# Interferon Effect on Cellular Functions: Enhancement of Virus Induced Inhibition of Host Cell Protein Synthesis in Interferon-Treated Cells

M. Suh, G. Bodo

Ernst-Boehringer-Institut für Arzneimittelforschung, Wien

and

W. Wolf, G. Viehhauser, and C. Jungwirth

Institut für Virologie, Würzburg

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*Herrn Professor* Dr. Otto Hoffmann-Ostenhof *zum 60. Geburtstag gewidmet*

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Total protein synthesis in mouse cells but not in confluent chick embryo fibroblasts (CEF) is inhibited shortly after infection with vaccinia virus. This inhibition by the infecting virus is enhanced drastically if the mouse cells have been pretreated with homologous interferon preparations. The enhanced reduction of protein synthesis also occurs if the cells are treated with actinomycin D and is therefore to a large extent caused by an enhanced inhibition of amino acid incorporation into host cell proteins. Enhanced inhibition of total protein synthesis during the early stages of infection may be a prerequisite for the complete degeneration of the cells (lysis) which occurs later. Various alterations of mouse cells and chick embryo fibroblasts due to exposure to homologous interferon preparations are discussed with respect to the antiviral state induced in these cells.

## Introduction

Studies with a variety of different cells led to the conclusion that pretreatment with interferon affects viral processes specifically and has little effect on cellular metabolism. Cells of murine origin seem to be an exception to this notion. Johnson reported that pretreatment of L-cells with a homologous interferon preparation resulted in the reduction of total amino acid incorporation into protein [1]. Partially purified interferon preparations also affected the growth properties of diverse cells of mouse-origin [2–5]. These effects could be caused by an alteration of cell metabolism or resulting from an effect of the interferon preparations on the RNA tumor virus population contained in these cells [3]. If mouse cells treated with interferon are exposed to various kinds of toxic effects such as infection with a variety of poxvirus strains or treatment with toxic compounds, an enhanced degeneration of these cells is observed [6, 7]. These observations imply that interferon preparations prepared in mouse cells contain components which affect the host cell. The connection between these effects and the antiviral state resulting from exposure to interferon preparations is at the present time controversial. The elimination of virus infection by destruction of the interferon-treated infected cells suggested by Joklik on the basis of studies with L-cells opens the possibility of an anti-viral effect of interferon not mediated by an inhibition of viral protein synthesis which may be of importance under certain conditions [6].

L-cells are of special interest for studies on the mechanism of interferon action because cell-free systems from these cells are active in the translation of cellular and viral m-RNA *in vitro* [8–12]. This makes a direct study of the effect of interferon on the various steps of viral protein synthesis possible [9–11]. However, because of the strong effects on host-cell functions in murine cells, it is not clear whether observations on the mechanism of interferon action obtained using these cells can be used to generalize about other systems.

The present investigation was undertaken to study the conditions necessary for the appearance of an enhanced inhibition of protein synthesis in

Requests for reprints should be sent to Dr. Ch. Jungwirth, Institut für Virologie der Universität, *D-8700 Würzburg*, Versbacher Landstr. 7.

the early phase of infection of interferon-treated cells by vaccinia virus. These conditions were compared with the conditions necessary for the complete "lysis" of the cells late in infection.

## Materials and Methods

*Cultivation of cells*

Chicken embryo fibroblast cultures (CEF) were prepared from 11-day old embryos by standard techniques. Growth medium was Parker's medium 199 supplemented with 10% inactivated calf serum. Primary mouse fibroblast cultures (MEF) were prepared from 15-day old embryos. Growth medium was Eagle's MEM plus 10% inactivated calf serum [13]. Mouse L-929 cells (Flow Lab., Scotland) were propagated in Eagle's MEM plus 10% inactivated calf serum.

