BIKEN JOURNAL Vol. 13, 353-364, 1970

VIRUS MULTIPLICATION IN CULTURE CELLS SYNCHRONIZED BY EXCESS THYMIDINE TREATMENT

I. EFFECT OF EXCESS THYMIDINE UPON GROWTH OF POXVIRUSES AND CHIKUNGUNYA VIRUS

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(Received on August 24, 1970)

SUMMARY The multiplications of cowpox virus (CPV) and Shope fibroma virus (SFV), as DNA viruses, and chikungunya virus (ChV), as an RNA virus, were studied in the presence of excess thymidine (E-Tdr). The concentrations of 2.5 mM and 8 mM E-Tdr, commonly used to produce cell-synchrony were adopted. Both CPV and SFV grew well in the presence of E-Tdr, but the maximum yield of these viruses was lower and the eclipse period was longer than those in the absence of E-Tdr. However, the presence of 8 mM E-Tdr had no effect on the multiplication of ChV. Neither increase in the eclipse period nor decrease in virus yield of CPV was observed in the presence of E-Tdr, when the growth curve of CPV was determined in FL cells which were resistant to E-Tdr. The mechanism of the inhibitory influence of E-Tdr on the multiplication of poxvirus was analyzed.

INTRODUCTION

There are many reports on the effects of E-Tdr on synthesis of nuclear DNA and cellular RNA (Morris and Fischer 1960; Xeros 1962; Bootsma et al. 1964; Galavazi et al. 1966). Thymidine is thought to act by inhibiting cellular DNA synthesis and is one of the agents used to produce synchronized cultures of mammalian cells. Kasten (1965) reported inhibition of nucleolar and cytoplasmic RNA synthesis by E-Tdr. Recent work by Studzinski (1968) showed that there is considerable DNA synthesis in the presence of E-Tdr and the rates of accumulation of RNA and protein are not affected. There are few reports on the effects of E-Tdr upon DNA- and RNA-virus replications. Groyon et al. (1967) showed that vaccinia virus replication was inhibited in the presence of 7.5 mM E-Tdr.

In this work, CPV and SFV, as DNA viruses, and ChV, as an RNA virus, were used to compare the effects of E-Tdr upon DNA virus replication with those upon RNA virus replication. For more precise analysis of the effects of E-Tdr itself upon virus multipli-

cation, FL cells which are resistant to E-Tdr were used.

MATERIALS AND METHODS

1. *Virus*

Cowpox virus (CPV) (LB red strain carrying the "A"v^- marker), Shope fibroma virus (SFV) (Madison strain) which was kindly supplied by Professor D. L. Walker of the University of Wisconsin, U.S.A. and chikungunya virus (ChV) (African strain) were used.

FL monolayer cultures were infected with CPV. After 24 hr, infected cell suspensions were made using a rubber-policeman and were sonicated (Kubota, 200 watts, 9 kc, 5 min). RK13 monolayer cultures were infected with SFV. Three days later, infected cell suspensions were made and sonicated in the same way as the suspension of CPV. After centrifugation at 3,000 rev/min for 10 min, both supernatant fluids were stocked at −70 C as virus materials. ChV stock material was prepared from the supernatant fluid of BHK21 monolayer cultures infected with ChV for 20 hr. After centrifugation of this supernatant fluid at 3,000 rev/min for 10 min, the supernatant fluid were stocked at −70 C.

2. *Preparation of cells*

Eagle's minimum essential medium (MEM) supplemented with 10% calf serum was used as growth medium (GM). FL cells were used to determine CPV- and SFV-growth curves, and BHK21 cells were used to determine the growth curve of ChV. FL cells resistant to E-Tdr (FL-r) were grown in GM containing 2.5 mM E-Tdr (Sigma Co.) for 4 months. Then these cells were passaged further for 4 weeks in the presence of 8 mM E-Tdr. FL-r cells were used for determination of the CPV-growth curve under conditions free from the inhibitory influence of E-Tdr on cellular DNA synthesis. All media contained penicillin (100 IU) and streptomycin (0.1 mg/ml).

