Protein Synthesis in HeLa Cells Double-infected with Encephalomyocarditis Virus and Poliovirus

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SUMMARY

The pattern of host, encephalomyocarditis (EMC) virus and poliovirus protein synthesis in HeLa cells double-infected with EMC virus and poliovirus has been examined. Both picornaviruses were able to block host translation after infection, although with different degrees of efficiency. In co-infection experiments, the inhibition of poliovirus protein synthesis by EMC virus and vice versa depended on the relative multiplicities of infection of each virus used. Under some conditions, double-infected HeLa cells simultaneously synthesized poliovirus and EMC virus proteins. In superinfection experiments, when the two viruses were added at different times, the pattern of the proteins synthesized depended on the time of addition of the second virus challenge. Poliovirus did not replicate when added 4 h after EMC virus. On the other hand, EMC virus replication was inhibited in cells preinfected with poliovirus. If co-infected cells were treated with guanidine from the beginning of the infection, only the synthesis of EMC virus proteins was apparent. However, if poliovirus was allowed to replicate for 4 h before guanidine addition, then the synthesis of EMC virus proteins was reduced, even though the translation of poliovirus mRNA was very much inhibited. EMC virus-infected HeLa cells exclusively synthesized cellular proteins in hypotonic media, whereas under hypertonic conditions only virus protein synthesis took place. In poliovirus-infected HeLa cells no cellular translation was detected under all ionic conditions tested. The ionic optimum of EMC virus and poliovirus protein synthesis was also different in cells infected with a single virus. However, in double-infected cells the monovalent ion optimum for translation of EMC virus and poliovirus mRNA was the same, although EMC virus protein synthesis was more resistant to inhibition by hypertonic media. No cellular protein synthesis was detected in double-infected HeLa cells under all the ionic conditions tested.

INTRODUCTION

Early studies of mutual interference with different picornaviruses of the genus Enterovirus lead to the hypothesis that interference is due to a competition between the two viruses for metabolites or replicating sites (Cords & Holland, 1964). These results also indicated that the extent of the interference is dependent on the ratio of multiplicities of infection used and the time of challenge of the second virus (Cords & Holland, 1964).

Further work with picornaviruses belonging to two different genera, mengovirus (a Cardiovirus) and poliovirus (an Enterovirus), indicated that non-replicating poliovirus inhibits host protein synthesis with no effect on mengovirus mRNA translation (McCormick & Penman, 1967). The authors concluded that mengovirus protein synthesis is immune to...
inhibition by poliovirus infection of HeLa cells. More recently, Detjen et al. (1981) reported that superinfection with poliovirus of HeLa cells already infected with encephalomyocarditis (EMC) virus does not inhibit translation of EMC virus mRNA, whereas residual host translation is completely inhibited.

Poliovirus and EMC virus inhibit host protein synthesis in HeLa cells to different degrees, so that higher multiplicities of EMC virus than poliovirus are required to inhibit protein synthesis in HeLa cells with similar kinetics. Because of this difference in inhibition of host protein synthesis between EMC virus and poliovirus, and because of the different ability of the extracts from EMC virus- and poliovirus-infected cells to translate capped mRNAs (Lawrence & Thach, 1974; Rose et al., 1978), Jen et al. (1980) suggested that the mechanisms used by both viruses to block host protein synthesis are different.

We have carried out a thorough analysis of the interference between EMC virus and poliovirus at the translational level, and the conditions of mutual interference between these two viruses are described. The effect of non-replicating poliovirus on EMC virus protein synthesis and the influence of the ionic conditions on the relative translation of EMC virus and poliovirus mRNA are also reported.

**METHODS**

**Cells and viruses.** HeLa cells were propagated in Falcon culture flasks containing 6 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (Gibco) and incubated at 37 °C in a 5% CO₂ atmosphere.

EMC virus was grown on L-929 cells in a mixture of Eagle's medium and phosphate-buffered saline, supplemented with 1% newborn calf serum. Poliovirus type I was grown on HeLa cells in DMEM supplemented with 1% calf serum. The fraction obtained after removal of cell debris by low-speed centrifugation was used as the source of the corresponding virus.