*Treatment of cells with interferon and inhibitors of RNA and DNA synthesis*

Confluent monolayers in 50 mm plastic petri dishes were washed with Parker's 199 (CEF) or Eagle's Minimum Essential Medium plus 2% calf serum (mouse cells), and incubated for 20 – 24 hours with 2 ml of interferon dilutions. Mouse interferon was diluted in Eagle's Minimum Essential Medium plus 2% serum; chick interferon in Parker 199 containing 0.5% bovine serum albumin and 0.005% Tween 80.

The interferon preparations used were either chick allantoic interferon or mouse L-929 cell interferon, which had been partially purified by the method described earlier [14]. (1 unit of mouse cell interferon corresponded to 15 units of the N.I.H. reference interferon; 160 CEF-interferon units corresponded to 100 units A 62/4 Medical Research Council, chick embryo reference standard. Specific activity of mouse interferon: 700 000 units/mg; specific activity of chick interferon; approximately $1 \times 10^6$ units/mg.) Inhibitors of DNA and RNA synthesis were present from the beginning of the adsorption period until the cells were harvested.

*Infection of cells*

Interferon dilutions were removed, and monolayers were covered with 0.5 ml of a virus suspension in Hank's balanced salt solution without glucose containing 0.5% DIFCO gelatin. After the adsorption period (20 min for mouse cells and one hour for CEF) 1.5 ml of maintenance medium was added (Parker's 199 without serum for CEF or Eagle's MEM for mouse cells) and incubation continued for the appropriate time. Cowpox virus strain Brighton, or vaccinia virus strain WR were used in the experiments. All virus preparations were purified by the method of Joklik [15]. Plaque forming units were determined in CEF as described by Lindemann and Gifford [16].

*Estimation of total protein synthesis*

At the times indicated, 1.25 $\mu$Ci of a [$^{14}$C]amino acid mixture (57 mCi/milliatom C, Amersham, England) were added to each dish and incubation continued for 15 or 30 min. Dishes were placed on crushed ice and the cells were scraped off and pelleted using cold phosphate buffered saline (PBS). If the cells tended to come off during the incubation period, the culture medium was centrifuged and the pellet combined with the cells scraped from the petri dish. After washing 2 times with PBS, cells were suspended in 1 ml of PBS and sonicated for 30 sec in a MSE 100 W ultrasonic desintegrator. The suspension was diluted with water to 2 ml and an equal volume of cold 10% trichloroacetic acid (TCA) was added. After standing for 1 hour at 4 °C, the precipitate was filtered onto 25 mm selectron filters 0.6 $\mu$m (Schleicher and Schüll, Germany) and washed with cold 5% TCA. In some experiments TCA precipitable material was heated for 10 min at 90 °C before filtration. No significant amount of radioactivity was solubilized. Under a variety of experimental conditions acid-soluble and insoluble radioactivity were measured simultaneously. No indication was obtained that the measurement of protein synthesis is significantly affected by variations in the penetration of radioactive amino acids into the cells. The radioactivity of each sample was counted in a Packard liquid scintillation spectrometer after dissolving the filter in 5 ml of Instagel. All figures show mean values of 3 replicates for each point.

*Pulse-labeling of viral and cellular proteins and preparation of extracts for electrophoresis*

The maintenance medium was removed and the cell cultures washed once with Hanks solution. 1 ml of Eagle's medium without methionin was added per petri dish (50 mm) containing 5 $\mu$Ci/ml [$^{35}$S]methionine (185 Ci/mmol). The cells were labeled for 15 min, placed on crushed ice and washed once with cold PBS. The cells were scraped from the petri dish and washed two times more with PBS. The cell pellets from 3 petri dishes were combined, suspended in 50 $\mu$l water and sonicated for 10 sec (Microtip of MSE 100 W Ultrasonic desintegrator). 10 $\mu$l of this extract were mixed with 10 $\mu$l 20 mM phosphate buffer (pH 7.2) containing

2% SDS, 4% mercaptoethanol and 8 M urea. The samples were heated for 5 min at 100 °C (50 $\mu$g protein and 200 000 cpm).