3. *Virus growth curve*

Volumes of 0.2 ml of each stock virus solution were inoculated onto monolayer cell cultures in 50 ml prescription bottles. An adsorption period of 1 hr was used for CPV and ChV, and of 2 hr for SFV with redistribution every 15 min at 37 C. After the adsorption period, excess inoculum was removed by washing the infected cells three times with Hanks' solution. After washing, 5 ml of GM were added to each bottle. At appropriate times, two bottles were taken out for duplicate determinations. Cells infected with CPV or SFV were sonicated and centrifuged as described above. In cultures infected with ChV, the supernatant fluid from infected cells was centrifuged. Plaque assays were carried out.

4. *Virus titration*

CPV and ChV were assayed on BSC1 cultures with agar overlay, containing 4 ml of a 1:1 mixture of 2% agar and double concentration MEM with 2% calf serum, using four prescription bottles for each dilution. Plaques were stained with 0.02% neutral red 72 hr after infection and were visible to the naked eye. The multiplicity of infection (moi) was then determined as the ratio of plaque-forming units (PFU) to the number of cells present at the time of infection.

SFV was assayed on RK13 cultures with agar overlay. Agars were overlaid twice, 2 and 4 days after infection. Plaques were visible to the naked eye as foci without staining with neutral red 6 days after infection.

5. *Autoradiography*

BHK21 cells in Leighton tubes with cover glasses on the bottom were infected with ChV at moi 50. After two hr adsorption period, 2 ml of GM containing 3 μg/ml actinomycin-S(ActMS), and 8 mM E-Tdr were added to the Leighton tubes. Eight hr later the GM in the Leighton tubes was replaced by MEM containing 2 μc/ml ^{3}H-uridine (specific activity, 6 c/mM). FL cells and FL-r cells in Leighton tubes were labeled with 2 μc/ml of ^{3}H-Tdr (specific activity 5 c/mM). During exposure of cells to ^{3}H-uridine solution or ^{3}H-Tdr solution for 1 hr, Leighton tubes were kept in a water bath at 37 C. Then the cover slips were washed with Hanks' solution and cells were fixed with methanol. After treatment with 2% perchloric acid at 4 C for 40 min, dipping autoradiography was carried out with Sakura NR-M_2 nuclear emulsion. The exposure time was 3 days. Giemsa was employed for post-staining.

6. *Staining of inclusion bodies of poxvirus with Giemsa solution, the Feulgen reaction and fluorescent antibody*

Since a high concentration of thymidine makes it difficult to study cellular and viral DNA synthesis with labeled precursors (Studzinski 1968), Feulgen staining, Giemsa staining and immunofluorescent staining were used to demonstrate poxvirus in-

clusion bodies.

FL cells infected with CPV were stained with anti-CPV rabbit gamma-globulin conjugated with fluorescein-isothiocyanate. Fluorescein-conjugated antibody was prepared as described previously (Mantani et al. 1967). The percentages of fluorescent cells and of cells bearing inclusion bodies stained with Giemsa solution were calculated.

RESULTS

1. *Growth of CPV and SFV in the presence and absence of E-Tdr*

FL monolayer cells were inoculated with 0.2 ml of CPV (moi 1) after 20 hr treatment with 2.5 mM E-Tdr. After washing the infected cells with Hanks' solution, 5 ml of GM with 2.5 mM E-Tdr were again added to one series of bottles. Five ml of GM were added to another series of bottles. As shown in Fig. 1, CPV could grow well in the presence of E-Tdr, but the maximum virus yield was slightly lower and the eclipse period was longer than those in the absence of E-Tdr.

