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CYTOPLASMIC RNA SYNTHESIS AND VIRAL ANTIGEN IN FL CELLS INFECTED WITH CHIKUNGUNYA VIRUS[1]

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SUMMARY Chikungunya virus is able to multiply in FL cells. One μg per ml of actinomycin S is sufficient to inhibit cellular RNA synthesis of FL cells. In the presence of this concentration of actinomycin, autoradiography of ^{3}H-uridine solution containing non-radioactive thymidine revealed that intensive RNA synthesis occurred in the cytoplasm of FL cells infected with chikungunya virus.

The fluorescent antibody technique also revealed that viral antigen was exclusively located in the cytoplasm of the infected cells.

INTRODUCTION

Actinomycin D inhibits DNA-dependent RNA synthesis in mammalian cells. However, it does not inhibit the synthesis of various kinds of RNA viruses (REICH *et al.*, 1962), including chikungunya virus (HELLER, 1963). Similarities have been found in the chemical structures and biological activities of actinomycin D and S (KAWAMATA and FUJITA, 1958; KAWAMATA, 1963). Both compounds almost completely inhibit the synthesis of cellular RNA. Therefore, RNA synthesized in cells infected with chikungunya virus in the presence of actinomycin is almost exclusively viral RNA.

This paper is on the intracellular localization of newly synthesized RNA and viral antigen in FL cells infected with chikungunya virus in the presence of actinomycin S.

MATERIALS AND METHODS

1. *Virus*

Chikungunya virus (ChV), prototype African strain at the 174th passage level in suckling mouse brain (SMB) were kindly supplied by Dr. Sompop Ahandrik of this Institute in Bangkok. The strain was used after 2 more passages in SMB and 3 more passages in cloned FL cells. Infected FL cells were frozen and thawed. The homogenate was centrifuged at 3,000 rpm for 15 min and the supernatant was kept at −70°C and used as the virus material in these experiments.

2. *Media*

Eagles minimum essential medium (MEM) sup-

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plemented with 10% calf serum (free from ChV neutralizing antibody) was used. Virus was grown in MEM without serum.

3. *Virus growth curve*

FL cells were cloned and passaged 8 times. The cells were grown in 2 ounce rubber-stoppered prescription bottles. The cell monolayers were used after 3 days incubation at 37°C. At the time of virus inoculation there were 2×10^6 FL cells per bottle. Cell monolayers were washed twice with 4 ml of PBS before infecting them with 0.2 ml of virus (1.1×10^8 PFU/ml). Virus was allowed to become adsorbed for 1 hour at 37°C. The multiplicity of infection (moi) was therefore 11. After adsorption of virus, the cultures were washed twice with 4 ml volumes of Hanks' solution.

Four ml of MEM (free from serum) were then added, and the bottles were incubated at 37°C. At intervals 2 bottles of culture were harvested, frozen and thawed three times, and centrifuged to remove cell debris. The supernatants were titrated for virus infectivity.

4. *Assay of infectivity of virus*

Assay of infectivity of virus was performed by the plaque technique in VERO cells essentially as described previously (IGARASHI *et al.*, 1967).

5. *Antibiotics*

Actinomycin S, kindly supplied by Dr. J. Kawamata (Research Institute for Microbial Diseases, Osaka University), was dissolved in a minimum volume of acetone, and diluted with MEM to appropriate concentrations.

6. *Radioactive isotope solution*

To demonstrate RNA synthesis, ^{3}H-uridine (The Radiochemical Center, England) with a specific activity 2.73 c/mM was used. Non-radioactive thymidine, at a concentration of 2×10^{-5} M, was added to the medium during incubation with ^{3}H-urdine to avoid incorporation of the isotope into DNA (YAMADA 1964).

7. *Infection of cells and labeling with ^{3}H-uridine for autoradiography*

FL cells were grown in Leighton tubes with cover slips. The cells were used after 2 days incubation at 37°C. At the time of virus inoculation there were 2×10^5 FL cells per tube. Cells were washed twice with 1 ml of PBS before infecting them with 1 ml of virus (1×10^7 PFU/ml). Tubes were gently shaken twice during the adsorption period. Virus was allowed to become adsorbed for 1 hour at 37°C. The moi was therefore 50. After adsorption of virus, the cultures were washed twice with 2 ml of Hanks' solution. Then 2 ml of MEM (free from serum) was added and the tubes were incubated at 37°C. As shown in Fig. 1, 1 ml of actinomycin solution (1 μg/ml) was added every 2 hours to 2 infected tubes. After 2 hours exposure to actinomycin, the medium was replaced by 1.5 ml of prewarmed MEM containing ^{3}H-uridine (2 μc/ml) and non-radioactive thymidine (2×10^{-5} M/ml).

Cultures were exposed to the isotope for 30 min in a water bath at 37°C. The cells on coverslips were taken out and fixed with methanol and extracted with 2% perchloric acid at 4°C for 40 min. Dipping autoradiography was carried out with NR-M2 emulsion (Konishiroku Photo Industry Co., Tokyo) with an exposure period of 3 days. The cells were then stained with Giemsa solution. Incorporation of ^{3}H-uridine into RNA was assayed by grain count.

8. *Preparation of anti-chikungunya serum*

The brain of a suckling mouse infected with Chikungunya virus was the source for immunization. Three days after intracerebral inoculation, the brain was removed aseptically. The supernatant fluid of the homogenized brain was used for intraperitoneal injection after appropriate dilutions; the first intraperitoneal injection into mice was made with 0.1 ml of the supernatant fluid diluted by 10^{-3}. After 10, 20, 30 and 40 days, four additional injections were made with ten fold dilutions of the same fluid used for the first injection. Two weeks after the last booster injection, animals were bled and the serum was separated. The hemagglutination inhibition antibody titer of this serum was $\times$10240.

