CYTOPLASMIC LOCALIZATION OF RIBONUCLEIC ACID SYNTHESIS INDUCED IN THE CELLS INFECTED WITH SOME GROUP A ARBOVIRUSES

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SUMMARY: Specific RNA synthesis induced in VERO cells by the infection with western equine encephalitis (WEE) virus or Chikungunya virus was studied by autoradiography in the presence of chromomycin A3 or actinomycin D allowing the viruses to propagate despite the suppression of cellular RNA synthesis. It was revealed that such virus-induced RNA pulse-labeled with 3H-uridine after infection of WEE virus or Chikungunya virus appeared dispersely in the cytoplasm of the infected cells. Chikungunya virus-induced RNA accumulated after chase in the restricted areas of the cytoplasm. This finding supports the general view that the loci of RNA synthesis induced by group A arboviruses are confined within the cytoplasm of the infected cells.

INTRODUCTION

It has been shown that virus-specific RNA synthesis in the cells infected with Newcastle disease, HVJ (Sendai) and measles viruses occurs in the nuclear region (Buklinskaya, Burducea and Vorkunova, 1966; Hosokawa et al., 1963; Parfanovich et al., 1966). On the other hand, RNA's of some of picorna viruses, such as polio, mengo and mouse encephalitis viruses, are synthesized in the cytoplasm (Darnell, 1962; Marcus and Freiman, 1966; Holland and Bassett, 1964; Franklin and Rosner, 1962; Hausen, 1962).

With respect to arboviruses, infectious RNA has been extracted from the cytoplasmic fraction of cells infected with WEE virus or eastern equine encephalitis virus during the period of RNA replication (Wecker and Richter, 1962). However, our previous report revealed that RNA synthesis of PS (Y-15) cells infected with Japanese encephalitis virus, a member of group B arboviruses, occurred in the nucleus when cellular RNA synthesis was suppressed by chromomycin A (Takeda, Yamada and Aoyama, 1965). Thus, two different cellular compartments, nucleus and cytoplasm, were proposed to be the sites of RNA synthesis induced by infections of different arboviruses. This may be interpreted as that specific RNA synthesis after the infec-
tion of a virus takes place in either nucleus or cytoplasm but not in both. Our interest is to examine the site of RNA synthesis with a number of arboviruses by means of autoradiography in relation to the antigenic grouping hitherto obtained. The present report describes the autoradiographic studies on RNA synthesis in VERO cells infected with two group A arboviruses namely WEE virus and Chikungunya virus.

**MATERIALS AND METHODS**

*Viruses:* WEE virus prototype strain (supplied by Dr. J. Casals) was used at the 12th passage through the suckling mouse brain. Chikungunya virus, BaH 306 strain at the 7th passage through the suckling mouse brain was further passed 6 times through VERO cells before the experiments. Each seed virus preparation contained $1.0 \times 10^9$ plaque forming units (PFU) of WEE or $3.6 \times 10^7$ PFU of Chikungunya in a 0.5-ml aliquot when determined on VERO cell monolayers. These preparations were dispensed in ampoules and kept at $-75^\circ$C till use.

*Cell culture:* VERO cells, an established cell line from African green monkey (*Cercopithecus*) kidney, were employed. Eagle's minimum essential medium (MEM)

![Graph](image)

**Fig. 1 Inhibition of cellular RNA synthesis in VERO cells with chromomycin A3.**

Coverslip cultures of VERO cells in logarithmic growth were kept in the media supplemented with chromomycin A3 at various concentrations for 5 and 10 hr. Each group of cultures was labeled with $^3$H-uridine ($0.5 \mu$Ci per ml.) for 30 min. This figure is corrected by subtraction of the control count of non-treated cell cultures and expressed as per cent of control.
supplemented calf serum in 10% was used for growth and maintenance of cells.

