Protein Synthesis and Membrane Integrity in Interferon-treated HeLa Cells Infected with Encephalomyocarditis Virus

By ALBERTO MUÑOZ AND LUIS CARRASCO*

Department of Microbiology, Centro de Biología Molecular, Universidad Autónoma, Canto Blanco, Madrid-34, Spain

(Accepted 1 May 1981)

SUMMARY

The survival of interferon (IFN)-treated cells after encephalomyocarditis (EMC) virus infection depends on both the concentration of interferon and the multiplicity of infection (m.o.i.) used. The cell survived EMC infection if a high IFN/m.o.i. ratio was used in the experiment, whereas cell death took place at low IFN/m.o.i. ratios, even if IFN is also present during infection. Analysis by polyacrylamide gel electrophoresis of the proteins synthesized in IFN-treated cells subsequently infected with EMC indicated that no virus proteins were detected at either low or high multiplicities of infection. However, at low m.o.i. the cell survived and continued synthesizing cellular proteins exclusively, whereas at high m.o.i. a drastic shut-off of host protein synthesis took place. Virus which had been inactivated by u.v. irradiation was unable to cause the shut-off of host protein synthesis, either in control or in IFN-treated cells. This result suggests that some virus gene expression occurs in cells treated with IFN, although no virus protein synthesis was detected. The synthesis of virus RNA was also strongly inhibited after treatment of cells with IFN.

The integrity of the cell membrane in control and in IFN-treated cells was studied by analysing the $^{86}$Rb$^+$ ion leakage, the thymidine pool, the choline uptake and the entry of the translation inhibitor hygromycin B, to which cells are impermeable, at different times after EMC infection. The results obtained indicate that the early membrane leakiness observed after virus infection is not prevented by IFN treatment. However, the development of late leakiness to $^{86}$Rb$^+$ ions, thymidine and hygromycin B was not observed in IFN-treated cells.

INTRODUCTION

Picornavirus infection of cultured cells leads to a profound inhibition of host protein synthesis (Carrasco & Smith, 1976, 1980; Lucas-Lenard, 1979). This inhibition is not prevented by treatment of cells with interferon (Levy, 1964; Gauntt & Lockart, 1968). Treatment of L cells with homologous interferon and subsequent infection with EMC virus produces a strong inhibition of both cellular and virus protein synthesis (Kerr et al., 1973). Similarly, infection of interferon-treated cells with vaccinia virus (Joklik & Merigan, 1966) or vesicular stomatitis virus (Yamazaki & Wagner, 1970) produces an indiscriminate inhibition of both cellular and virus protein synthesis, which leads to cell death. However, under some conditions, an early shut-off of host protein synthesis is observed in interferon-treated L cells after mengovirus infection. This is followed by recovery of the cellular translation capacity (Falcoff & Sanceau, 1979). In some of these virus–cell systems it is not clear whether the inhibition of virus production by interferon, thought to be a consequence of the interference with virus translation, leads to cell death or whether the cell recovers its translational capacity.
and survives after virus infection. We report here an analysis of this inhibition and of the cellular functions which are altered after virus infection.

**METHODS**

**Cells and virus.** HeLa cells and L929 cells were grown in Dulbecco's modified Eagle's medium (E4D) supplemented with 10% newborn calf serum (Difco) (E4D10 medium) and incubated at 37 °C in a 5% CO₂ atmosphere. Encephalomyocarditis (EMC) virus was grown on L929 cells in a mixture of Eagle's medium, phosphate-buffered saline (PBS) solution and E4D medium (80:15:5) supplemented with 1% newborn calf serum. 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 grown on 16 mm diam. 24-well plates (Falcon Plastics) were infected with EMC virus at the m.o.i. described in each experiment. After 1 h incubation at 37 °C the medium was removed and 1 ml E4D2 was added. Time of virus addition was considered — 1 h and 0 time was taken when the virus was removed. The incubation at 37 °C was continued until the labelling period. For this purpose 0.5 ml methionine-free E4D1 medium and 0.11 μCi [35S]methionine (1100 Ci/mmol; 5-4 mCi/ml, The Radiochemical Centre, Amersham) were added to the cells for 1 h pulse. The medium was then removed, the cells were washed with PBS solution and precipitated with 5% trichloroacetic acid (TCA). After 5 min the TCA was removed and the cell monolayer washed three times with ethanol, dried under an infrared lamp and dissolved with 250 μl 0.1 M-NaOH plus 1% SDS. 125 μl were counted in an Intertechnique scintillation spectrometer.

