); the reactions were linear with respect to the amount of enzyme.

Enzyme activities in mock-infected cells were assayed in parallel in every experiment. These endogenous enzyme activities were not subtracted from values obtained in HSV-1-infected cells. Endogenous DNA polymerase activity in uninfected cells (at zero time of infection) represents residual cellular DNA polymerase activity (5 to 10%) not inhibited by the KCl (0.2 M) included in the assay.

TK activity in uninfected L<sub>n</sub> cells represents the level of cell-coded enzyme activity. In the biochemically transformed LTK<sup>+</sup> cells, endogenous TK activity represents constitutive expression of the cloned HSV-1 tk gene.

Protein content of cell extracts was determined according to Lowry <i>et al.</i> (1951), and enzyme activities (radioactivity incorporated in 30 min) were calculated per μg protein. All results use the average of duplicate cultures.

**RESULTS**

To investigate the mechanisms of HSV-1 inhibition by IFN, we chose L-cells which presented several advantages for such a study. (i) L-cells are very sensitive to the antiviral activity of IFN and replication of viruses such as encephalomyocarditis (EMC) and vesicular stomatitis (VSV) is efficiently inhibited. (ii) Enzyme systems induced by IFN, namely, the double-stranded (ds)RNA-dependent protein kinase, (2',5')oligoadenylate synthetase and RNase F are well defined in L-cells (Kimchi <i>et al.</i>, 1979; Knight <i>et al.</i>, 1980; Epstein <i>et al.</i>, 1981). (iii) L-cells deficient in thymidine kinase (TK<sup>−</sup>) may be transformed to the TK<sup>+</sup> phenotype with a cloned tk gene of HSV-1 (Wigler <i>et al.</i>, 1977) and thus effects of IFN on the expression of a single virus gene can be analysed.

To investigate the effects of IFN on the early stages of HSV-1 replication, we measured the induction of HSV-1 DNA polymerase and thymidine kinase, both β-proteins, which precede the synthesis of virus DNA. While the two HSV-1 enzymes could be detected 4 to 5 h post-infection in control cells, their specific activities were significantly reduced in the IFN-treated cultures (Fig. 1). These results could indicate inhibition of virus β-protein synthesis at an early stage of virus replication, or, alternatively, formation of an inhibitor in the IFN-treated cells which interferes with the DNA polymerase and TK enzyme assays. The latter possibility can be excluded, since in mixing experiments, extracts from IFN-treated cells had no inhibitory effect on the two enzyme activities assayed in the extracts of HSV-1-infected cells (data not shown).

To quantify the sensitivity of HSV-1 replication to IFN, cultures were treated with increasing concentrations of IFN and the induction of DNA polymerase and TK was analysed following infection (Fig. 2). Reductions in specific activities of both enzymes were identical but as much as 1000 units/ml of IFN were needed to inhibit enzyme induction by 50%. Treatment with 25 units/ml of IFN resulted in no inhibition of HSV-1 enzyme induction. On the other hand,
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Fig. 1. Effect of IFN on the kinetics of induction of HSV-1 DNA polymerase and TK. Lb cells pretreated with IFN (1000 units/ml) (○) and control cells (●) were infected with HSV-1 at an m.o.i. of 2, as described in Methods. At various times, cells were harvested and TK (a) and DNA polymerase (b) activities were determined.

Table 1. Inhibition of HSV-1 and EMC virus production: yield reduction by plaque assays*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Virus titre (p.f.u./ml)</th>
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<tbody>
<tr>
<td></td>
<td>HSV-1</td>
<td>EMC</td>
<td></td>
</tr>
<tr>
<td>−IFN</td>
<td>$2 \times 10^7$</td>
<td>$1 \times 10^9$</td>
<td></td>
</tr>
<tr>
<td>+IFN</td>
<td>$5 \times 10^3$</td>
<td>$1 \times 10^4$</td>
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* Lb cell cultures were treated with IFN (1500 units/ml) for 18 h. Cells were infected with HSV-1 or EMC virus at an m.o.i. of 1 and, after 24 h, cells together with the growth media were collected under sterile conditions and sonicated. HSV-1 was titrated in BSC-1 cells, in duplicate, for plaque formation. Plaques were counted 4 to 5 days after infection. EMC virus was titrated by endpoint dilution on Lb cells in 96-well microtitre plates.

EMC virus replication, as measured by virus RNA synthesis, was inhibited to 50% at low concentrations of IFN (25 units/ml). In this experiment EMC virus was more sensitive to IFN in spite of the fact that infection with EMC virus was at a higher multiplicity.

To compare further the sensitivities of EMC and HSV-1 to IFN, we measured virus yield by plaque assay (Table 1). HSV-1 replicated efficiently in L-cells and gave high virus yields ($10^7$ p.f.u./ml). Treatment with high concentrations of IFN (1500 units/ml) resulted in a reduction of 1 to 2 log10 in the yield of HSV-1. Under similar conditions, the EMC virus yield was reduced by 5 log10 units. These results again indicate that, relative to EMC virus, HSV-1 replication exhibited low sensitivity to IFN.

