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INTERFERON ACTION ON MAYARO VIRUS REPLICATION

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Summary. - Treatment of TC7 cells with interferon (IFN) drastically reduced the yield of infectious Mayaro virus under experimental conditions that virus attachment and penetration into the cells were not affected. In IFN-treated cells, synthesis of Mayaro virus proteins was inhibited and cellular protein synthesis was restored. This phenomenon is dependent on IFN concentration and multiplicity of infection. Electron microscopy of these cells revealed normal and anomalous viral particles inside cytoplasmic vacuoles. This suggests that IFN also interferes with Mayaro virus morphogenesis and inhibits the release of virions from cells.

Key words: Mayaro virus; virus replication; interferon

Introduction

Interferon (IFN) can inhibit virus replication by interfering with different steps in virus replicative cycle. These effects vary primarily with the virus-cell system and the IFN used (Pestka *et al.*, 1987).

Mayaro virus (Alphavirus genus, Alphaviridae family isolated in Brasil) is an arthropod-borne virus, antigenically closely related to Semliki Forest virus (Casals and Whitman, 1957). Clinical manifestations of human infection were described as a feverish illness accompanied by headache, epigastric pain, backache, chills, nausea and photophobia (Causey and Maroja, 1957).

The replication of Mayaro virus in vertebrate cells produces acute cytocidal effects leading to cell death in approximately 48 hr. However, in *Aedes albopictus* cells there is a development of a persistent infection (unpublished results).

The alphaviruses are enveloped RNA viruses with a wide host range. The viral RNA functions as a mRNA molecule for the synthesis of a RNA-dependent RNA polymerase which transcribes the viral genome (42 S) as well as a subgenomic 26 S RNA molecule which serves as a mRNA for the synthesis of the structural proteins (Kaariainen and Soderlund, 1978). Three structural virus proteins have been identified in Semliki Forest virus (Kaariainen and Soderlund, 1978) as well as in Mayaro virus. The relative molecular weights of structural

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proteins of Mayaro virus are 54 K, 50 K and 34 K (unpublished data). The nucleocapsid of alphaviruses contains a single RNA molecule and only one type of protein, the C protein (Kaariainen and Soderlund, 1978). Little is known about the effects of IFN on alphaviruses except for few works with Semliki Forest virus (Friedman *et al.*, 1967; Friedman, 1968; Munoz and Carrasco, 1984).

In this paper we have undertaken a study on the effects of recombinant IFN alpha-2b on some aspects of Mayaro virus replicative cycle, especially on the synthesis of the major virus 34 K protein (p_{34}).

Materials and Methods

Cell cultures and virus. The TC7 cell line, a clone of CV-1 cell line derived from the kidney of a male adult African green monkey was used. The cells were grown in 60 cm^2 glass bottles at 37 °C in Dulbecco's modified Eagle's medium supplemented with 2 % foetal bovine serum plus 8 % bovine serum. Mayaro virus was obtained from the American Type Culture Collection, Rockville, MD, USA. The virus stock was prepared from BHK-21 cells and stired at -70 °C. Infectivity titrations of Mayaro virus were performed by plaque assay in L-A9 cells. Virus dilutions (0.5 ml) were added to cell monolayers in 60 mm Petri dishes that had just reached confluency. After 60 min at 37 °C, virus inoculum was removed and the monolayers were overlaid with 4 ml of medium 199 supplemented with 10 % foetal bovine serum and 0.95 % agarose, and were further incubated in an atmosphere of 5 % CO_2 at 37 °C. Two days later, the monolayers were stained with neutral red (25μg/ml) and the virus plaques were counted.

IFN. Recombinant human IFN alpha-2b (10^8 IU/mg of protein) was a generous gift from Dr. P. H. Leal from Schering-Plough. Concentrations of IFN are expressed in international reference units.