**Antibiotics:** Chromomycin A₃, an antibiotic inhibiting the cellular RNA synthesis (Michi, 1964; Takeda, Yamada and Aoyama, 1965), was kindly supplied by Dr. K. Kaziwara, Takeda Research Laboratory. The drug dissolved in a minimum volume of methanol was diluted with MEM to the appropriate concentrations.

As shown in Fig. 1, the rate of cellular RNA synthesis was reduced by chromomycin A₃ depending on its concentration. It was reduced to less than 10% following the treatment with 4 µg of chromomycin A₃ per ml.

Actinomycin D was used instead of chromomycin A₃ in the experiments with Chikungunya virus when a satisfactorily purified lot of chromomycin A₃ was not in our hands. This antibiotic was supplied by Merck Sharp & Dohme, West Point, Pa. A preliminary experiment revealed that the cellular RNA synthesis in VERO cells was reduced by more than 95% following the treatment with 4 µg of actinomycin D per ml. (Takeda, in preparation). Based on these results, the following standard condition for antibiotic treatment was determined: 4 µg per ml of medium for 3 hr.

**Radioisotope:** Uridine generally labeled with tritium (the Radiochemical Center, Amersham, Buckinghamshire, England) with a specific activity of 760 mCi per mM was used.

**Incorporation experiments:** Procedures employed in labeling virus-induced RNA are illustrated in Figs. 2 and 3.

The medium was supplemented with 2×10⁻⁵ M unlabeled thymidine in all experiments to inhibit incorporation of the label into DNA. Coverslip cultures of VERO cells (2×10⁴ cells per coverslip) were treated with actinomycin D or chromomycin A₃, each at 4 µg per ml, for 3 hr prior to virus inoculation. WEE virus or Chikungunya virus was inoculated at a multiplicity of 10 or 100 PFU per cell, respectively. The cultures were washed once with phosphate buffer saline (PBS) after the adsorption period (60 min) of virus, and further incubated in a medium containing excess unlabeled thymidine with or without the antibiotic. Mock infected cultures were prepared in the same manner. At various intervals, 6 coverslips in 2 dishes were taken out from each of the following culture groups; non-infected and infected cultures with and without the antibiotic-treatment. They were pulse-labeled for 30 min with ³H-uridine 0.5 µCi per ml.
In the chase experiments shown in Fig. 3, half of the labeled cultures was fixed immediately after the termination of pulse-label and the other incubated further at 37°C for 120 min in a chase medium containing unlabeled uridine (2×10^{-5} M per ml) until fixation. The fixation was carried out with an acetic acid-alcohol mixture (1:3) for 10 min. The acid-soluble material of these cell specimens was extracted with four changes of chilled 2% perchloric acid for 10 min, rinsed in running water for 20 min and air dried. Coverslip cultures processed in this way were submitted to the measurement of the radioactivity and to autoradiography. The radioactivity measurement was performed by a windowless gas-flow counter.

**RESULTS**

**Effect of Chromomycin A₃ on WEE Virus Growth**

VERO cell cultures in bottles were divided into two groups. Culture fluid was replaced with fresh medium with or without chromomycin A₃. They were incubated at 37°C for 4 hr, then washed once with PBS before infection. Chromomycin-treated and control cultures were inoculated with WEE virus at the multiplicity of 10 PFU per cell and kept for 60 min in a 37°C incubator. Cells were washed again with PBS and further incubated in fresh medium with or without the antibiotic. The
cells of 2 bottles were harvested by trypsinization at intervals of 3 hr. The harvested cells were disrupted with a sonic oscillator (Kubota Ltd., SW-250) at 10 KC for 10 min. Cell-associated viruses were measured by plaque assay on VERO cells.

Figure 4 demonstrates growth curves of WEE virus in chromomycin-treated and control cell cultures.