To study factors that may influence the inhibitory activity of IFN on HSV-1, the effects of IFN when added before or after virus infection were compared. IFN, when added after
infection, had no effect on the induction of DNA polymerase and TK; maximal inhibition required pretreatment for at least 6 h (data not shown). The extent of HSV-1 inhibition by IFN appears to be largely dependent on the multiplicity of infection, and at multiplicities lower than 2, HSV-1 inhibition by IFN was most efficient (Fig. 3). In repeated experiments, infection of IFN-treated cells with very high multiplicities (m.o.i. of 10 or more) did not result in the inhibition of TK induction and the HSV-1 DNA polymerase was only slightly affected.

The results presented in Fig. 1 to 3 indicate that IFN inhibits the synthesis of β-proteins (TK and DNA polymerase) during productive HSV-1 infection. Moreover, they provide simple and rapid enzyme assays to follow the anti-HSV-1 activity of IFN. To investigate the effects of IFN on a single virus gene in the absence of virus replication, we measured HSV-1 TK activity in L-cells biochemically transformed from the TK- to the TK+ phenotype by a cloned HSV-1 tk gene. Culturing the LTK+ cells for 7 days (about 14 generations) in the presence of a high concentration of IFN (2000 units/ml) resulted in no significant alteration of HSV-1 TK specific activity (Table 2). The proliferation of LTK+ cultures was only slightly inhibited by the IFN and their protein content was 10 to 15% lower than control cultures. The LTK+ cell line, however, retained its normal sensitivity to IFN, since infection of IFN-treated cultures with HSV-1 resulted in the inhibition of DNA polymerase induction (Fig. 4b). The extent of HSV-1 inhibition after infection of IFN-treated LTK+ cells was similar to the inhibition observed with the standard Lb cells (Fig. 3b). The induction of TK activity was also inhibited during HSV-1 infection of IFN-treated LTK+ cells (Fig. 4a). In the infected LTK+ cells, however, it was not possible to differentiate between the endogenous TK and the replicating virus TK activities.
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Fig. 3. Dependence of IFN inhibition on the multiplicity of HSV-1 infection. L929 cells pretreated with IFN (1000 units/ml) (■) and control cells (○) were infected with HSV-1 at increasing multiplicities. After 10 h of virus infection, cells were harvested and TK (a) and DNA polymerase (b) activities were assayed.

Table 2. Thymidine kinase specific activity in LTK+ cells grown in the presence of IFN*

<table>
<thead>
<tr>
<th>Day of harvest after IFN added</th>
<th>TK (ct/min/μg protein)</th>
<th>Ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>-IFN</td>
<td>+IFN</td>
</tr>
<tr>
<td>1</td>
<td>54</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
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<td>43</td>
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<tr>
<td>7</td>
<td>56</td>
<td>50</td>
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</table>

* LTK+ cells (200 000) were plated and, after 24 h, IFN (2000 units/ml) was added. Cells grown in the presence or absence of IFN were collected each day. After 4 days, cultures were re-plated (200 000 cells/dish) in the presence or absence of IFN (2000 units/ml) and harvested as indicated. At the end of the experiment, cell extracts were assayed for TK and protein content.

Note that the specific activity of endogenous TK in the mock-infected LTK+ cells was not inhibited by IFN (Fig. 4a), in accordance with the observation presented in Table 2.

Some of the antiviral factors present in cells treated with IFN may require a replicating virus for their activation. One such example is the synthesis of (2',5')oligoadenylate in IFN-treated cells, which is dependent upon EMC virus infection. The replicating EMC virus appears to provide dsRNA for activation of the enzyme (2',5')oligoadenylate synthetase (Knight et al., 1980).

To test whether replicating HSV-1 could potentiate an antiviral state in LTK+ cells that would also result in inhibition of the endogenous TK activity, we infected the LTK+ cells with a mutant
Fig. 4. Inhibition of HSV-1 and HSV-1 TK- mutant replication in LTK+ cells. LTK+ cells pretreated with IFN (1500 units/ml) (●) and control cells (○) were infected with HSV-1 (a, b) or HSV-1 TK- (c, d) at increasing multiplicities. After 10 h of virus infection, cells were harvested and TK (a, c) and DNA polymerase (b, d) activities were assayed. In control experiments where HSV-1 TK- was analysed by infecting LTK- cells, TK specific activity was negligible, <25 cT/min/µg protein, whereas DNA polymerase specific activity was 480 cT/min/µg.

HSV-1 TK-, defective for the induction of TK. Transactivation of the endogenous TK specific activity after infection with HSV-1 TK- was seen in the control cultures (Fig. 4c). The transactivation phenomenon appears to be dependent on HSV-1 α-proteins made early after infection (Leiden et al., 1976; Kit et al., 1978; Preston, 1979) which were provided in this experiment by the replicating HSV-1 TK- mutant. IFN treatment suppressed the transactivation phenomenon (Fig. 4c). Under these conditions, replication of the TK- virus was also inhibited, as indicated by reduction of the DNA polymerase specific activity in IFN-treated LTK+ cultures (Fig. 4d). In repeated experiments with LTK+ cells infected by HSV-1 TK-, we never observed inhibition of the TK specific activity below the endogenous level found in the mock-infected cultures (Fig. 4c).