*Gel electrophoresis*

The proteins were fractioned by gel electrophoresis according to Pereira and Skehel using 7.5% acrylamide gels containing 0.1% SDS and 5 M urea [17]. Samples were mixed with 10 $\mu$l of 50% sucrose and the gels (5 × 130 mm) run for 16 h at 25 V at room temperature. The gels were stained with Coomassie blue. Excess dye was removed overnight with 7% acetic acid. The gels were sliced longitudinal and dried as described by Fairbanks, Levinthal and Reeder [18]. The dried gels were exposed to X-ray film (Agfa Gevaert Osray X) for 3 to 10 days.

## Results

*Switch-off of protein synthesis in interferon-treated and infected cells*

Inhibition of protein synthesis, after infection with the poxvirus strains vaccinia WR or cowpox, was studied in a permanent mouse L-cell line, in primary mouse cells and in chick embryo fibroblasts (CEF). In mouse L-cells, the pretreatment with homologous interferon led to a marked accentuation of the switch-off of total amino acid incorporation into acid insoluble material (Fig. 1). The enhanced switch-off phenomenon in the L-929 cells is observed reproducibly approximately 20 min after contact of virus with the cells. No effect is seen if the L-cells are exposed to heterologous interferon which implies that it is actually the interferon component which is responsible for the enhanced switch-off phenomenon (Table I)). Under the same conditions, the morphological degeneration of interferon-treated L-cells is with certainty detected by microscopic inspection 5 – 6 hours p.i. At this time cell-bound $^{51}Cr$ was first released into the medium [7]. An inhibitory effect on total protein synthesis was also seen in infected primary mouse fibroblasts (Table I).

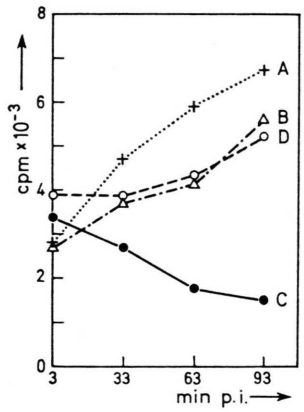


Fig. 1. Inhibition of [$^{14}C$]amino acid incorporation into acid-insoluble material in vaccinia infected L-cells with and without interferon treatment. Multiplicity of infection: 20 PFU/cell; interferon concentrations: 200 units/ml. For experimental conditions, see Materials and Methods. A, uninfected; B, uninfected and interferon treated; C, infected and interferon treated; D, infected.

The "early" inhibition of amino acid incorporation in interferon-treated cells might be due to an enhanced inhibition of cellular protein synthesis or a reduction of viral protein synthesis or both. We have noted previously that "early" vaccinia virus specific protein synthesis in L-cells treated even with high doses of interferon could still be detected by immunological techniques [19]. Analysis of total cytoplasmic fractions by acrylamid gel electro-

Table I. Enhanced inbibition of protein synthesis in continuous mouse L-929 and primary mouse fibroblasts: Effect of homologous and heterologous interferon.

| | | L-929 | | MEF | |
|---|---|---|---|---|---|
| | | TCA-precipitable material labeled with [$^{14}C$]amino acid mixture at various times post infection [cpm per petri dish] | | | |
| Virus | Interferon | 0 – 30 min | 120 – 150 min | 0 – 30 min | 120 – 150 min |
| – | – | 30.741 | 46.736 | 21.264 | 23.238 |
| infected | – | 17.908 | 29.850 | 17.414 | 12.802 |
| infected | mouse interferon [1000 units/ml] | 11.961 | 11.877 | 11.225 | 9.711 |
| infected | chick interferon [500 units/ml] | 17.183 | 29.653 | – | – |

phoresis also reveals that viral proteins are still synthesized in interferon-treated L-cells (Fig. 2).