The infective virus titers in both cultures started to increase at the fourth hour and reached a peak at the eighth hour of the infection. The growth rate of virus in chromomycin-treated cells was the same as that in the control, indicating that the production of WEE virus was not affected by chromomycin A₃ at a concentration of 4 μg per ml.
Quantitative Study of RNA Synthesis in VERO Cells Infected with WEE Virus

RNA synthesis in cultures infected with WEE virus was measured by pulse labeling as described in Materials and Methods. Mock infected cultures were examined in parallel.

Results are shown in Fig. 5. RNA synthesis in the infected cultures rose from 2.5 hr post infection (p.i.) and increased steadily throughout the observation period up to 7.5 hr p.i., while the incorporation rate of $^3$H-uridine in control cultures remained unchanged. Therefore, the increment of uridine incorporation in infected cultures may probably have resulted from the virus-induced RNA synthesis.

Intracellular Site of RNA Synthesis in WEE Virus-infected Cells

Autoradiographic studies were performed to determine the site of RNA synthesis following the infection with the same specimens of culture groups as employed in the measurement of radioactivity. Thirty cells of each specimen were counted for the developed silver grains and mean numbers of grains on the nuclear and cytoplasmic regions were plotted against hours p.i. in Fig. 6 (a, b).

Grain numbers on the nucleus after WEE virus infection are depicted in Fig. 6 a. There was neither significant change with time p.i. nor significant difference between the control and infected cultures. Fig. 6 b shows the increase of grain numbers on the cytoplasm through the course of WEE virus infection in VERO cells. Grains on the cytoplasm in non-infected cells should be regarded as the background since cellular RNA is said to be synthesized in the nucleus and should not be able to appear in the
Fig. 6 Shift of grain number in autoradiograph on the nucleus (6a) and the cytoplasm (6b) of VERO cells infected with WEE virus. Open circles and dotted lines represent of grain numbers on non-infected control cells with chromomycin treatment. The cross bar on each point gives the standard error of the mean grain number of 30 cells.

cytoplasm within one hour. In fact, the grain number in the cytoplasm of the infected cells appeared to increase in parallel with the increase of total radioactivity as shown in Fig. 5. Grains appeared dispersely over the cytoplasmic region any time through the course of the infection (Fig. 9 B). Those results suggested that RNA synthesis caused by WEE virus infection occurred in the cytoplasm through the period of viral RNA synthesis.

**RNA Synthesis in Chikungunya Virus-infected VERO Cells**

Preliminary studies showed that the production of Chikungunya virus was not affected by actinomycin D at 4 μg per ml. (Takeda, unpublished data). RNA synthesis of actinomycin-treated cells infected with Chikungunya virus arose at 3 hr and reached
Fig. 7 RNA synthesis of VERO cells infected with Chikungunya virus. Horizontal band denotes the ranges of standard deviation for the count of non-infected control without actinomycin treatment. Dashed line with open circles and solid line with filled circles represent RNA syntheses of control cells and Chikungunya virus-infected cells with actinomycin treatment.

a maximum level at 9 hr p.i. as shown in Fig. 7.

Autoradiographic studies were carried out with these infected cells as well as non-infected controls. Mean grain numbers on the nuclear and cytoplasmic regions of 30 cells each were plotted in Fig. 8 a and 8 b. Grain numbers on the nucleus of the infected cells were almost the same as those of the non-infected control cells. Grain numbers on the cytoplasm of Chikungunya virus-infected cells, however, began to increase at the third hour of the infection.

A large number of grains appeared to be distributed dispersedly over the cytoplasm any time through the course of the infection (Fig. 9 C). These results suggested that RNA synthesis caused by Chikungunya virus took place in the cytoplasm of the infected cells.

