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**Na⁺ and K⁺ Concentration and Regulation of Protein Synthesis in L-A9 and Aedes albopictus Cells Infected with Marituba Virus (Bunyaviridae)**

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**SUMMARY**

Infection of L-A9 cells with Marituba virus produces a severe inhibition of protein synthesis. This inhibition is temporally correlated with an increase in the intracellular Na⁺ concentration and a decrease in the intracellular K⁺ concentration. However in Marituba virus-infected Aedes albopictus cells the intracellular level of Na⁺ and K⁺ ions and protein synthesis remained unaltered. Incubation of both cell types at high NaCl concentration facilitated the translation of viral RNA whereas the cellular protein synthesis was inhibited. Using a hypotonic medium, the opposite was found. Results are discussed in terms of a possible involvement of these ions in the viral translational process.

Marituba virus (MTBV) is an arthropod-borne virus first isolated in the Oriboca Forest, in the Amazon region of Brazil (Causey et al., 1961) and is a member of the serologically distinct group C of the large Bunyaviridae family. This virus has a lipoprotein envelope containing two glycoproteins: G1 (Mᵦ 120000) and G2 (Mᵦ 26000). The internal nucleocapsid is composed of the L protein (Mᵦ 190000) and the N protein (Mᵦ 20 000) which are the minor and the major viral components respectively (Frugulhetti et al., 1983).

The most distinctive feature of arboviruses is their ability to multiply in both vertebrate and invertebrate cells (Elliott & Wilkie, 1986). However the end result of an arbovirus replicating in cells of disparate phylogeny is often noticeably different (Verani et al., 1984). The replication of MTBV in L-A9 mouse cells is paralleled by an inhibition of host macromolecular synthesis and c.p.e. (Rebello et al., 1981). In contrast infection of Aedes albopictus mosquito cells with MTBV is characterized by a persistent infection in which no changes in host cell macromolecular synthesis were observed (Carvalho et al., 1986). In both cell types, the levels of infectious virus production are similar. However in L-A9 cells maximum virus production is obtained after about 40 h post-infection (p.i.) and in A. albopictus, it is observed 96 h p.i. (Volkmer et al., 1983).

Several reports have shown that virus infection results in an alteration of the intracellular ionic concentration (Lacal & Carrasco, 1982; Garry et al., 1982) and, curiously, the mRNAs specified by a number of DNA and RNA viruses are efficiently translated under altered ionic conditions which block cellular protein synthesis (Carrasco & Smith, 1976; Saborio et al., 1974; Cherney & Wilhelm, 1979). In this work we studied the possible effect of Na⁺ and K⁺ ions on the replication of MTBV during a lytic infection and during a persistent infection.

*A. albopictus* cells, clone C6/36 (Igarashi, 1978) were obtained from Dr R. E. Shope, Arbovirus Research Unit, Yale University, New Haven, Conn., U.S.A. The cells were maintained at 28 °C in Dulbecco's modified Eagle's medium supplemented with 0·2 mM each of non-essential amino acids, 2·25% NaHCO₃, penicillin (500 U/ml), streptomycin (100 µg/ml), amphotericin B (Fungizone, 2·5 µg/ml) and 2% foetal bovine serum. Mouse L-A9 fibroblasts were maintained as described previously (Volkmer & Rebello, 1981). MTBV (strain Be Anl5) was obtained as a

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Fig. 1. Na⁺ and K⁺ concentration and protein synthesis in MTBV-infected L-A9 cells (a) and *A. albopictus* cells (b). Protein synthesis was expressed as the percentage of [³⁵S]methionine incorporation into TCA-precipitable material by infected cells (at 50 p.f.u./cell) in reference to mock-infected cells. The data are presented as (the amount of radioactivity measured in c.p.m. in infected cells)/(radioactivity incorporated into mock-infected cells) x 100. The intracellular Na⁺ and K⁺ concentrations were determined by atomic absorption spectroscopy. K⁺ concentrations (▲); Na⁺ concentrations (●); protein synthesis (●).

lyophilized mouse brain suspension from Dr F. Pinheiro (Instituto Evandro Chagas, Pará, Brazil) and was adapted to and propagated in L-A9 cells. Infectivity titrations of MTBV were performed by plaque assay in L-A9 cells as described by Volkmer & Rebello (1981).