9. *Preparation of fluorescein conjugated antibody*

The antiserum was fractionated with 50 percent saturation with ammonium sulfate. The resulting precipitate was separated by refrigerated centrifugation at 9,000 rpm for 20 min and dissolved in the original volume of saline. This procedure was repeated three times. To remove ammonium ions from the final solution a Sephadex G25 column was used. This mouse globulin was conjugated with fluorescein isothiocyanate (Baltimore Biological Laboratories) essentially by the method of MARSHALL

et al. (1958). Then, 0.01 mg of fluorescein isothiocyanate per 1 mg of protein was added to the globulin solution, which had been adjusted to pH 9.5 with 0.5 M carbonate buffer and the mixture was stirred at room temperature for about 3 hours by a magnetic stirrer. To remove unconjugated dye from the solution a Sephadex G25 column was used. The effluent was passed through a column of DEAE-cellulose and the globulin was fractionated further. The virus antigen was specific for fluorescent antibody since; 1) labeled antibody did not stain uninfected cultures, 2) the specific fluorescence was blocked by preincubation of the coverslip preparations with mouse antiserum, but not with normal mouse serum and 3) infected cultures were not stained with conjugated anti-cowpox rabbit globulin. The staining titer was 1 : 4.

10. *Staining procedures with fluorescent antibody*

Cells on coverslips were fixed with acetone at room temperature for 5 min.

Each preparation was overlaid with the conjugate. After one hour of contact at 37°C in a vapor saturated chamber, the samples were washed thoroughly with PBS and covered with a coverslip with a drop of mounting medium (glycerin diluted with PBS). For observation, fluorescence was generated by a 250 W high pressure mercury vapor lamp (Tiyoda Optical Co.).

RESULTS

1. *Growth curve of virus in FL cells*

Fig. 2 represents the growth curves of chikungunya virus over a 25-hour period. The latent period was approximately 4 hours. The virus titer reached about 2×10^7 PFU at 9 hours after infection. At 24 hours after infection the virus titer reached a maximum.

2. *Concentration of actinomycin S sufficient to inhibit cellular RNA synthesis*

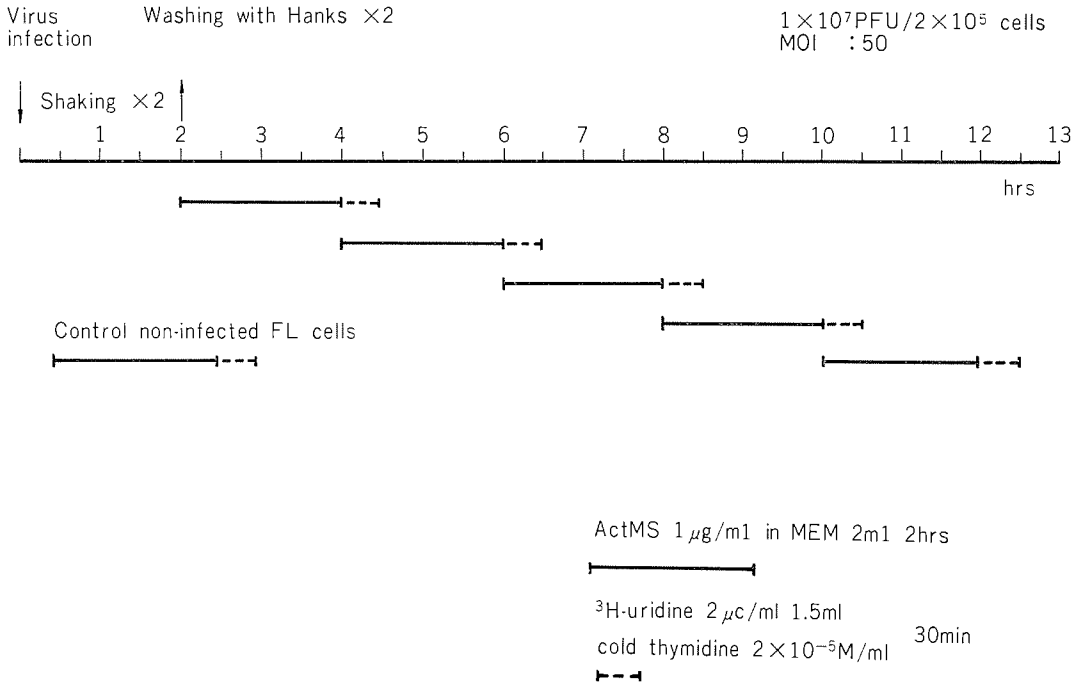

FIGURE 1 Chronological order of virus infection, treatment with actinomycin S(AcMS) and labeling with ^{3}H-uridine. Two Leighton tubes were prepared for samples at each point.

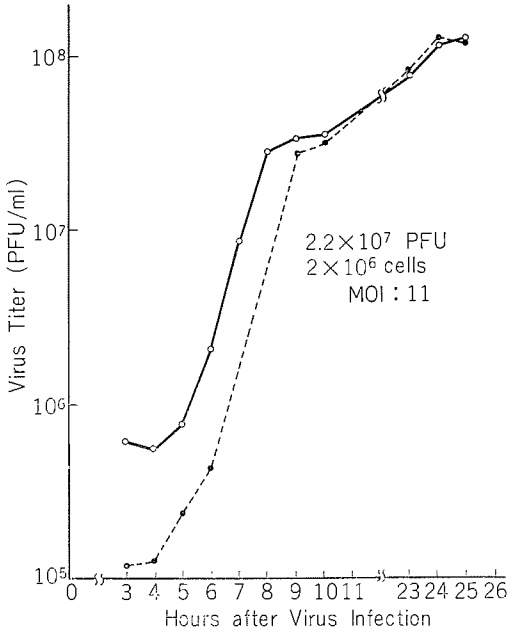

FIGURE 2 Growth curve of chikungunya virus in FL cells.
—○—○—: cell phase
--●---●--: medium phase

FL cells on the coverslips were kept in the presence of various concentrations of actinomycin S for two hours. Then medium was replaced with ^{3}H-uridine solution (1 μc/ml) containing non-radioactive thymidine. Thirty minutes later the samples were taken out and autoradiographed. As shown in Fig. 3, in non-treated control cells, grains appear predominantly in the nuclei (Fig. 13). In actinomycin-treated cells, 1 μg/ml of the compound is shown to be enough to inhibit cellular RNA synthesis (Fig. 14). Fig. 4 shows the distribution of the number of silver grains per nucleus in 100 cells.