**Conditions of infection and protein labelling.** HeLa cells were grown in 3.5 cm diam. Petri dishes containing 2 ml DMEM supplemented with 10% newborn calf serum. The medium was removed from confluent monolayers and the cells infected with EMC virus and/or poliovirus for 1 h at 37 °C. The inoculum was then replaced by 0.5 ml DMEM supplemented with 1% newborn calf serum. Poliovirus type I was grown on HeLa cells in DMEM supplemented with 1% calf serum. The fraction obtained after removal of cell debris by low-speed centrifugation was used as the source of the corresponding virus.

**Polyacrylamide gel electrophoresis (PAGE).** After incubation of cells in the presence of [³⁵S]methionine, the medium was removed and the cell monolayer washed with 2 ml phosphate buffer; the cells were dissolved in 0.1 ml 0.2 M-NaOH containing 0.1% SDS, plus 0.2 ml sample buffer (62.5 mM-tris pH 6.8, 2% SDS, 0.1 M-dithiothreitol, 17% glycerol). Each sample was sonicated to reduce viscosity, heated at 90 °C for 2 min and 5 µl samples were analysed by PAGE using 15% acrylamide gels. The gels were electrophoresed overnight at 30 V, stained and destained, and labelled proteins visualized by fluorography. Densitometric profiles of the gel were made in a microdensitometer Optronics P1700.

**Measurement of protein synthesis.** A 10 µl portion of each sonicated sample was precipitated with 1 ml 10% trichloroacetic acid, heated for 10 min at 90 °C and filtered through Whatman GF/C glass fibre filters. The radioactivity retained on the filters was determined in an Intertechique scintillation spectrometer.

**RESULTS**

To obtain further insight into the mechanisms used by picornaviruses to shut-off host translation, we carried out mixed-infection experiments and analysed interference between
Translation in double-infected cells

Fig. 1. Time course of protein synthesis in HeLa cells co-infected with a fixed m.o.i. of EMC virus and different m.o.i.s of poliovirus. Cell cultures were simultaneously infected with EMC virus at an m.o.i. of 40 and the indicated m.o.i.s of poliovirus (0, 10, 20 and 60). Protein synthesis was estimated at the indicated times after infection (1, 3, 5 and 7 h) as described in the text. The positions of poliovirus VP3 protein and the γ protein of EMC virus are indicated on the autoradiographs of the gels. Panel (a) shows the VP3/γ ratios with the different m.o.i.s of poliovirus used. These were calculated from the areas of the densitometric scans of autoradiograms obtained from polyacrylamide gel analysis of the proteins synthesized 3 to 4 h post-infection. Panels (b to e) show the time course of incorporation of [35S]methionine into protein under the different conditions of infection used, measured as described in Methods.

poliovirus, EMC virus and the host cell at the level of protein synthesis. Fig. 1 shows the kinetics of protein synthesis in HeLa cells co-infected with 40 p.f.u./cell of EMC virus and varying m.o.i. of poliovirus. A high m.o.i. of poliovirus reduced the synthesis of EMC virus proteins. Even the addition of 10 p.f.u./cell of poliovirus had a depressing effect on EMC virus protein synthesis. The ratio of poliovirus VP3 and EMC virus γ proteins synthesized at 5 h after infection increased when the m.o.i. of poliovirus was raised (Fig. 1a). There are, however, conditions in which the co-infected HeLa cells simultaneously synthesized both poliovirus and EMC virus proteins. Fig. 1 (b to e) shows that under all m.o.i.s of poliovirus used this virus imposed its profile of inhibition of protein synthesis.

In the experiment shown in Fig. 2 HeLa cells were infected with 10 p.f.u./cell of poliovirus and co-infected at varying m.o.i.s with EMC virus, and the proteins synthesized throughout the infection period were analysed by PAGE. The ratio between the synthesis of EMC virus γ
Fig. 2. Time course of protein synthesis in HeLa cells co-infected with a fixed m.o.i. of poliovirus and different m.o.i.s of EMC virus. Cell cultures were simultaneously infected with poliovirus at an m.o.i. of 10 and the indicated m.o.i.s of EMC virus (0, 10, 30 and 80). Protein synthesis was estimated at the indicated times after infection (1, 3, 5 and 7 h) as described in the text. The positions of poliovirus VP3 protein and the γ protein of EMC virus are indicated on the autoradiographs of the gels. Panel (a) shows the γ/VP3 ratios with the different m.o.i.s of EMC virus used, calculated as in Fig. 1. Panels (b to e) show the time course of incorporation of [35S]methionine in protein under the different conditions of infection.

protein and poliovirus VP3 protein increased when increasing m.o.i.s of EMC virus were used. These results suggest that the relative synthesis of the proteins of each virus in co-infection experiments is strongly dependent on the m.o.i. of the two viruses. There are conditions in which poliovirus strongly depresses the synthesis of EMC virus proteins, and conditions in which the simultaneous expression of the genome of both viruses takes place.