To determine the Na⁺ and K⁺ intracellular concentration in L-A9 cells and *A. albopictus* cells infected with MTBV, the following experiments were performed. The procedure used was the same as described by Garry et al. (1982). Essentially, cultures of *A. albopictus* and L-A9 cells were grown in scintillation vials (New England Nuclear) to confluence. Media were removed by aspiration and cells were washed quickly with double-distilled, deionized (ddd) H₂O. This procedure was completed in less than 5 s. The cells did not change in size or morphology even after several minutes in ddd H₂O. Cells were then allowed to swell in 1 ml of ddd H₂O for 1 h, scraped with a rubber policeman, disrupted with a 1 ml syringe, and finally frozen at −20 °C. Appropriate dilutions of the cell suspensions were made in ddd H₂O, and Na⁺ and K⁺ levels were determined using a Varian atomic absorption spectrophotometer, Model AA 1475. The number of cells in the sample and the cell volume were determined by using a Coulter counter (Model 7B) connected to an automatic cell size analyser. The instrument was calibrated with 20 μm diameter latex beads. Total cell volume (assuming spherical shape) was calculated from the modal diameter of the cells.
The level of Na\(^+\) and K\(^+\) ions in mock-infected cells was determined. For L-A9 cells we found 20 mM Na\(^+\) and 150 mM K\(^+\) and for A. albopictus cells we found 17 mM Na\(^+\) and 130 mM K\(^+\). In both uninfected cell lines these concentrations did not change significantly during the experimental period (data not shown).

Fig. 1 shows our determination of the intracellular amount of Na\(^+\) and K\(^+\) during the course of infection. During the first 2 h there were no differences in Na\(^+\) and K\(^+\) levels between mock-infected and infected L-A9 cells (Fig. 1a). The first significant change was detected 4 h p.i., when the Na\(^+\) concentration increased to 38 mM and finally to 50 mM at 6 h p.i. We also observed that at 6 h p.i. the content of K\(^+\) declined from 160 mM to 110 mM. As previously shown, MTBV inhibits host cell protein synthesis in vertebrate cells (Rebello et al., 1981). Since exact knowledge of the timing was essential to the interpretation of subsequent experiments, the time course of the inhibition of protein synthesis was examined. The overall rate of protein synthesis in MTBV-infected L-A9 cells began to decline 4 h p.i. and by 6 h p.i. the level was only 30 to 45\% of that of the uninfected cells. The results show that the decline in protein synthesis was temporally associated with an alteration of the intracellular monovalent cation concentration. On the other hand, experiments with MTBV-infected A. albopictus cells revealed that during the replicative cycle the intracellular level of Na\(^+\) and K\(^+\) ions and the protein synthesis remained unaltered (Fig. 1b).

The observation above indicated that MTBV infection induced modifications of the levels of Na\(^+\) and K\(^+\) in L-A9 cells. In order to establish whether the inhibition of protein synthesis could be due to these alterations, we carried out the following set of experiments. L-A9 and A. albopictus cultures infected and mock-infected with MTBV were placed in media containing various concentrations of NaCl (116 to 250 mM) and protein synthesis was estimated by \(^{35}\)S methionine incorporation. In L-A9 infected cells (Fig. 2a), a substantial decrease in \(^{35}\)S methionine incorporation can be seen in normal medium (116 mM-NaCl). However the inhibition of protein synthesis was partially restored when we increased the NaCl concentration.

As previously reported, MTBV replicates in A. albopictus cells without any change in the host protein synthesis (Carvalho et al., 1986). In Fig. 2(b) we show that at 116 mM-NaCl the incorporation of \(^{35}\)S methionine has the same value in both mock-infected or infected cells.
Fig. 3. Reversibility of the hypotonic medium effect on protein synthesis in MTBV-infected L-A9 cells. L-A9 cells mock-infected (lanes 1, 3, 5 and 7) or infected (lanes 2, 4, 6 and 8) with MTBV (1 p.f.u./cell) were maintained for 19 h in isotonic medium. After this period the cells were treated with hypotonic medium (50 mM-NaCl) or with hypertonic medium (220 mM-NaCl) for 1 h and further labelled with [35S]methionine (10 μCi/ml) for 1 h. Cellular extracts were subjected to PAGE and adjusted for constant radioactivity as described by Carvalho et al. (1986). Cells were maintained in isotonic medium and labelled in isotonic media (lanes 1 and 2) or in hypotonic media (lanes 3 and 4), or cells were treated with hypotonic media and labelled in isotonic media (lanes 5 and 6) or in hypotonic media (lanes 7 and 8).

However in infected cells, the addition of excess NaCl in the media results in a stimulation of protein synthesis to 150% at 210 mM-NaCl.