Localization of The Newly Synthesized RNA in Cytoplasm

Actinomycin-treated cultures infected with Chikungunya virus were labeled with $^3$H-uridine for 30 min and chased for 120 min as shown in Fig. 3 in order to follow the transfer of the synthesized RNA. Most of grains were shown to be distributed
dispersedly over the cytoplasm till the 8 hr p.i. when approximately 30% of cells were labeled. However, some grains appeared to accumulate on a few inclusions in the perinuclear region at 8 hr p.i. (Fig. 9 D). The number of labeled cells increased progressively from 8 to 14 hr p.i. The largest portion of grains was accumulated near the cell surface when a majority of the cells were heavily labeled with tritium at 14 hr p.i. (Fig. 9 E and F). These phenomena were not observed in the non-infected cells treated with actinomycin D and processed at the same time.

**DISCUSSION**

The autoradiographic studies reported herein seem to demonstrate that WEE virus- and Chikungunya virus-induced RNA syntheses take place in the cytoplasm of the infected VERO cells. Mantani et al. (1967) demonstrated by autoradiography that intensive RNA synthesis occurred in the cytoplasm of FL cells infected with Chikungunya virus African strain in the presence of actinomycin S. Higashi et al. (1967) reported that, in the electron microscopic investigation, Chikungunya virus precursors
appeared to be formed in the cytoplasmic matrix and then aligned along the regions of cell membrane or apposed to vacuolar membranes. The inclusions with accumulated grains in the cytoplasm described in the present report may correspond to the sites of formation of virus precursors in their report.

Mifune and Hayashi (1969) showed the autoradiographic evidence that RNA synthesis induced by Sindbis virus took place in the cytoplasm of the infected PS cells. The unique cytoplasmic structures designated as type I cytopathic vacuoles (CPV-I) were found in chick embryo cells early in the logarithmic phase of Semliki forest virus replication (Grimley, Berezovsky and Friedman, 1968). High resolution autoradiography demonstrated that the CPV-I were the loci of $^3$H-uridine incorporation. In conclusion, all members of group A arboviruses so far examined seem to behave similarly in the way that RNA synthesis induced by the infection, presumably viral RNA synthesis, took place in the cytoplasm but not in the nucleus of cells.

The time interval between the onset of RNA synthesis and the appearance of infective particles seems in general to be shorter with group A arboviruses (Wecker and Richter, 1962; Mantani et al., 1967; Higashi et al., 1967; Ben-Ishai et al., 1968; Grimley et al., 1968; Mifune and Hayashi, 1969) than with group B arboviruses (Takeda et al., 1965; Takeda, unpublished data). While RNA synthesis induced by the infection of the group A arboviruses, probably viral RNA synthesis, was shown in the present report to take place in the cytoplasm, viral RNA of some of group B arboviruses was suggested to be synthesized in the nucleus (Takeda et al., 1965; Takeda unpublished data). The viral maturation of group A arboviruses of which viral components are all synthesized in the cytoplasm may progress faster than that of group B arboviruses of which RNA is synthesized in the nucleus and transferred to cytoplasm to form mature particles.

REFERENCES


Holland, J. J. AND Basnett, D. W. (1964): Evidence for cytoplasmic replication of polio-
Explanation of photographs

Autoradiograph of VERO cells after 30 min-labeling with $^3$H-uridine.

Fig. 9A
Non-infected VERO cells under actinomycin treatment. Incorporation of tritiated uridine into the nuclei was inhibited with actinomycin D.

Fig. 9B
WEE virus-infected VERO cells under chromomycin treatment at the 8th hour of the infection. Grains were observed dispersedly on the cytoplasm of the cells.

Fig. 9C
Chikungunya virus-infected VERO cells under actiomyacin treatment. Grains were scattered all over the cytoplasm of the cells at the 12th hour of the infection.
Autoradiography of Chikunya virus-infected VERO cells in pulse-chase experiment.

Fig. 9D
Most grains were dispersedly distributed over the cytoplasm at 8 hr p.i., but some were accumulated in the restricted parts of the perinuclear region (arrows).

Fig. 9E
Cells at 14 hr after infection. Note the accumulation of grains near the cell surface (arrows).

Fig. 9F
Cells at 17th hours after infection. Note the large masses of grains in cytoplasm (arrows).
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