3. *Effect of actinomycin S on ChV multiplication*

Cells in 2 ounce prescription bottles were infected with ChV under the similar condition of infection to the experiment for virus growth curve. After adsorption of virus and washing, half of the cultures were kept in the presence of actinomycin S(1 μg/ml). At various time intervals one control and one actinomycin-treated culture were harvested, frozen and

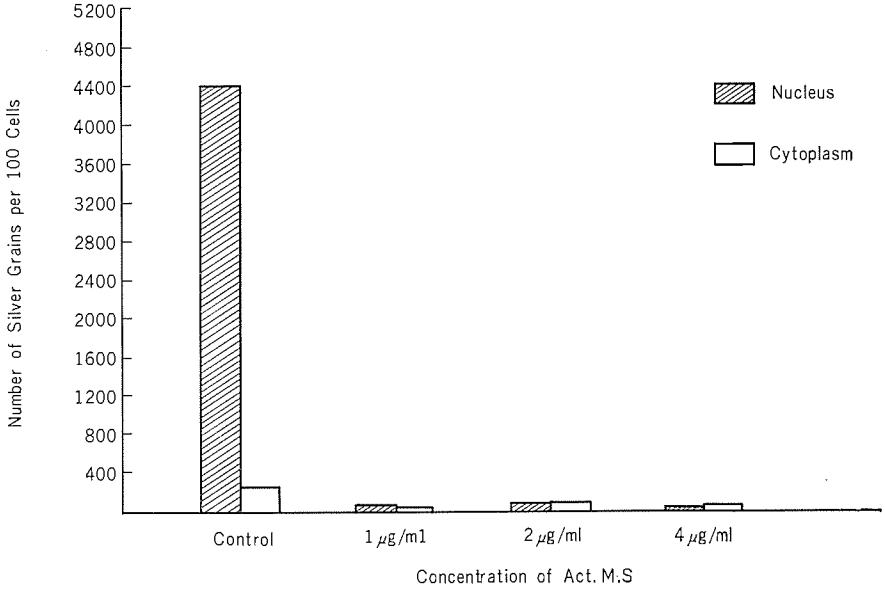

FIGURE 3 Effect of actinomycin S upon cellular RNA synthesis of control FL cells.

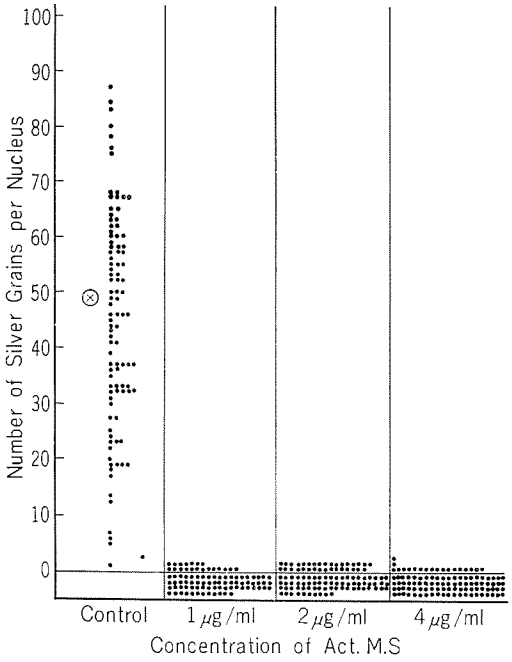

FIGURE 4 Effect of actinomycin S upon nuclear RNA synthesis of control FL cells. ⊗ shows the average number of silver grains per nucleus. The dots below zero indicate cells with cytoplasm without silver grains.

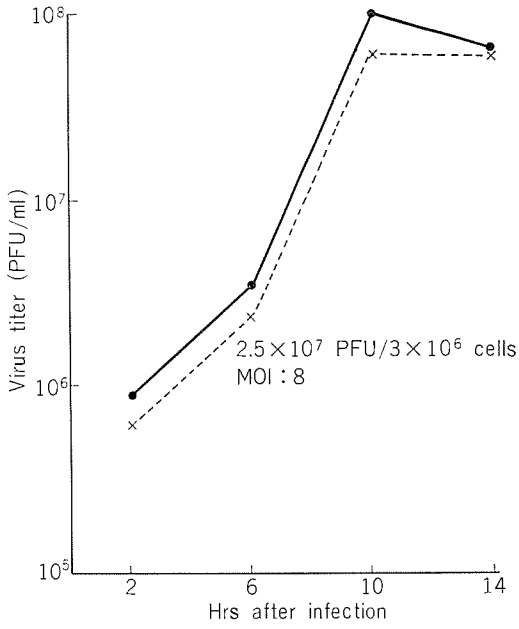

FIGURE 5 Growth curve of chikungunya virus in FL cells in the presence of Actinomycin S.
●——●: control
×------×: ActMS I μg/ml

thawed three times, and centrifuged to remove cell debris, the supernatants were titrated for virus infectivity. As shown in Fig. 5, no remarkable difference in the virus yield could be observed between two groups at least until 14 hours after infection.

4. *Location of RNA synthesis in chikungunya virus-infected FL cells*

Experiments were carried out as described in materials and methods and in Fig. 1.

(a) Qualitative autoradiographic findings.

Cells bearing cytoplasmic grains could be seen distinctly after 6 hours. The number of silver grains per cytopasm and the number of cells bearing cytoplasmic grains increased gradually. The silver grains in the cytoplasm were not localized in any particular area but scattered rather diffusely. However there was a tendency for dense areas of silver grains corresponding to the areas staining more strongly with methylene blue (which stains RNA exclusively in Giemsa staining).

Generally the nuclei of cells were free from silver grains. However, in samples 10 hours and 12 hours after infection, silver grains were found in the nuclei of some cells, and most of these also had cytoplasmic silver grains. The silver grains in the nuclei were often found in the peripheral part of the nucleus next to areas of rather dense cytoplasmic silver grains. Figs. 15 and 16 show typical cells bearing silver grains in the cytoplasm.

(b) Quantitative autoradiographic analysis.