Measurement of infectious virus yields. Confluent monolayers of TC7 cells growing in scintillation vials (4×10^5 cells/vial) were treated with various concentrations of IFN for 18 hr. Then, the medium was removed and the cells were infected with Mayaro virus at a multiplicity of infection (MOI) 1 PFU/cell. After 1 hr incubation at 37 °C, the inoculum was replaced by fresh medium. Incubation at 37 °C continued until 48 hr when medium was collected and virus was assayed by plaque test.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Confluent monolayers of TC7 cells, growing in scintilation vials, treated or not treated with IFN, were infected with Mayaro virus as described above. At indicated times, the medium was removed and the cells were pulse labelled for 30 min with 50 μCi/ml of ^{35}S-methionine. The cellular proteins were analyzed by SDS-PAGE as described by Laemmli (1970). Gel slabs were autoradiographed on Kodak XK-1 films. Mayaro virus proteins were designated by their relative molecular mass estimated by reference to standard proteins (Carvalho *et al.*, 1989).

^{35}S-labelling and purification of Mayaro virus. Samples of TC7 cells were grown in 150 cm^2 flasks to confluence. One group was treated with 10^3 IU/ml of IFN and the other, untreated, served as control. After 18 hr, the cultures were infected with Mayaro virus at MOI 10 PFU/cell during 1 hr at 37 °C. The inoculum was removed, growth medium containing ^{35}S-methionine was added, and incubation continued until cell lysis. The supernatant fluid was collected and sedimented at 10 000 x g to remove cell debris. Virus from the supernatant fluid was sedimented at 100 000 x g for 90 min at 4 °C; the pellet was ressuspended in PBS containing 0.4 % serum albumine and further purified on a 5-50 % potassium-tartarate gradient in PBS-albumine by centrifugation at 100 000 x g for 90 min at 4 °C. The virus band was collected, diluted in PBS-albumine and sedimented by centrifugation at 100 000 xg for 90 min. The pelleted virus was resuspended in PBS.

Virus binding and penetration were studied as described by Belkowski and Sen (1987). Briefly, the monolayers were infected at MOI 10 using purified virions. After virus adsorption at 37 °C for 1 hr, the cultures were washed 3 times with a buffer containing 150 mmol/l NaCl and 10 mmol/l Tris pH 7.4, and lysed in the same buffer containing 0.5 % SDS. The radioactivity of each lysate was determined in TCA precipitable material in a liquid scintillation counter.

In the penetration experiments the monolayers were infected and washed as described above. Then a fresh medium was added and the incubation continued for 90 min at 37 °C. The monolayers were washed with the same buffer and the remaining virus on the cell surface was removed by incubation with 0.25 % trypsin-0.25 % EDTA for 5 min at room temperature. The trypsinization was stopped by adding a fresh medium. Cells were harvested by centrifugation, washed with culture medium and the cell pellet was solubilized in lysis buffer as described above. The radioactivity was determined by scintillation counting.

Electron microscopy. TC7 cells were processed for electron microscopy as previously described (Mezencio *et al.*, 1989).

Results

Effect of IFN on initial events of Mayaro virus infection

The effect of IFN treatment on infectious Mayaro virus production is shown in Table 1. Treatment of TC7 cells with IFN concentrations as low as 10 IU/ml led to a decrease of more than 90 % in the production of infective particles, although cells lysed 48 hr after infection as did the controls. When higher concentrations of IFN were used in conditions of low MOI (5 PFU/cell), cell death was delayed for approximately 36 hr. If the drug was present continuously in the culture medium after infection, CPE could be preserved. At high MOI (20 PFU/ml) we observed cell death in all concentrations of IFN tested (data not shown).

In order to test the effect of IFN on Mayaro virus binding and penetration, TC7 cells were pretreated for 18 hr with IFN. The IFN containing medium was removed and the cells were infected with ^{35}S-labelled Mayaro virus. The results indicated that the adsorption and penetration of Mayaro virus in TC7 cells were not influenced by IFN treatment. The averages of three independent experiments showed that the values of adsorption and penetration of Mayaro virus into

Table 1. Inhibition of Mayaro replication in TC7 cells

IFN treatment (IU/ml)	Virus yield (PFU/ml)	% of inhibition
-	2.6×10^7	
1	1.95×10^7	75
10	1.6×10^6	94
10^2	8.0×10^5	97
10^3	4.0×10^5	99
10^4	n.d.	>99

TC7 cells were treated with different concentrations of IFN for 18 hr, then infected with Mayaro virus at MOI 1 PFU/cell. The supernatant was collected 48 hr p. i.
n.d. - not detected

TC7 cells were 98 % and 96 % of control (infected, IFN-untreated cells), respectively.