Subsequently, we analysed the interference of virus translation in superinfection experiments. Fig. 3 shows that in HeLa cells infected with 40 p.f.u./cell of EMC virus, virus protein synthesis was apparent from 3 h post-infection until 7 h post-infection. When poliovirus (10 p.f.u./cell) was added to these cells 1 h after EMC virus infection (time 0), an inhibition of EMC virus protein synthesis occurred. If EMC virus was allowed to replicate for 2 h (time 1) or 4 h (time 3) then the inhibition of EMC virus mRNA translation was much smaller and poliovirus replication was inhibited. In the experiment shown in Fig. 4, HeLa cells infected with 10 p.f.u./cell of poliovirus were superinfected at different times with 40 p.f.u./cell
Translation in double-infected cells

Fig. 3. Time course of protein synthesis in EMC virus-infected (m.o.i. 40) HeLa cells superinfected with poliovirus (m.o.i. 10) at different times. Conditions of infection and protein labelling were as described in Methods. Protein synthesis was estimated at the indicated times after infection (1, 3, 5 and 7 h).

of EMC virus. Under co-infection conditions, the synthesis of proteins of both viruses was apparent. However, if poliovirus was allowed to replicate for 2 h (time 1) or 4 h (time 3) before EMC virus infection, then EMC virus protein synthesis did not occur (Fig. 4). This result indicates that poliovirus replication is more rapid than EMC virus replication and a 2 h advantage for poliovirus is enough to take over all the cellular components necessary for its replication and to inhibit EMC virus reproduction. If HeLa cells were co-infected with both viruses and poliovirus replication was inhibited by guanidine, then EMC virus protein synthesis occurred and lasted until 8 h after infection (Fig. 5). However, if the enterovirus was allowed to replicate for 4 h before guanidine addition, then the synthesis of EMC virus proteins was reduced, even though the synthesis of poliovirus proteins was severely inhibited (Fig. 5).

Finally, we examined the influence of different ionic concentrations in the extracellular medium on protein synthesis in HeLa cells infected with EMC virus, with poliovirus and in double-infected cells (Fig. 6). In agreement with our previous results (Alonso & Carrasco, 1981), HeLa cells infected by EMC virus preferentially synthesized cellular proteins under hypotonic conditions, whereas in hypertonic medium cellular protein synthesis was
preferentially inhibited. In poliovirus-infected HeLa cells, this was not so. The ionic optimum in vivo for protein synthesis was smaller than in EMC virus-infected HeLa cells (Fig. 6a, b). No cellular protein synthesis was apparent under hypotonic conditions, even though virus protein synthesis was inhibited. On the other hand, maximum poliovirus protein synthesis was observed at 90 mM-NaCl in the external medium, whereas the optimum for EMC virus protein synthesis was around 150 mM-NaCl. The pattern of protein synthesis in double-infected cells under different ionic conditions was particularly striking. The optimum poliovirus mRNA translation in double-infected cells was raised to around 150 mM, which was similar to the optimum for EMC virus protein synthesis. The ratio of EMC y protein to VP3 protein of poliovirus increased by increasing the NaCl concentration. This means that EMC virus protein synthesis is more resistant to inhibition by hypertonic medium than is poliovirus protein synthesis. However, cellular protein synthesis was not observed under hypotonic conditions in these double-infected cells, as occurred in cells infected by EMC virus (Alonso & Carrasco, 1981).
Fig. 5. Effect of guanidine on protein synthesis in HeLa cells double-infected with EMC virus and poliovirus. Cell cultures were simultaneously mixedly infected with EMC virus at an m.o.i of 20 and with poliovirus at an m.o.i of 10. 1 mM-guanidine was added at the indicated times post-infection (−1 h indicates preinfection with poliovirus). Protein labelling was carried out as described in Methods. Protein synthesis was estimated at the indicated times after infection (1, 3, 5 and 7 h).