Fifty cells on a coverslip were chosen at random. Since two Leighton tubes were prepared at each point, 100 cells were studied quantitatively. The numbers of silver grains

per cytoplasm and per nucleus were counted and plotted as shown in Fig. 6 and Fig. 7, respectively. The total numbers of silver grains in the cytoplasms and nuclei of 100 cells are plotted in Fig. 8. The number of silver grains in the cytoplasm definitely increased from 6 hours to 12 hours, while the number of silver grains in the nuclei did not increase remarkably. To find the significance of the silver grains appearing in the nuclei of cells, the relationship between the number of silver grains in the nucleus of a cell and that in the cytoplasm of the same cell was studied on 3 samples at 8 hours, 10 hours and 12 hours after infection. As shown in Figs. 9, 10 and 11, cells without any silver grains in the cytoplasm rarely had silver grains in the nucleus. The line in each figure was calculated by the method of least squares. The gradients of these lines were 0.15, 0.12 and 0.199 at 8 hours, 10 hours and 12 hours, respectively.

From this together with the location of the nuclear silver grains mentioned above, some of the grains, especially in the peripheral parts of the nuclei seem to be silver grains in the cytoplasm covering the nuclei.

These results suggest that in the presence of actinomycin S, intensive RNA sythesis occurs in the cytoplasm in FL cells infected with chikungunya virus.

5. *Production of chikungunya virus antigen in the presence of actinomycin S*

In the experiments on RNA synthesis, the synthesis of viral antigen was also followed by the fluorescent antibody technique in ChV-

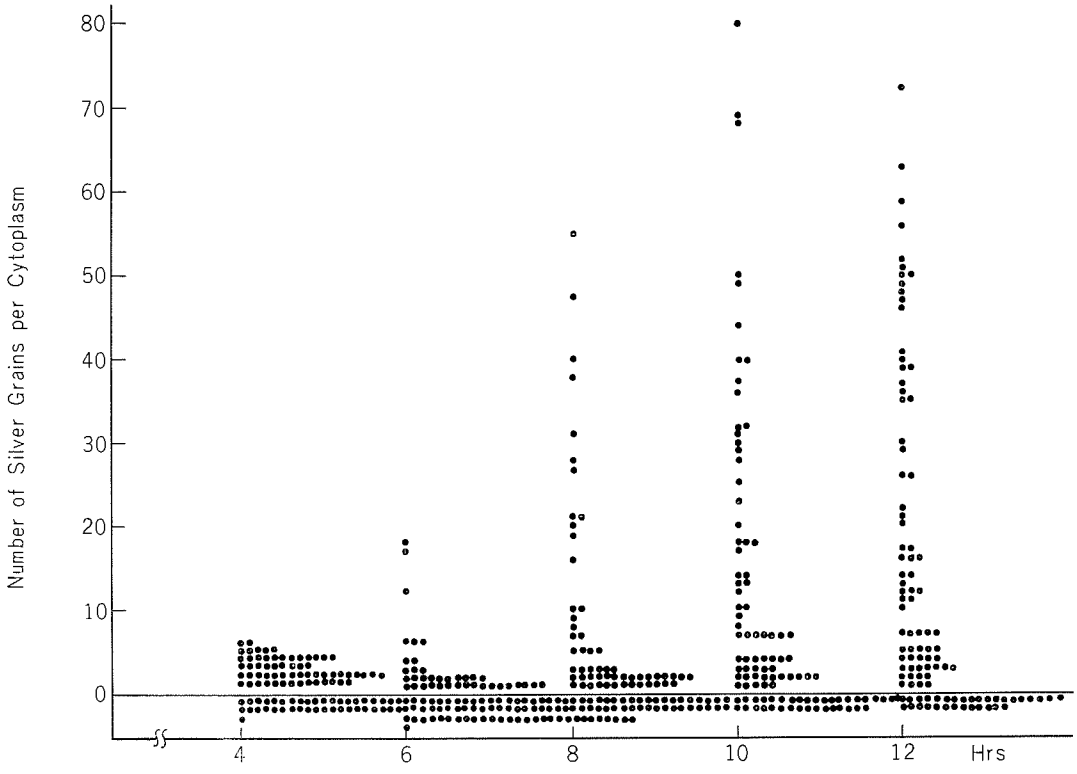

FIGURE 6 Quantitative autoradiogram of ^{3}H-uridine in cytoplasm of FL cells infected with chikungunya virus in the presence of actinomycin S. The dots below zero indicate cells with cytoplasm without silver grains.

infected cells. The specific fluorescence of ChV antigen was clearly seen 4 hours after infection. All the antigens were localized exclusively in the cytoplasm. In the early stage of infection fluorescence was seen rather diffusely around the nuclei (Fig. 17). Later the fluorescence became localized to certain perinuclear areas (Fig. 18). The kinetics of the percentage of fluorescent cells is shown in Fig. 12. The percentage of fluorescent cells increased rapidly up to 76% by 13 hours after infection.