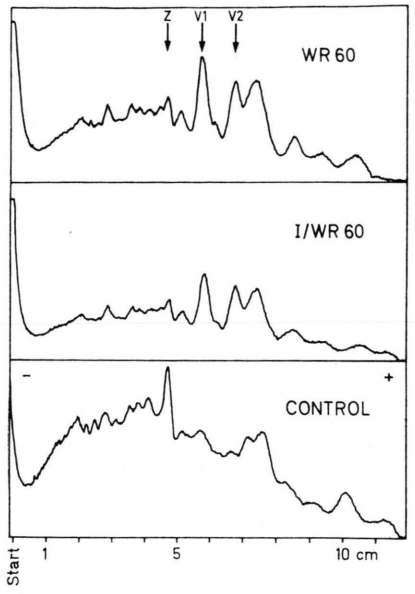


Fig. 2. Microdensitometer tracings of SDS polyacrylamide gel autoradiographs of L-929 cell extracts. Cells were labeled 60 min after infection with [$^{35}$S]methionine for 15 min. Infection: 20 PFU/cell purified vaccinia WR; Interferon treatment: 1000 units/ml. For conditions of electrophoresis see Materials and Methods. The gels were sliced longitudinally, dried and exposed to X-ray film; microdensitometer tracings were made with a Joyce Loebl, Chromoscan; WR 60: infected with vaccinia for 60 min; I/WR 60: interferon-treated and infected with vaccinia WR for 60 min. V, viral proteins; Z, cellular proteins.

Shatkin measured the inhibition of HeLa cell protein synthesis by vaccinia virus by preventing viral protein synthesis by the addition of actinomycin D[20]. As has been described for HeLa cellls, inhibition of host cell protein synthesis also occurs in L-cells in the presence of actinomycin D and inhibition of cellular protein synthesis by the virus is more drastic in interferon-treated cells (Table II). Enhanced inhibition of amino acid incorporation into acid insoluble material can be seen also in interferon-treated primary mouse fibroblasts in the presence of actinomycin D (3 $\mu$g/ml). The reduced incorporation of amino acids into total proteins in the "early" phase of infection is therefore not only due to the inhibition of the "early" viral proteins by interferon treatment but to an increased switch-off of host protein synthesis. It is evident from these results that in interferon-treated and vaccinia virus-infected mouse cells, host-cell functions are affected long before morphological destruction becomes detectable[21].

Table II. Inhibition of protein synthesis in infected and interferon-treated mouse L-cells in the presence of actinomycin D.

| Virus [10 PFU/cell] | Interferon [200 units/ml] | Actinomycin D * [$\mu$g/ml] | TCA-precipitable protein [cpm per petri dish] Time of [$^{14}$C]amino acid pulse 30—45 min | 90—120 min |
|---|---|---|---|---|
| — | — | 0 | 3899 | 14590 |
| — | + | 0 | 3508 | 13742 |
| — | — | 3 | 3137 | 8785 |
| — | + | 3 | 3396 | 9210 |
| + | — | 0 | 4147 | 12777 |
| + | — | 3 | 3204 | 4482 |
| + | + | 0 | 3136 | 8487 |
| + | + | 3 | 2674 | 2286 |

* Early viral RNA synthesis was inhibitest 85% by Actinomycin D.

The replication of cowpox virus in L-cells is only partially reduced by pretreatment with interferon and the viral proteins synthesized in the "early" phase are still made to a large extent[14]

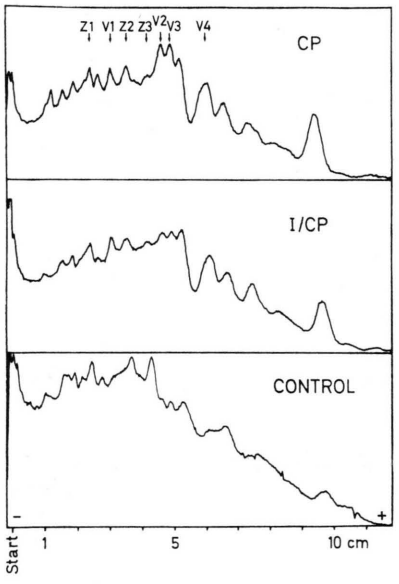


Fig. 3. Microdensitometer tracings of SDS polyacrylamid gel autoradiographs of total cytoplasmic extracts from uninfected and cowpox infected L-cells treated with interferon. Cells were labeled with [$^{35}$S]methionine from 4—5 h p.i. Treatment of cells with homologous interferon as in the experiments described in Table I. For preparation of cytoplasmic extracts and conditions of electrophoresis see Materials and Methods. After destaining, the gels were sliced longitudinally, dried and exposed to X-ray film. CP, infected with 25 PFU/cell; I/CP, 1000 units mouse interferon and infected with 25 PFU/cell. V, viral proteins, Z, cellular proteins.