Effect of IFN on protein synthesis

TC7 cells were pretreated with different concentrations of IFN, infected with Mayaro virus at MOI 5 PFU/cell and the proteins were analyzed by SDS-PAGE at 24 and 48 hr p. i. The results are shown in Fig. 1. In IFN-untreated cells we could identify at 24 hr p. i. only the p_{34} viral protein, while cellular proteins were not affected. At 48 hr p. i. there was a drastic inhibition of cellular protein

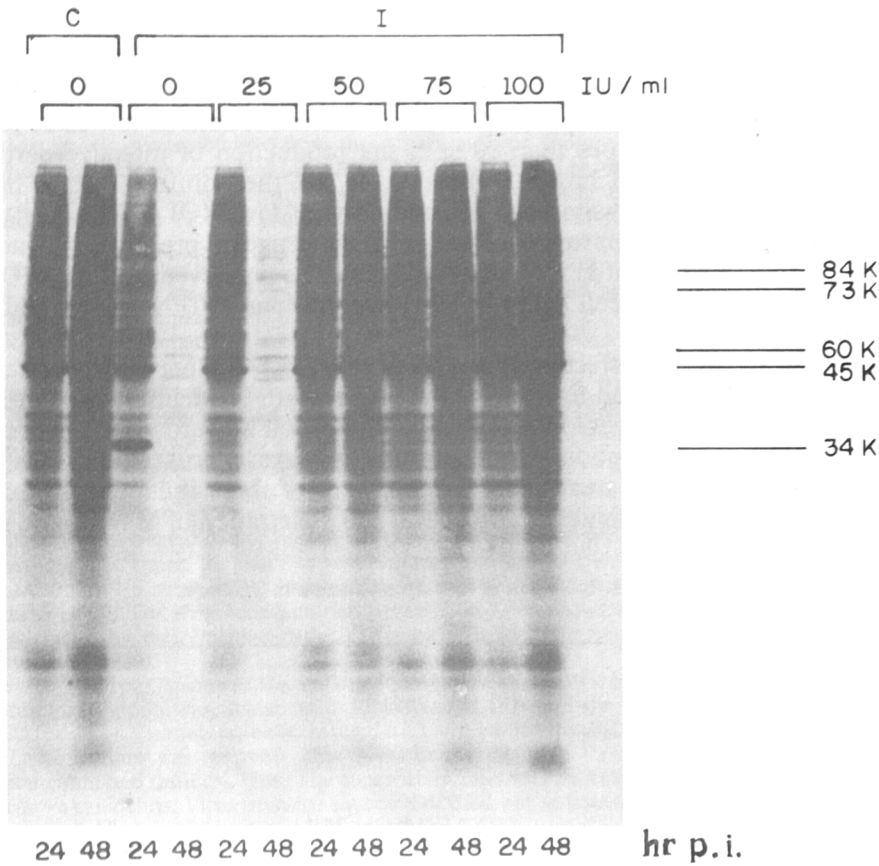

Fig. 1

Effect of different concentrations of IFN on protein synthesis

TC7 cells were treated with IFN (25, 50, 75, 100 IU/ml) and infected with Mayaro virus at MOI 5 PFU/cell. At 24 and 48 hr p. i. the proteins were analyzed by SDS-PAGE and autoradiography. C – control, non-infected cells; I – infected cells.

synthesis and all the viral proteins were clearly distinguished. In the cells treated with low concentrations of IFN (25 IU/ml) the synthesis of p_{34} was inhibited at 24 hr, but at 48 hr the profile of all proteins resembled that of the IFN-untreated cells. Higher concentrations of IFN, however, suppressed apparently all viral protein synthesis. In these conditions we could also observe no inhibition of cellular proteins. These results indicate that IFN can block viral protein synthesis and protect cell protein synthesis from inhibition.