**DISCUSSION**

Based on the different kinetics of the inhibition of HeLa cell protein synthesis by EMC virus and poliovirus, and on the different activity of the EMC virus- and poliovirus-infected cell extracts to translate capped mRNAs (Lawrence & Thach, 1974; Rose et al., 1978), it was proposed that the shut-off mechanism of translation by these two picornaviruses was different (Jen et al., 1980); EMC virus blocks host protein synthesis by modification of the intracellular ionic conditions, thus favouring virus mRNA for translation (Carrasco & Smith, 1976; Carrasco, 1977), whereas poliovirus infection modifies the activity of some initiation factors involved in translation (Helentjaris & Ehrenfeld, 1978; Rose et al., 1978; Helentjaris et al., 1979; Trachsel et al., 1980). Our results on the kinetics of the shut-off of translation by EMC virus indicated that EMC virus is also able to shut down host protein synthesis with kinetics similar to those observed after poliovirus infection, if a high enough m.o.i of EMC virus is used (Muñoz & Carrasco, 1981), and that the kinetics also depend on the type of cell infected, e.g., the shut-off of translation in BHK cells is much quicker than in HeLa cells (J. C. Lacal &
Fig. 6. Effect of varying concentrations of NaCl on protein synthesis in HeLa cells single-infected with EMC virus and poliovirus and double-infected with both viruses. Infections were carried out using an m.o.i. of EMC virus and of poliovirus of 10 and 80 respectively. At 3 h post-infection the medium was removed and replaced by 0.5 ml methionine-free DMEM with the indicated final concentrations of NaCl (mM). Labelling of newly synthesized proteins was carried out 30 min later by incubating the cell cultures with 5 μCi [35S]methionine for 30 min. The positions of the cellular protein actin (Ac), the poliovirus protein VP3 and the protein γ of EMC virus are indicated on the autoradiograms. (a) Area of the γ protein of EMC virus calculated from the densitometric scans of the autoradiograms corresponding to EMC virus single-infected cells. (b) Area of poliovirus VP3 protein calculated from the autoradiograms corresponding to poliovirus single infected cells. (c) Area of EMC virus γ protein (O) and poliovirus VP3 protein (●) calculated from the autoradiograms corresponding to double-infected cells. (d) VP3/γ (O) and γ/VP3 (●) ratios calculated from the data shown in (c).

L. Carrasco, unpublished results). In cells co-infected by poliovirus and EMC virus, the former determines the kinetics of inhibition of host translation.

The main conclusion from these results is that simultaneous translation of EMC virus and poliovirus mRNA in HeLa cells is possible. This indicates that whatever the mechanisms used by both viruses to shut off host protein synthesis are, they do not interfere with each other’s protein synthesis. These results are in agreement with the findings of McCormick & Penman (1967), in which mengovirus protein synthesis took place in cells co-infected with poliovirus in
the presence of guanidine, and also with the results of a co-infection experiment between EMC virus and poliovirus described by Detjen et al. (1981).

Another conclusion of our findings is that the extent of interference at the level of translation between the two picornaviruses depends on the conditions of infection, i.e. on the relative multiplicity of each virus used and the time when the second virus is added. This is in agreement with other reports on mixed infection experiments between enteroviruses (Cords & Holland, 1964) and with other virus systems in which two viruses that possess mRNAs with cap structures are involved (Marcus & Carver, 1967), and also to virus systems in which the translation of a capped and an uncapped virus mRNA are examined (Otto & Lucas-Lenard, 1980; Alonso & Carrasco, 1982). If HeLa cells are co-infected by Semliki Forest virus (SFV) and EMC virus, the simultaneous translation of the capped 26S mRNA from SFV and the uncapped picornavirus mRNA can take place. The relative translation of each mRNA in this system depends on the m.o.i. of each virus used and the time of superinfection by the second virus (Alonso & Carrasco, 1982).

The effect of different monovalent ion concentrations in the culture medium of cells double-infected with EMC virus and poliovirus indicated that virus protein synthesis in these two viruses responds in a different way. In EMC virus-infected HeLa cells, a hypotonic medium inhibited virus protein synthesis, whereas some poliovirus translation took place under these conditions. On the other hand, a hypertonic medium was more inhibitory for poliovirus than for EMC virus protein synthesis. Finally, no cellular protein synthesis was observed in poliovirus-infected HeLa cells under all conditions tested. These findings taken together point out new differences in the behaviour of EMC virus and poliovirus protein synthesis.

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REFERENCES


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