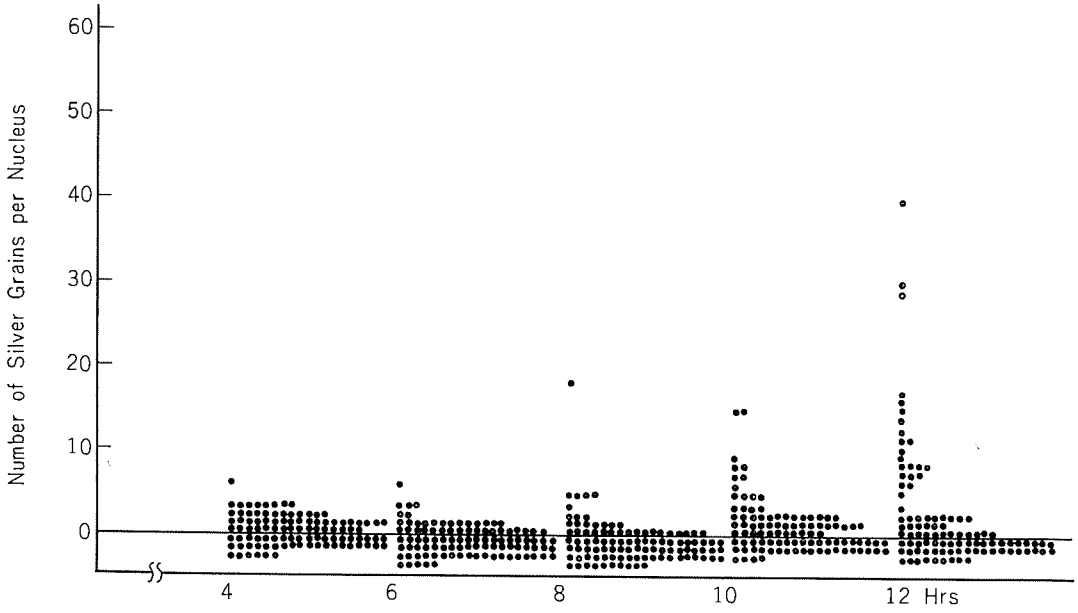

FIGURE 7 Quantitative autoradiogram of ^{3}H-uridine in nuclei of FL cells infected with chikungunya virus in the presence of actinomycin S. The dots below zero indicate nuclei without silver grains.

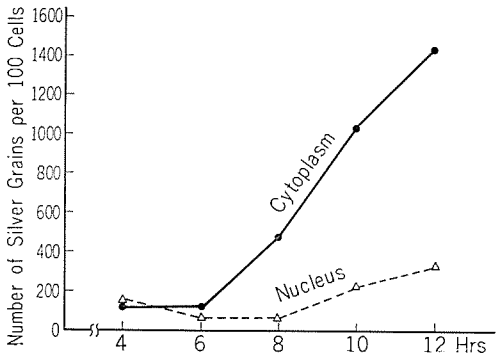

FIGURE 8 Kinetics of the total number of silver grains in cytoplasm and nuclei of 100 cells infected with chikungunya virus, in the presence of actinomycin S.

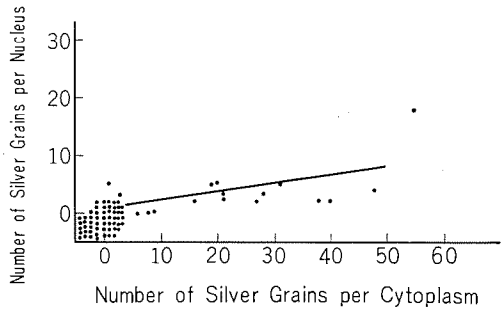

FIGURE 9 Relationship between number of silver grains in cytoplasm and that in nucleus. The line was calculated by the method of least squares. 8 hour sample.

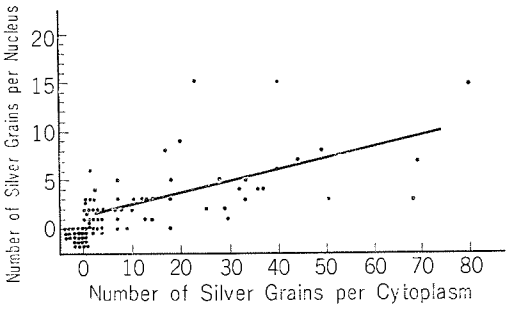

FIGURE 10 Relationship between number of silver grains in cytoplasm and that in nucleus. The line was calculated by the method of least squares. 10 hour sample.

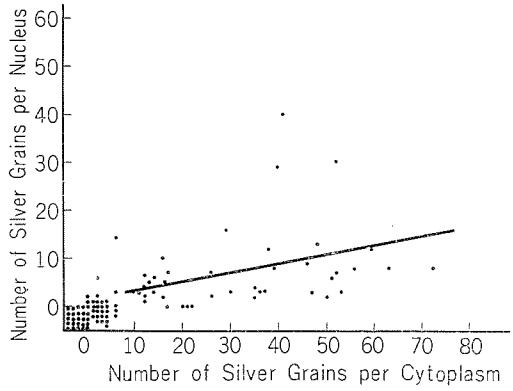

FIGURE 11 Relationship between number of silver grains in cytoplasm and that in nucleus. The line was calculated by the method of least squares. 12 hour sample.

DISCUSSION

The site of synthesis of viral nucleic acids varies from virus to virus. Among DNA viruses, viral DNA synthesis of poxvirus is induced in localized intracytoplasmic areas: "B" type inclusions (KATO *et al.*, 1961), while other viruses, such as herpes virus synthesize, their own DNA exclusively in the nucleus (NII *et al.*, 1961). Among RNA viruses the site of viral nucleic acid synthesis again depends upon the group and sometimes even the subgroup or member of virus group. Viral

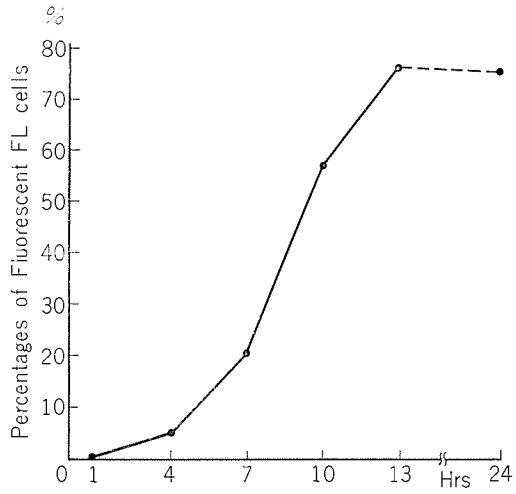

FIGURE 12 Kinetics of the percentage of fluorescent cells in 500 cells.

RNA synthesis of Mengo virus takes place in the cytoplasm (REICH *et al.*, 1962), while Japanese B encephalitis virus is considered to synthesize its own RNA in the nucleus of the infected cells (TAKEDA *et al.*, 1965). Curiously, Western Equine Encephalitis (WEE) virus, which is an arbor virus like Japanese B encephalitis, synthesizes viral RNA in the cytoplasm. The present experiments showed that in FL cells infected with chikungunya virus RNA is synthesized in the cytoplasm. Since cellular RNA synthesis is inhibited by actinomycin and the increase in newly synthesized cytoplasmic RNA is parallel with the increase of viral antigen and viral infectivity, this cytoplasmic RNA may be regarded as viral RNA. Both WEE virus and chikungunya virus are type A arbor virus, while Japanese B encephalitis virus is type B arbor virus. If the site of viral RNA synthesis of arbor virus reflects the serological marker of the type of arbor virus, it could be another type specific marker of arbor virus.