The effects of IFN on protein synthesis as described for others virus cell-systems are dose and MOI dependent (Pestka *et al.*, 1987). Thus, when we analyzed viral and cellular proteins in conditions of high MOI, we observed that low concentrations of IFN (under 200 IU/ml) did not protect the cell (data not

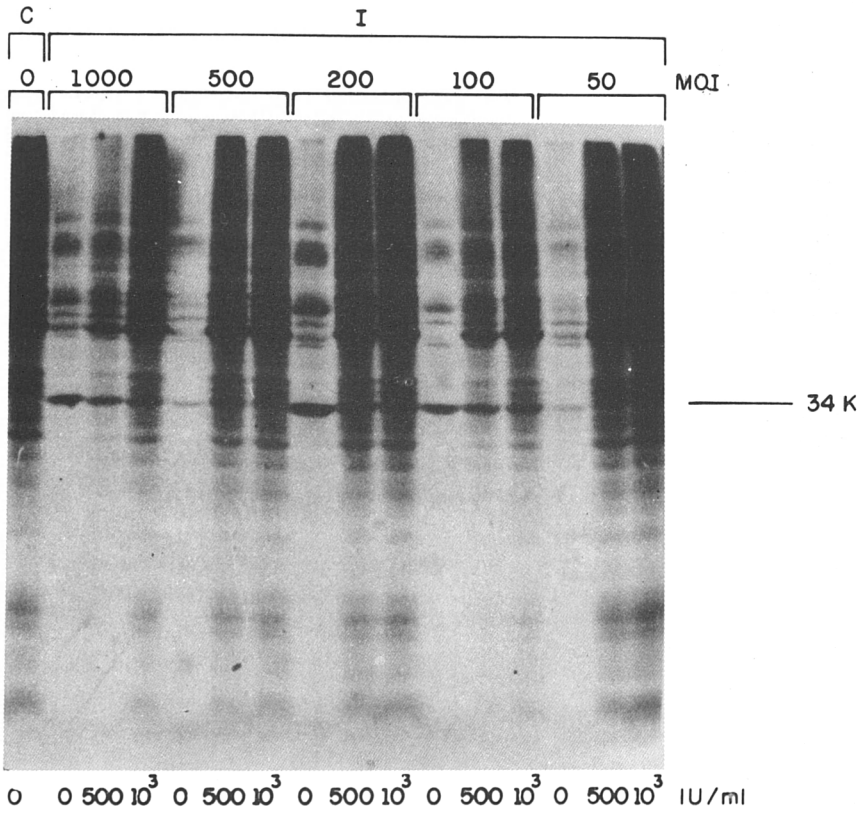

Fig. 2
Effect of different concentrations of IFN on protein synthesis in cells infected at different MOI TC7 cells treated with IFN (500 and 1000 IU/ml) 24 hr p. i. with Mayaro virus in conditions of high MOI. C – control, non-infected cells; I – infected cells.

shown). In other experiments, cells were treated with high concentrations of IFN (500 and 1000 IU/ml) and infected with high MOI (50–1000 PFU/cell). At 24 hr p. i., the cells were pulse labelled for 30 min with ^{35}S-methionine and the proteins were analyzed by SDS-PAGE and autoradiography. The results are shown in Fig. 2. In IFN-treated infected cells, no inhibition of cellular protein synthesis was observed except for MOI 1000, but even in this case, this phenomenon was not so drastic as compared to the control infected cells. However, in these conditions, high concentrations of IFN could not block viral proteins synthesis since p_{34} was clearly distinguished.

Effect of IFN in the morphogenesis of Mayaro virus

In TC7 cells infected with Mayaro virus it was observed that the virions mature by budding from the plasma membrane, either to the extracellular medium (Fig. 3A) or to the intracellular spaces (Fig. 3B). The presence of vacuoles containing virus particles was not detected.