**Analysis of the proteins synthesized in virus-infected cells.** HeLa cells were grown on 30 mm Petri dishes and pulsed with 5.4 μCi [35S]methionine. At the end of the pulse period the cells were washed with 1 ml PBS solution and dissolved in 200 μl 0.02 M-NaOH plus 1% SDS and 200 μl sample buffer (62.5 mM-tris pH 6.8; 2% SDS; 0-1 M-dithiothreitol; 17% glycerol and 0-024% bromphenol blue as indicator). Each sample was sonicated to reduce viscosity and heated to 90 °C for 5 min. Ten μl were applied to a 15% polyacrylamide gel and run overnight at 30 V. Fluorography of the gel was carried out with 2,5-diphenyloxazole/dimethyl sulphoxide (20%, w/w). The dried gels were exposed using XS-5 X ray films (Kodak).

**Interferons.** Human lymphoblastoid interferon [HuIFN-α (Ly); 1-6 × 10⁶ IU/mg protein] was a generous gift of Drs Finter, Fantes and Johnston, Wellcome Research Laboratories, Beckenham, U.K.

**Nucleic acid synthesis and thymidine pool in EMC virus-infected HeLa cells.** Cellular DNA synthesis was measured by estimating the incorporation of methyl-[3H]thymidine (40 to 60 Ci/mmol, 1 mCi/ml; The Radiochemical Centre, Amersham) into TCA precipitate material. At the times indicated after infection 1 μCi/ml of the isotope was added and after a 1 h pulse the cells were processed as described for protein synthesis. The thymidine pool was measured by estimating the cell radioactivity soluble in 5% TCA, after incubation of cells with 1 μCi/ml methyl-[3H]thymidine for 1 h at 37 °C, in a culture medium supplemented with 2% calf serum.

**Measurement of 86Rb⁺ content.** HeLa cells grown in E4D10 medium were placed in 280 μl of a mixture of methionine-free E4D1 medium-E4D10 medium (3:1); 0-2 μCi 86Rb⁺ (1 mCi/ml; The Radiochemical Centre, Amersham) was added, and the cells were incubated for 17 h at 37 °C. Virus infection was then carried out while maintaining constant the 86Rb⁺ concentration. At the times indicated the cells were pulsed with 0-14 μCi [35S]methionine. After 1 h incubation, the medium was removed and the cells were washed three times with 1 ml PBS, and 0.5 ml 5% TCA was added to extract the 86Rb⁺ from the cells. The radioactivity of 0.4 ml of the TCA extract was determined by estimating the Cerenkov radiation in a liquid
Fig. 1. Influence of interferon concentration, multiplicity of infection and cell confluence on the antiviral state induced by human interferon in HeLa cells. Interferon was added 18 h before EMC virus infection and the level of protein synthesis at 24 h after infection was measured by a 1 h pulse of [35S]methionine. Control were uninfected cells treated with the corresponding interferon concentration. Numbers indicate the m.o.i. employed. (a) 4.4 × 10^6 cells/16 mm diam. well; (b) 5.3 × 10^5 cells/16 mm well; (c) 6.1 × 10^5 cells/16 mm well.

RESULTS

We first analysed protein synthesis after treatment of HeLa cells with different interferon concentrations followed by infection with different multiplicities of EMC virus. Fig. 1 shows the analysis of protein synthesis in HeLa cells 24 h after infection with EMC virus. A high m.o.i. led to cell death as assessed by their appearance under the microscope and loss of the capacity to synthesize proteins, whereas if the cells were treated with HuIFN-α (Ly) and infected at low multiplicities, they survived virus infection. The percentage of cells that survive at a given multiplicity depended on the concentration of interferon used. The prevention of cell death depends on the ratio of interferon to m.o.i. The higher this ratio the better the protection achieved. The influence of cell confluence is also noted in Fig. 1. An increased cell survival after EMC virus infection is observed in the very confluent cell cultures (6.1 × 10^5 cells/16 mm diam. well; Fig. 1 c). We next analysed the pattern of the proteins synthesized at different times after infection after treatment with 200 IU/ml HuIFN-α (Ly) and infection with EMC virus at either 20 or 200 p.f.u./cell. Inhibition of protein synthesis was apparent in control cells not treated with interferon using either m.o.i. (Fig. 2), whereas if cells pretreated with IFN were infected with 20 p.f.u./cell only a slight decrease in protein synthesis 3 to 4 h after infection was observed, followed by a recovery, the infected cells continuing to synthesize proteins at control levels. When the IFN-treated cells were infected at 200 p.f.u./cell the inhibition of protein synthesis was pronounced and irreversible. Analysis by polyacrylamide gel electrophoresis of the proteins synthesized showed that no virus proteins could be detected in IFN-treated cells (Fig. 3). These results indicate that two different
Fig. 2. Kinetics of protein synthesis in human HeLa cells infected with EMC virus at high and low multiplicity, untreated (○) or treated (●) with human interferon (200 IU/ml) 18 h before infection. The cell monolayers were labelled and processed as indicated in Methods. (a) M.o.i. 20; (b) m.o.i. 200.