It is also of interest that the site of the synthesis of viral nucleic acids does not always correspond with the site of viral antigen or the site of viral maturation. However, our fluores-

scent antibody study revealed that viral antigen was also exclusively located in the cytoplasm. HIGASHI *et al.* (1967) found by electron microscopy that the virion of chikungunya virus growing in VERO cells appeared in the cytoplasm.

It may be concluded that all events in the synthesis of viral RNA and viral antigen and maturation of the virions of chikungunya virus take place exclusively in the cytoplasm of the infected cells.

ACKNOWLEDGEMENTS

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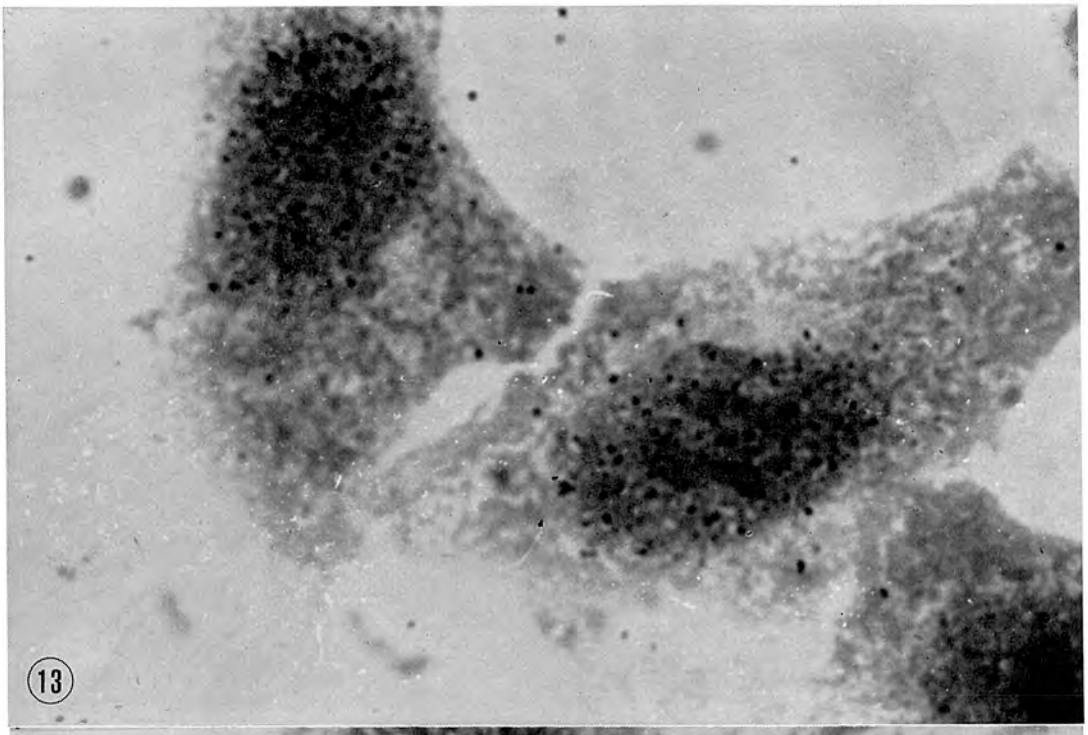
ADDENDUM

During preparation of manuscription of this paper, we heard from Dr. A. Oya (personal communication, 1967) that similar autoradiographic results had been obtained independently by him and his colleagues. Although the details of their experiments are not known, their findings seem to agree essentially with ours. Dr. I. Gaidamovich (1967), recently studied chikungunya virus-infected cells by the fluorescent antibody technique and obtained similar results to ours on the appearance of fluorescence.

FIGURE 13 Autoradiogram of ^{3}H-uridine incorporated into control FL cells, stained with Giemsa solution. Silver grains were located in the nuclei.