In contrast in IFN-treated infected cells budding of Mayaro virus from the plasma membrane was scarcelly detected (Fig. 4A). However, the presence of cytoplasmic vesicles containing viral nucleocapsids and mature viral particles was observed (Fig. 4B). Together with complete, enveloped particles 43 nm in diameter we also observed particles 69 nm in diameter containing electron-lucent centers (Fig. 4B, 4C). In addition we observed in these cells vacuolization with aberrant forms and inclusion bodies scattered in the cell cytoplasm (Fig. 4C).

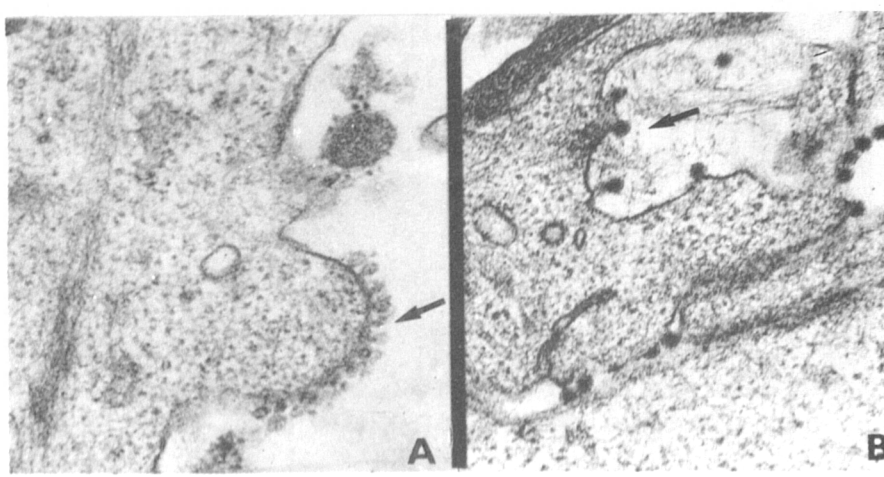

Fig. 3

Electron microscopy of TC7 cells infected with Mayaro virus

(A) The cell membrane shows a number of budding virions in spherical form (arrow) (magn. 21 000×). (B) Typical virus budding into the intercellular space (arrow) (magn. 42 000×).

Discussion

The aim of the present study was to identify the step s of the replicative cycle of Mayaro virus sensitive to IFN-mediated inhibition. In some experiments we used relatively high doses of IFN which are presumably well within physiological limits. In the vicinity of IFN producing cells *in vivo* the local IFN concentrations has been estimated as high as 10^7 IU/ml (Whitaker-Dowling *et al.*, 1983). On the other hand, concentrations of IFN between 1 and 1000 units had no significant effect on cellular growth (data not shown).

As described by Munoz and Carraso (1984) for Semliki Forest virus, very low concentrations of IFN reduced drastically virus yields and prevented CPE. However, we did not find protection against CPE but only a delay in the time of