FL cells were inoculated with 0.2 ml of SFV (moi 4) after 20 hr treatment with 8 mM E-Tdr. After washing the infected cells, GM with 8 mM E-Tdr was again added to one series of bottles. Five ml of GM were added to the other series. As shown in Fig. 2, SFV grew in the presence of E-Tdr, but the maximum yield of SFV was lower and the eclipse period was longer than those in the absence of E-Tdr. As shown in Figs. 1 and 2, the eclipse period in the presence of E-Tdr seemed to be about 4 hr longer than in the absence of E-Tdr. There are three explanations of the extended eclipse period and the low yield of poxvirus in the presence of E-Tdr.

1) The growth of poxviruses which are sensitive to E-Tdr may be inhibited in the presence of E-Tdr, so that poxviruses which are resistant to E-Tdr grow selectively.

2) Adsorption of poxvirus may be inhibited by E-Tdr.

3) Prolongation of the eclipse period may be related with the inhibitory influence of E-Tdr on cellular DNA synthesis.

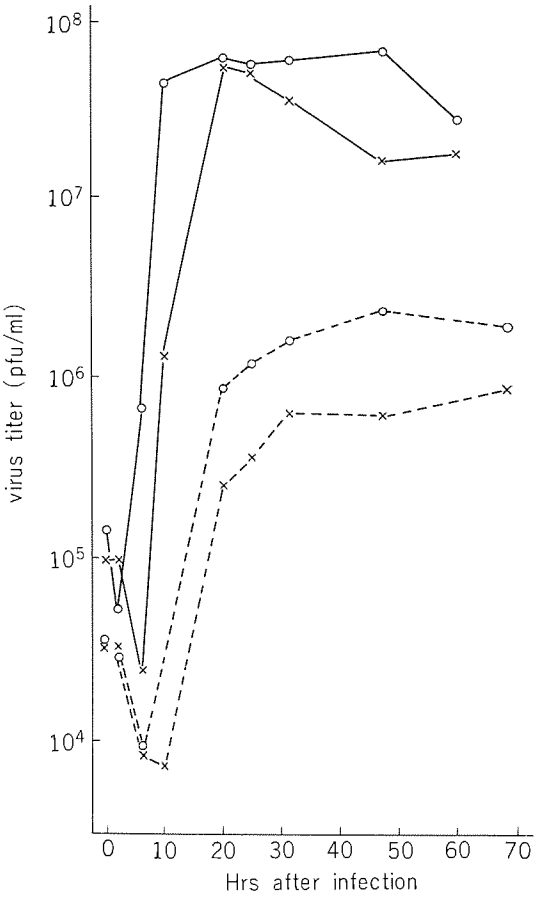

FIGURE 1. *Growth curves of CPV in the absence and presence of 2.5 mM E-Tdr after treatment with 2.5 mM E-Tdr for 20 hr.*
moi 1. in the absence of E-Tdr: ○———○ *cell phase,* ○------○ *medium phase. in the presence of 2.5 mM E-Tdr:* ×———× *cell phase,* ×------× *medium phase.*

2. *Percentages of fluorescent cells and inclusion-bearing cells infected with CPV*

To obtain morphological evidence at a cellular level for virus growth in the presence of E-Tdr, the immunofluorescent technique, the Feulgen reaction and Giemsa staining were employed, instead of autoradiography with ^{3}H-thymidine, since cellular and viral DNA synthesis could not be studied with labeled DNA precursors in the presence of E-Tdr.