FIGURE 14 Autoradiogram of ^{3}H-uridine exposed FL cells in the presence of actinomycin S(1 μg/ml), stained with Giemsa solution. Silver grains were scarce both in the nuclei and in the cytoplasm.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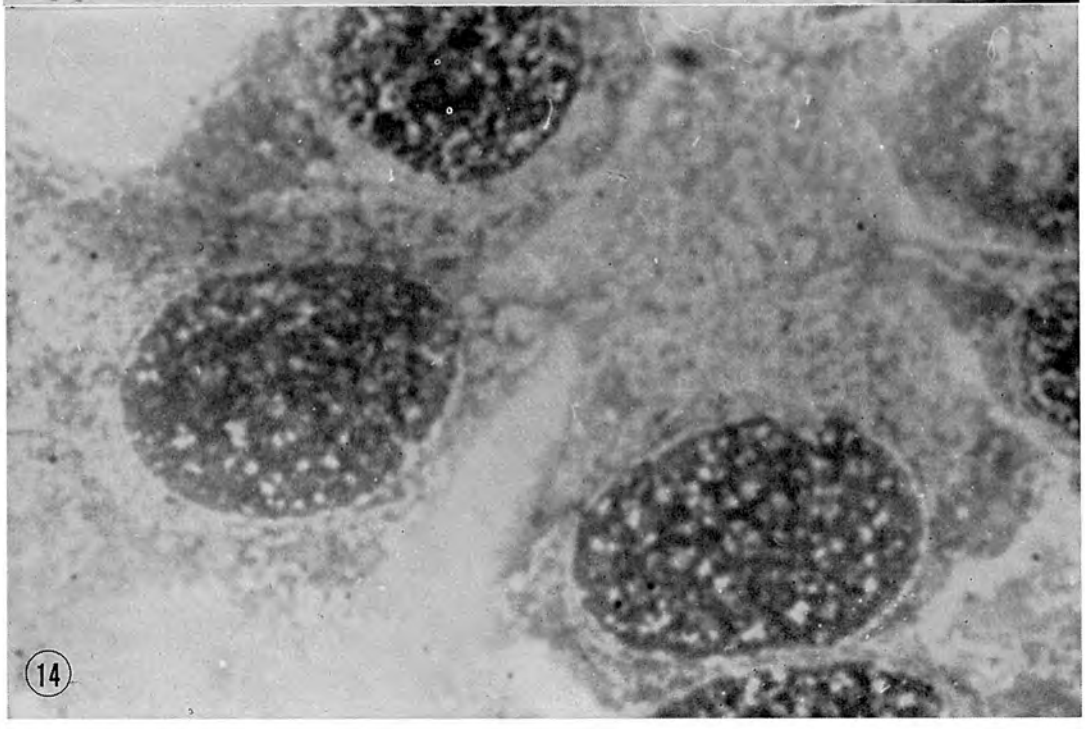
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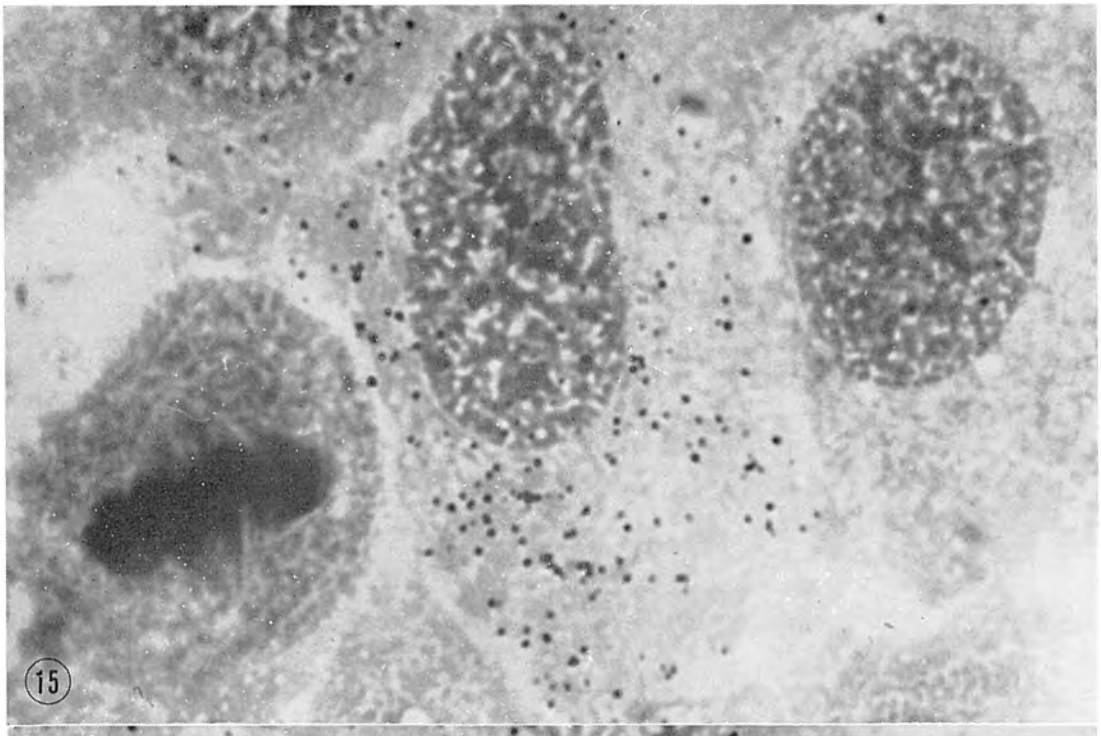
FIGURE 15, 16 Autoradiograms of ^{3}H-uridine incorporated into FL cells infected with chikungunya virus (10 hours after infection) in the presence of actinomycin S (1 μg/ml), stained with Giemsa solution. Cells with many silver grains in the cytoplasm were seen.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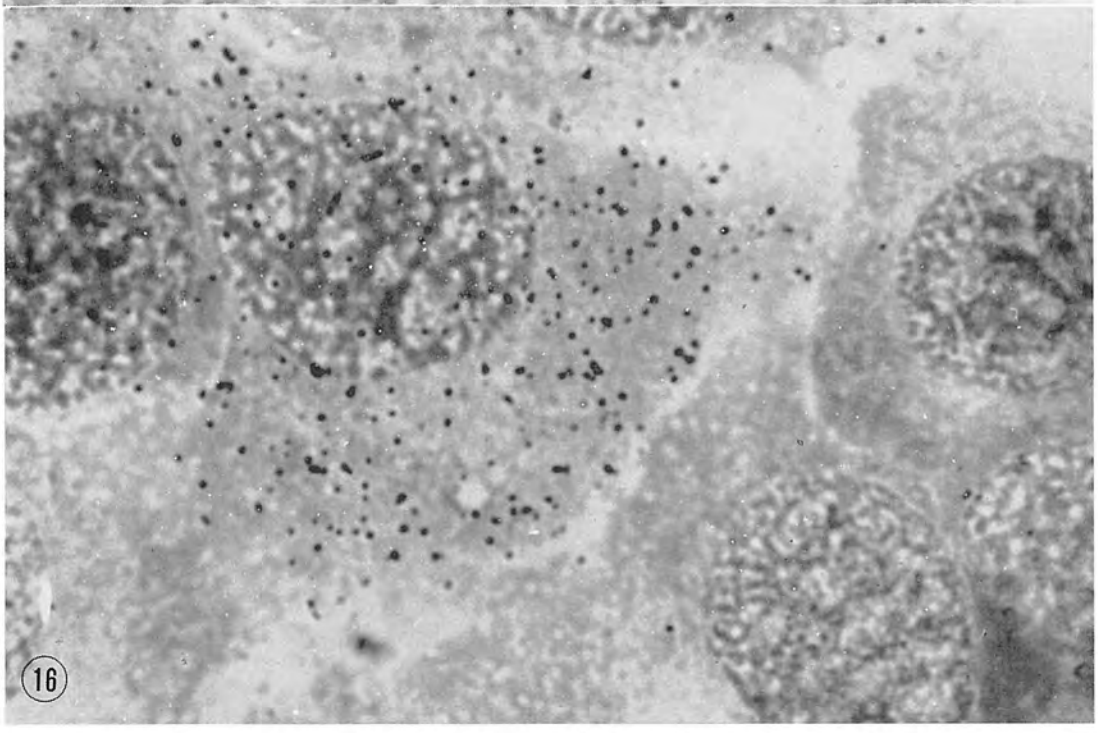