situations could result after EMC virus infection of IFN-treated HeLa cells. If the IFN/m.o.i. ratio was high, no inhibition of host protein synthesis was observed, the cells synthesizing cellular proteins exclusively and surviving the viral infection. At low IFN/m.o.i. ratios inhibition of host protein synthesis occurred but still no virus proteins were detected. However, the cytopathic effect developed and the cell died, as did control cells not treated with interferon. Similar results were obtained with mouse L cells infected with EMC virus and mouse IFN (data not shown), suggesting that these observations were not specific for human cells and HuIFN-α (Ly). The results can be reconciled with the previous findings by Kerr et al. (1973) and Esteban (1975) who found that EMC virus infection of IFN-treated L cells led to the total inhibition of protein synthesis because they used a low IFN concentration (40 IU/ml). On the other hand, the results reported by Falcoff & Sanceau (1979) in L cells treated with 500 IU/ml IFN and infected with mengovirus at 20 p.f.u./cell are more similar to those observed by us in Fig. 3 (a). In conclusion, the effect of virus infection on protein synthesis varies considerably and depends on the IFN/m.o.i. ratio.

In order to establish whether the continuous presence of IFN could prevent cell death at low IFN/m.o.i. ratios, HuIFN-α (Ly) was added either 22 h before infection and then removed from the medium, or was continuously present before and after EMC virus infection. The level of protein synthesis in both instances was similar at 8 h and at 30 h after infection, suggesting that cell death does, in fact, take place if a low IFN/m.o.i. ratio is used and the continuous presence of IFN does not influence this result (results not shown).

In IFN-treated RK cells infected with vesicular stomatitis virus the inhibition of protein synthesis is due to a component of the virion (Yamazaki & Wagner, 1970), while in mengovirus-infected cells it has been suggested that the inhibition of protein synthesis observed in IFN-treated cells was a consequence of virus double-stranded RNA production (Falcoff & Sanceau, 1979). It was of interest to determine whether virus gene expression was necessary for the inhibition of protein synthesis observed at low IFN/m.o.i. ratios. When EMC virus was inactivated by more than two log units using u.v. irradiation, no inhibition of protein synthesis was observed in IFN-treated HeLa cells (Fig. 4). This result suggests that although no virus proteins could be detected, virus gene expression was necessary for the shut-off to occur, both in control and IFN-treated HeLa cells.

In a number of virus–cell systems, the cellular membrane becomes permeable to ions some time after infection, when the bulk of virion proteins are synthesized (Carrasco & Smith, 1976; Egberts et al., 1977; Nair et al., 1978; Garry et al., 1979). The effect of IFN treatment
on $^{86}\text{Rb}^+$ leakage after EMC virus infection is shown in Fig. 5. If a low multiplicity of EMC virus was used, there was a correlation between the inhibition of protein synthesis and the decrease in $^{86}\text{Rb}^+$ content in control cells not treated with interferon (Fig. 5 a, b). If the cells were infected with a higher multiplicity of infection, there was an earlier inhibition of host protein synthesis but $^{86}\text{Rb}^+$ leakage did not occur until 5 h after infection when the bulk of virus protein synthesis takes place (Fig. 5 c, d). If the cells were treated with IFN, the $^{86}\text{Rb}^+$
leakage was prevented, both when inhibition of host protein synthesis did not occur because a low multiplicity was used and also when the inhibition of host protein synthesis took place at the higher multiplicity of infection.

Membrane integrity was also analysed by means of the translation inhibitor hygromycin B, which does not enter normal cells, but which readily penetrates virus-infected cells both early after infection and also late in virus infection. Fig. 6 indicates that the early membrane leakiness to hygromycin B developed in both interferon-treated and control cells, but the modification of membrane permeability late in infection is prevented if the cells are pretreated with interferon. These results are in agreement with those illustrated in Fig. 5 for $^{86}\text{Rb}^+$ leakage.
Translation in interferon-treated cells

Fig. 6. Effect of interferon treatment on EMC virus-induced permeability to hygromycin B (H.B.). Permeability to H.B. of HeLa cells untreated (O) or treated (●) with 50 IU/ml IFN 18 h before infection was estimated by measurement of the level of protein synthesis in cells treated with [35S]methionine (including 1 mM-H.B.) for 1 h. The controls were cells not treated with the inhibitor and infected at the indicated multiplicity. (a) M.o.i. 5; (b) m.o.i. 100. The radioactivity incorporated in uninfected IFN- and H.B.-untreated cells was 44 835 ct/min, in uninfected IFN-untreated H.B.-treated cells 38 441 ct/min, in uninfected IFN-treated H.B.-untreated cells 36 854 ct/min and in uninfected IFN- and H.B.-treated cells 31 116 ct/min.