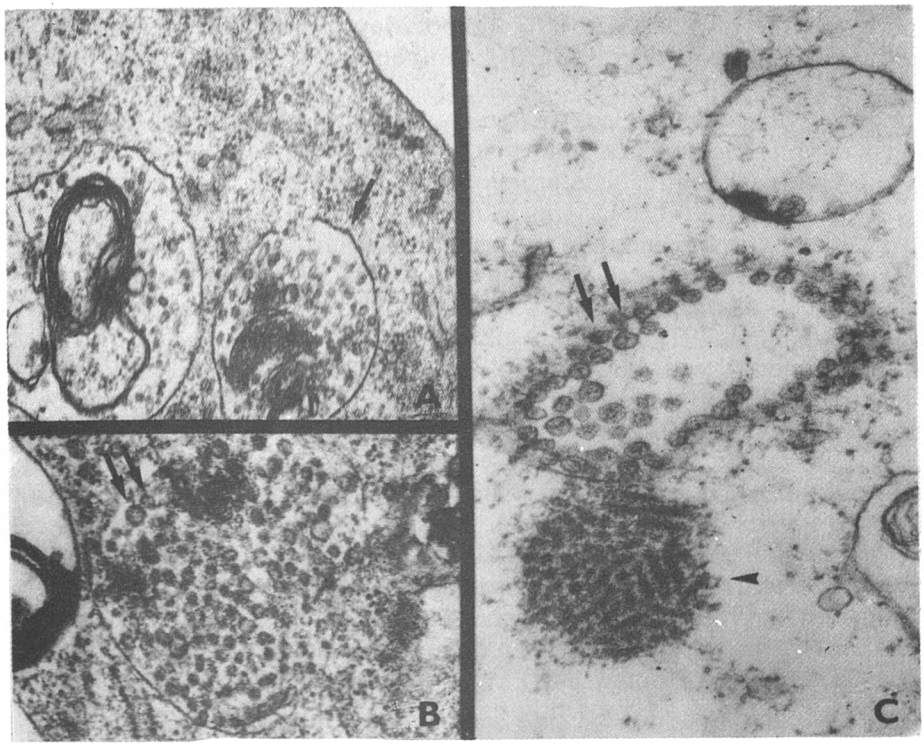

Fig. 4

Electron microscopy of TC7 cells treated with IFN and infected with Mayaro virus (A) The absence of virus budding (magn. 37 500×). (B), (C) Vacuoles with complete virus particles (46 nm diameter, arrow), virions with electron luscent center (61 nm diameter, double arrow), and aberrant forms and inclusion bodies (arrow head) (magn. 47 600× (B), 57 800× (C)).

cell lysis. The protection occurred only if IFN was continuously present in the culture medium.

Our present results show that when TC7 cells are infected with Mayaro virus in conditions of low MOI, cellular proteins are gradually replaced by viral proteins and the inhibition of total protein synthesis takes place relatively late in infection. In cells treated with IFN we found an inhibition of virus protein synthesis only in conditions of low MOI. When higher MOI were employed at least the p_{34} was synthesized efficiently even in the presence of high concentration of IFN. Comparing our results with those described by Munoz and Carrasco (1984) for Semliki Forest virus, we find that for both viruses (in IFN-treated cells) there is no shut-off. The authors also found an impairment in Semliki Forest virus protein synthesis, but in contrast with our results this inhibition was observed with all MOI tested and with relatively low concentrations of IFN.

The data presented here do not provide any molecular explanation for a putative effect of IFN on the inhibition of Mayaro virus protein synthesis. It is possible that the two systems (oligo-A synthetase and protein kinase) described for other virus-cell systems (Staeheli, 1990) operate also in our case. IFN-induced proteins can have a restricted antiviral properties as oligo-A synthetase which inhibits picornavirus and possibly vaccinia virus. On the other hand protein kinase P1 can regulate protein synthesis affecting the multiplication of a great number of unrelated virus (Staeheli, 1990; Samuel, 1991).

As described in the literature the process of alphavirus assembly seems to be extremely fast and efficient. Generally, viral particles free in the cytoplasm are not seen; only mature particles budding from plasma membrane can be observed (Koblet, 1990). These results are in agreement with our findings for TC7 cells infected with Mayaro virus.

It is also well known that IFN interferes with the process of virus morphogenesis in many virus-cell systems (Chaterjee *et al.*, 1985; Chaterjee, 1987; Staeheli, 1990). Our results suggest that IFN inhibition of Mayaro virus replication involves blockade at a late stage in viral morphogenesis and interferes with the process of virus budding from cell membrane. In our study the electron microscopy did not reveal virus budding but numerous cytoplasmic vacuoles containing mature and defective virions. At present we do not know whether our findings results from IFN-induced changes in plasma membrane or are due to alterations in the expression of viral proteins (Aboud *et al.*, 1982; Chaterjee *et al.*, 1985; Chaterjee and Hunter, 1987).

Many laboratories are presently engaged in precise definition of the IFN-sensitive multiplication steps of different viruses (Staeheli, 1990). Here we find that Mayaro virus morphogenesis is affected by IFN. Viral protein synthesis is also inhibited in certain conditions, but it is also possible that this phenomenon is a consequence of alterations in the process of viral RNA synthesis. We are now developing studies in order to determine the IFN action on Mayaro RNA synthesis.

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