Since transactivation of the tk gene depends on the synthesis of α-proteins, these results suggest that IFN may inhibit production of HSV-1 α-proteins. Moreover, replicating HSV-1 does not appear to supply a factor which would activate the IFN system to suppress endogenous levels of HSV-1 TK in biochemically transformed cells.

**DISCUSSION**

There are several studies on the sensitivity of HSV to IFN but little is known about the mechanisms of inhibition of HSV. In the present work, we have analysed the effects of IFN on the expression of HSV-1 β-proteins; induction of both thymidine kinase and DNA polymerase after virus infection was inhibited in cells pretreated with IFN. This observation provides,
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among other things, a quantitative, rapid assay for the analysis of herpesvirus sensitivity to IFN in different cells. The inhibition of enzyme induction is a more sensitive assay for IFN than assays based on plaque reduction or inhibition of cytopathic effect (Glasgow et al., 1967; Lerner & Bailey, 1976). The enzyme assay described in this work is nearly as sensitive as the assay based on HSV-1 yield reduction, which is determined by titrating the virus yield from the IFN-treated cultures on indicator cells (Rasmussen & Farley, 1975; Table 1). The latter assay, however, is time-consuming and laborious. The sensitivity of a biochemical assay in the analysis of virus inhibition by IFN is usually lower than that of virus yield reduction. For example, inhibition of EMC virus RNA synthesis by 60% (Fig. 2) was followed by a 3 log10 reduction in EMC virus yield. However, the yield of HSV-1, which is reduced by only 1 to 2 log10 in IFN-treated cells, is preceded by a significant suppression (two- to tenfold) in the HSV-1 early gene products.

Our results suggest that the effect of IFN on TK induction in HSV-1-infected cells may be a consequence of inhibition of an earlier step and not a direct suppression of β-gene expression. Transactivation of the tk gene in biochemically transformed cells appears to be dependent on an immediate-early α-polypeptide (Kit et al., 1978; Preston, 1979). This α-protein, which was provided in our experiments by the replicating HSV-1 TK−, might have been suppressed by IFN and thus transactivation of the endogenous tk gene in LTK+ cells was inhibited. We have attempted to analyse possible effects of IFN on α-protein synthesis by pulse-labelling cells 3 to 6 h after infection with [35S]methionine and identifying virus proteins in cell extracts after electrophoresis on SDS-polyacrylamide gels (Hones & Roizman, 1974). We could not detect significant differences between the patterns of virus proteins obtained from IFN-treated and control cells. Two problems, however, precluded an accurate analysis. (i) Relatively high multiplicities of infection (>10) were needed to observe HSV α-protein synthesis (Hones & Roizman, 1974). Under these conditions, the effect of IFN was negligible on DNA polymerase and TK induction (Fig. 3), as well as on α-protein synthesis (data not shown). (ii) HSV-1 infection of L-cells resulted in a weak shut-off of cellular protein synthesis 4 to 6 h post-infection and, thus, the background of host protein synthesis was rather high. Since HSV-2 has been shown to produce, in a human cell line, a stronger shut-off than HSV-1 (Powell & Courtney, 1975), the effect of IFN on α-protein synthesis is being investigated with HSV-2-infected cells.

The finding that IFN has no effect on the expression of the HSV-1 tk gene when stably integrated in the cell chromosome indicates a differential inhibition of the replicating virus gene. However, the tk mRNA and its polypeptide product are indistinguishable whether produced during HSV-1 infection or in the biochemically transformed cells (Wigler et al., 1977; McKnight et al., 1981).

The effects of IFN on TK described in this work are similar in several respects to the inhibition of simian virus 40 (SV40) T-antigen by IFN. The synthesis of this antigen early in SV40 productive infection is inhibited by IFN (Oxman & Levin, 1971; Yakobson et al., 1977; Mozes & Defendi, 1979). In fact, the inhibition of T-antigen synthesis early in SV40 infection might be a consequence of suppression of a very early step in infection, prior to T-antigen mRNA synthesis (Yamamoto et al., 1975).

Using a virus mutant (HSV-1 TK−), we demonstrated that the replicating virus was unable to activate the IFN system for the suppression of the HSV-1 tk gene when stably integrated in the cell DNA. Whether the replicating HSV-1 produces a factor which is required to promote its own inhibition in IFN-treated cells is not clear. The inhibition of RNA viruses, such as EMC and reovirus, in IFN-treated cells has been shown to be dependent on a factor (dsRNA) provided by the replicating virus for the activation of the (2′,5′)oligoadenylate synthetase–RNase F pathway (Knight et al., 1980; Nilsen et al., 1982). It should be noted here that induction of these two enzyme pathways in the L-cells used in this work required relatively low concentrations of IFN (25 units/ml) (Epstein et al., 1981). These concentrations of IFN which inhibited EMC virus had no effect on HSV-1 replication. It remains to be investigated as to what extent HSV-1 can activate the (2′,5′)oligoadenylate synthetase–RNase F pathway and whether the replication of HSV-1 is susceptible to RNase F activity.

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REFERENCES