Fig. 3 shows the PAGE analysis of cytoplasmic extracts of MTBV-infected L-A9 cells pulse-labelled (as described above) in isotonic, hypotonic and hypertonic media. In isotonic media (lanes 1 and 2) we can identify two MTBV proteins, G1 and N. When cultures were transferred to hypotonic media (lanes 3 and 4), virus protein synthesis was inhibited. When the cells maintained in hypotonic media were returned to isotonic media (lanes 5 and 6) we observed a recovery of virus proteins. Finally when the cells previously treated with hypotonic media were transferred to hypertonic media (lanes 7 and 8) the synthesis of the N protein was stimulated. In A. albopictus cells infected with MTBV, proteins G1 and N were detected 25 h p.i., as reported previously (Carvalho et al., 1986). At a later time (50 h) the synthesis of the G1 protein was too low to allow quantification and at this time only the N protein was visible. In Fig. 4 we show that
the N protein was also stimulated when the *A. albopictus* cells were incubated in hypertonic medium.

In this study we have shown an alteration in intracellular Na\(^+\) and K\(^+\) concentrations in L-A9 cells infected with MTBV (Fig. 1). These changes were temporally correlated with the selective inhibition of protein synthesis, and we can assume that they mediate this inhibition. On the other hand, *A. albopictus* cells infected with MTBV did not show any change in intracellular Na\(^+\) and K\(^+\) concentrations, and also no inhibition of host protein synthesis was observed in these conditions. However MTBV-infected *A. albopictus* cells responded to changes in intracellular levels of Na\(^+\) and K\(^+\) ions (in hypertonic medium) which stimulated the synthesis of virus proteins.

Nair (1981) showed that in poliovirus-infected HeLa cells the levels of both Na\(^+\) and K\(^+\) were substantially altered and these changes were associated with a decrease in cellular volume. Schaefer *et al.* (1982) demonstrated in HeLa cells infected with poliovirus that a rise in ATPase activity and a decrease in cell volume coincided with the onset of protein synthesis inhibition. In our experiments, alterations in cell volume were observed in neither L-A9 cells nor *A. albopictus* cells infected with MTBV (data not shown).

The observed influx of sodium ions in MTBV-infected L-A9 cells suggests the possibility that changes in intracellular Na\(^+\) concentrations might play a role in determining the extent of host translational inhibition observed in MTBV infection. When L-A9 cells and *A. albopictus* cells infected with MTBV were incubated in media containing different NaCl concentrations an increase in \(^{35}\)S\text{methionine} incorporation was observed in high NaCl concentrations. Analysis of the pattern of viral proteins under these conditions revealed that viral protein synthesis is more resistant than cellular protein synthesis to the hypertonic medium (210 mM NaCl). We also
observed a twofold stimulus in N protein synthesis (data not shown). Evidence from in vitro systems indicates that an increase in the monovalent ion concentration inhibits the translation of cellular mRNAs but stimulates the translation of viral mRNAs (Alonso & Carrasco, 1981). The selective inhibition by high intracellular [Na+] was predicted by Carrasco (1977). He proposed that shut off of host cell protein synthesis is often caused, at least in part, by an increase in intracellular Na+ concentration. A number of viruses have been reported to alter cell membrane permeability, causing an imbalance in the transmembrane Na+/K+ concentration gradient (Lacal & Carrasco, 1982). In contrast to cellular mRNA, whose translation is inhibited by high Na+ concentration, a number of viral mRNAs function with increased efficiency under these conditions, e.g. those of poliovirus (Saborio et al., 1974), encephalomyocarditis virus (Carrasco & Smith, 1976) and vesicular stomatitis virus (Nuss et al., 1975). It seems likely that altered salt concentrations affect translation at least in part by altering the conformational states of mRNAs, or the case with which their conformation can be changed (Kozak, 1986).

Our analysis of the reversibility of protein synthesis inhibition caused by medium containing altered NaCl concentration demonstrated that viral components synthesized in the infected cells under hypertonic conditions were not affected by this treatment (Fig. 3). Our results show that viral protein synthesis could be switched on and off by manipulating two ionic conditions in the medium. The shift to hypotonic conditions reduced the synthesis of viral protein. However, when the cells that had been exposed to hypotonic medium for 1 h were placed in a hypertonic medium, viral protein synthesis resumed immediately. These results indicate that the viral components synthesized in the infected cells were not affected by this treatment and reinforced the belief that ions play a role in the control of translation of protein synthesis.

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