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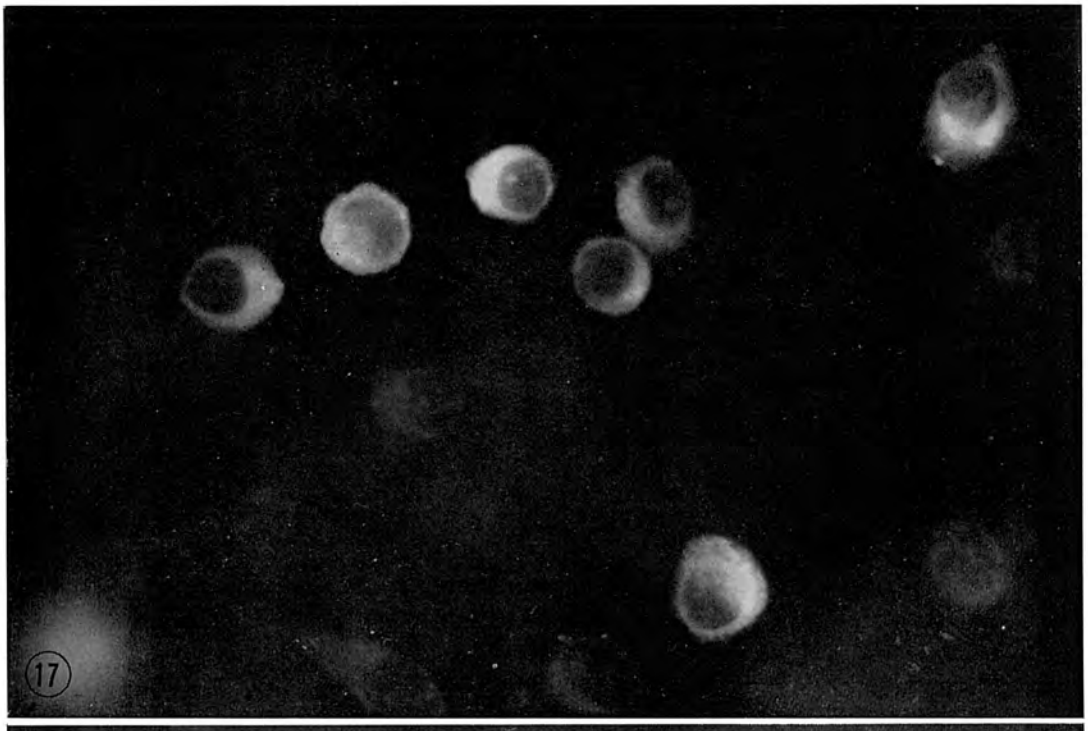
FIGURE 17, 18 FL cells infected with chikungunya virus in the presence of actinomycin S (1 μg/ml), stained with fluorescein iso-thiocyanate coupled with anti-chikungunya virus mouse γ-globulin. Fig. 17, 18, were cells taken 7 hours and 10 hours, respectively, after infection. Fluorescence was first found rather diffusely around the nuclei (Fig. 17) and then localized to certain perinuclear areas (Fig. 18).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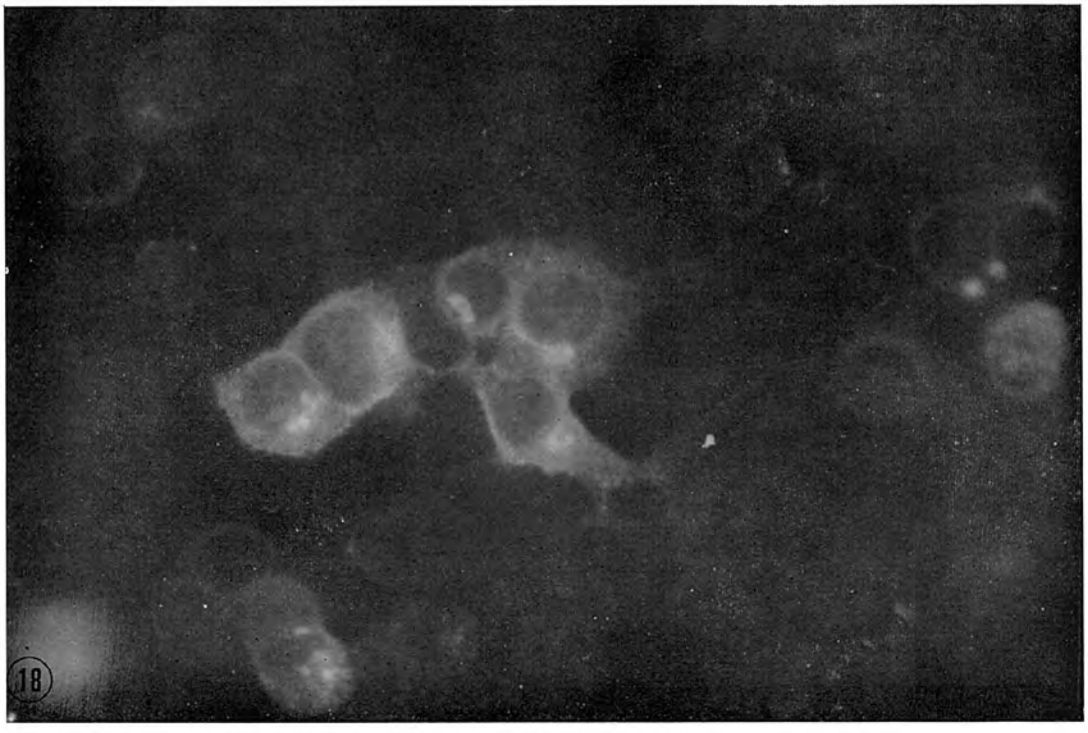
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