Fig. 7. Time course of (a) DNA synthesis and (b) entry of radioactivity into the thymidine pool in HeLa cells infected with the EMC virus at high and low multiplicities and untreated or treated with IFN (50 IU/ml) 18 h before infection. △, Untreated cells, m.o.i. 5; ▲, IFN-treated cells, m.o.i. 5; ○, untreated cells, m.o.i. 100; ●, IFN-treated cells, m.o.i. 100.

The synthesis of cellular DNA and the thymidine content of the cells was analysed both in control and IFN-treated cells (Fig. 7). DNA synthesis is drastically inhibited in EMC-infected cells perhaps as a consequence of inhibition of protein synthesis. If the cells were pretreated
with IFN, a lower level of DNA synthesis occurred. However, the inhibition only occurred if a high EMC virus input was used. The thymidine pool decreased in EMC virus-infected cells from 5 h after infection, which is about the time when membrane integrity is damaged as measured by $^{86}\text{Rb}^+$ content and hygromycin B entry. However, in IFN-treated EMC virus-infected cells the thymidine pool stayed constant, except at a long time after infection with a high multiplicity of EMC virus.

Finally, the capacity of the cells to take up choline was analysed. In several virus-infected cells there is an increased membrane proliferation, accompanied by an increase in the uptake of choline, which is used in the synthesis of several membrane constituents (Penman, 1965; Plagemann et al., 1970). Such an increase was observed in EMC virus-infected HeLa cells (Fig. 8). Surprisingly enough, if the cells were pretreated with IFN and infected with EMC virus, a similar increase is observed, suggesting that membrane proliferation probably also takes place in interferon-treated EMC virus-infected cells.

**DISCUSSION**

Human cells treated with IFN do not support the growth of a number of animal viruses (Friedman, 1977; Burke, 1979). The failure of picornaviruses to multiply in IFN-treated cells is believed to lie in an impairment of protein synthesis. However, it was not clear from previous studies whether the IFN-treated cells were able to overcome viral infection and survive (Falcoff & Sanceau, 1979), or whether the cell dies (Esteban, 1975; Metz, 1975). Our results indicate that both situations can take place, and that the cellular response depends on the IFN/m.o.i. ratio used. When treated with a high concentration of IFN and infected at a low multiplicity, the cell survived virus infection, at least for the first 24 h, whereas when they were treated with a low concentration of interferon and later infected at a high multiplicity with EMC virus, the cells died, although the production of new infectious virus was strongly inhibited in both situations.

In our system, no virus protein synthesis was detected in IFN-treated cells, whereas in L cells treated with mouse IFN and infected with mengovirus (Falcoff & Sanceau, 1979) and also in IFN-treated HeLa cells infected with VSV (Simili et al., 1980) the synthesis of some virus proteins was apparent. Since u.v.-inactivated virus was unable to induce the inhibition of host protein synthesis, it seems reasonable to suggest that some translation of the EMC RNA
Translation in interferon-treated cells

takes place, and that a virus product is responsible for the inhibition. It remains to be established whether the inhibition of host protein synthesis at high multiplicities is triggered off by the formation of double-stranded RNA. In any case, this system provides a good model to test whether the blockade of translation is mediated by the degradation of cellular mRNA via the 2,5 A system, or whether the modification of a protein factor involved in translation is the cause of that inhibition.

Modification of membrane permeability takes place after virus infection (Carrasco & Smith, 1980; Imprain et al., 1980; Foster et al., 1980). A correlation between such modification and the shut-off of protein synthesis exists in Sindbis virus-infected cells (Garry et al., 1979) and also in EMC virus-infected cells at low multiplicities of infection (Carrasco & Smith, 1976; Fig. 5b). However, at high multiplicities the membrane becomes modified when the bulk of viral proteins are synthesized, but the inhibition of host protein synthesis occurred earlier than the release of 86Rb+ ions from the cells (Fig. 5d). In IFN-treated HeLa cells, the inhibition of host protein synthesis observed at high multiplicities, took place in a cell with an integral membrane, as measured by means of the unpermeant translation inhibitor hygromycin B and by the release of 86Rb+ ions. These results suggest that the shut-off of translation in IFN-treated cells is not a consequence of the modification of the ionic composition in the cell cytoplasm.

The expert technical assistance of Ms M. A. Ramos is acknowledged. We also acknowledge CAICYT, Asociación Española Contra el Cáncer and Plan Concertado de Investigación no. 5/78 for financial support.

REFERENCES


*(Received 9 March 1981)*