(Fig. 3). In contrast to vaccinia virus (WR) infection, cellular destruction is not observed. Total protein synthesis is not inhibited in the "early" phase of infection in the presence or absence of interferon treatment (Fig. 4).

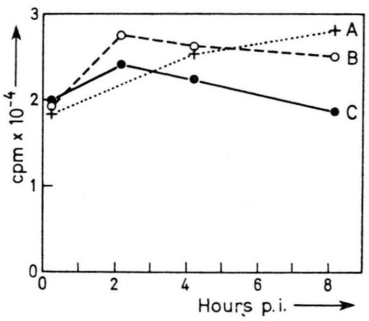


Fig. 4. Amino acid incorporation into total protein in L-cells infected with cowpox virus: Effect of homologous interferon: A, uninfected; B, infected (25 PFU/cell); C, interferon-treated (1000 units/ml) and infected. All experimental procedures as in Fig. 1 and Materials and Methods. A similar experiment was carried out in the presence of actinomycin D (5 $\mu$g/ml). No inhibition of host cell protein synthesis could be observed in cells with or without interferon treatment.

Previously it was noted that after infection of confluent CEF-monolayers with poxviruses, no enhanced destruction of the cells comparable to the lysis of interferon-treated and vaccinia-infected L-cells was observed if they had been treated with homologous interferon [7]. If the cells are exposed to high doses of chick interferon to establish the antiviral effect, no reduction of amino acid incorporation into total protein is observed after infection (Fig. 5). Under the same conditions, the total amount of viral protein 3 and 6 hours after infection reacting with an anti-vaccinia serum is reduced by at least 90% [19].

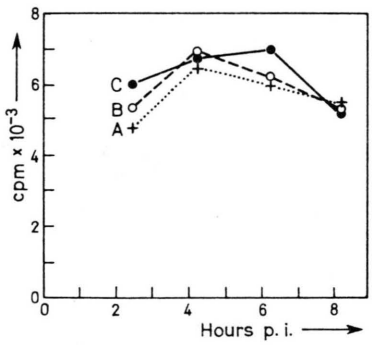


Fig. 5. Total protein synthesis in vaccinia virus infected CEF with and without interferon treatment. A, uninfected; B, infected 25 PFU/cell; C, pretreated with chick interferon (500 units/ml) and infected 25 PFU/cell. A similar experiment was carried out in the presence of actinomycin D (5 $\mu$g/ml). Under these conditions incorporation of [$^3$H] amino acids into total protein was found to be slightly reduced in infected cells. In interferon-treated cells this inhibition was not enhanced significantly.

Table III shows the effect of increasing interferon concentrations on the switch-off of total protein synthesis in vaccinia WR virus infected L-929 cells. At a multiplicity of 20 PFU/cell, as little as 10 units/ml of mouse interferon are sufficient to produce a significant enhancement of protein synthesis inhibition, and the effect is maximal at 100 units/ml.

Table III. Effect of increasing amounts of homologous interferon on enhanced swich-off of total protein synthesis in mouse L-cells *.

| Vaccinia virus [PFU/cell] | Mouse interferon [units/ml] | TCA-precipitable material [cpm per petri dish] Time of pulse post infection 0—30 min | 120—150 min |
|---|---|---|---|
| — | — | 16.167 | 25.901 |
| 20 | — | 5.596 | 7.899 |
| 20 | 1 | 4.433 | 7.153 |
| 20 | 10 | 3.435 | 4.325 |
| 20 | 100 | 2.549 | 1.450 |
| 20 | 1000 | 1.916 | 1.829 |

* A similar experiment has been carried out in the presence of actinomycin D. Experimental conditions were the same as in the experiment described in Table II. The enhanced inhibition of amino acid incorporation into host cell protein by vaccinia infection measured in the presence of actinomycin D was found to be dose-dependent between 10 and 1000 units/ml of interferon.

## Discussion

In mouse L-cells pretreated with homologous interferon and infected with vaccinia virus, two phenomena can be observed: A very rapid shut-off of cellular protein synthesis immediately after adsorption of the virus to the cells and, later, in the infectious cycle, an enhanced morphological destruction of these cells ("lysis"). Compared to the effects of interferon treatment on uninfected cells, the effects on the vaccinia virus infected host cell are much more drastic and the severity seems to increase with the sequential expression of the viral functions [1—6]. A similar enhanced inhibition of hemoglobin RNA- and viral RNA translation was noted in cell-free extracts from interferon-treated cells infected with vaccinia virus when compared to

uninfected cells [9]. These various phenomena may be related since the conditions for the lysis of L-cells and the "early" occurring enhanced switch-off of total protein synthesis were closely correlated, it is possible that the enhanced inhibition of protein synthesis is required for the lysis of interferon-treated cells which occurs late in infection. Additional viral functions, however, are necessary to establish the latter phenomenon, since DNA and RNA synthesis are required for the lysis phenomenon but are not required for the enhanced inhibition of protein synthesis in interferon-treated cells (Table II) (unpublished).

The requirements for the early inhibition of HeLa cell protein synthesis by vaccinia infection have been eluicidated by Moss, who showed that a viral constituant of the infecting particle is responsible for the early inhibition of host cell protein synthesis and that no newly synthesized viral product has to be made [22]. Since, however, "early" viral RNA synthesis in vaccinia infected L-cells could not be completely inhibited by treatment with actinomycin D or cordicepin (10 $\mu$g/ml) confirming Metz and Esteban's observations, the conclusion from this experiment that no "early" viral functions are necessary for the enhanced switch-off has to be made with certain reservations [21].

If the interferon induced effects on host-cell functions in murine cells are contributing to the antiviral effect of interferon and a general mechanism for interferon is assumed, similar effects in other cells treated with homologous interferon preparations could be expected. Since the degree of enhanced switch-off of protein synthesis and the lysis phenomenon depend very much on the type of cell used, it is likely that the possible effects of interferon-treatment on host-cell functions may vary. Confluent CEF monolayers have also been used extensively for studies of the interferon-effect on poxvirus-specific functions. No enhanced early switch-off of protein synthesis or enhanced destruction of these cells after infection has been observed [7, 19]. However, CEF cells seem to be changed by interferon treatment in a subtle way. The experiments carried out by Metz and Esteban demonstrated clearly that in interferon-treated CEF cells infected in suspension cellular protein synthesis "early" after infection was inhibited at an enhanced rate similar to the situation found in mouse L-cells [21].

Another observation made previously might possibly be explained by assuming alterations induced by interferon in CEF cells. Over-production of "early" vaccinia RNA is observed in CEF pretreated either with interferon or other protein synthesis inhibitors. An overproduction of early viral RNA is not seen if interferon-treated cells are exposed simultaneously to both inhibitors [14]. Reduced RNA synthesis of vesicular stomatitis virus in interferon-treated cells treated also with inhibitors of protein or RNA synthesis has been reported [23]. These data might be interpreted as an indication that the interferon-treated CEF cell is in a subtle way altered and that this becomes manifest in a variety of ways if the cells are exposed to unfavorable growth conditions.

So far even the highest purified interferon preparations from mouse and chick cells contain several proteins without antiviral activity. Furthermore, there are indications that the antiviral activity consist of several components [24, 25]. It remains therefore to be shown whether the effects on the host-cell are caused by the interferon protein itself and whether the anti-cellular activity is related to the antiviral effect.

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