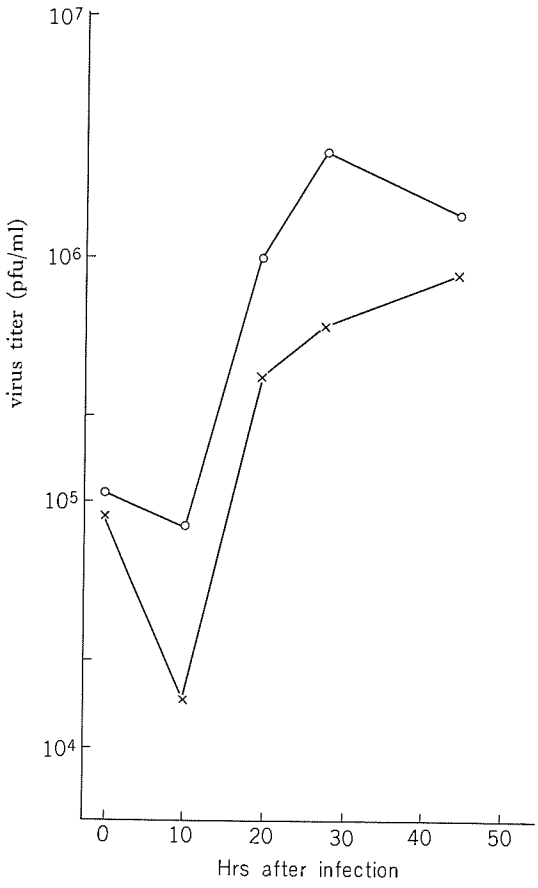

FIGURE 2. *Growth curves of SFV in the absence and presence of 8 mM E-Tdr after treatment with 8mM E-Tdr for 20 hr. moi 4.*
in the absence of E-Tdr: ○———○
in the presence of 8 mM E-Tdr: ×———×

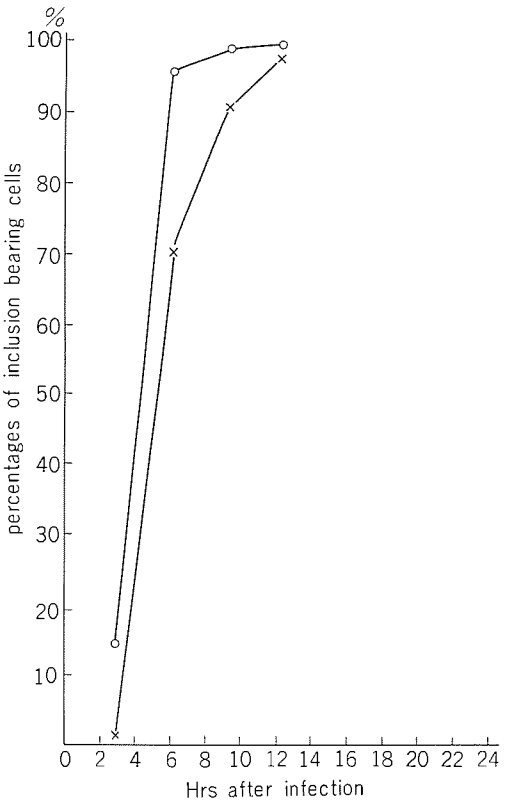

FIGURE 3. *Effect of E-Tdr upon inclusion body formation in FL cells infected with CPV.*
FL cells were infected with CPV (moi 50) after treatment with 2.5 mM E-Tdr for 20 hr. in the absence of E-Tdr after treatment: ○———○ *in the presence of 2.5 mM E-Tdr after treatment:* ×———×.

As reported by Kato et al. (1964), inclusions and the viral antigen of CPV were found exclusively in the cytoplasm of infected cells in the presence of E-Tdr (Fig. 12). As shown in Figs. 3 and 4, the number of inclusion-bearing cells and fluorescent cells increased rapidly with time after infection in the presence of E-Tdr. However, the increase started about 2 hr later in the presence of E-Tdr than in its absence. Fig. 13 shows inclusion bodies giving a positive Feulgen reaction in the cytoplasm of FL cells infected with SFV in the presence of E-Tdr. This sample was taken 24 hr after infection.

3. *Effect of E-Tdr on adsorption of CPV onto cells*

Four experimental groups (A, B, C, D) were prepared using the FL cell-CPV system, as shown in Fig. 5a. In group A, 4 mM E-Tdr was added to the FL culture 20 hr before virus infection, and the cells were exposed to E-Tdr throughout the experimental period. In group B, the cells were brought into contact with the E-Tdr before virus inoculation and during virus adsorption. In group C, infected

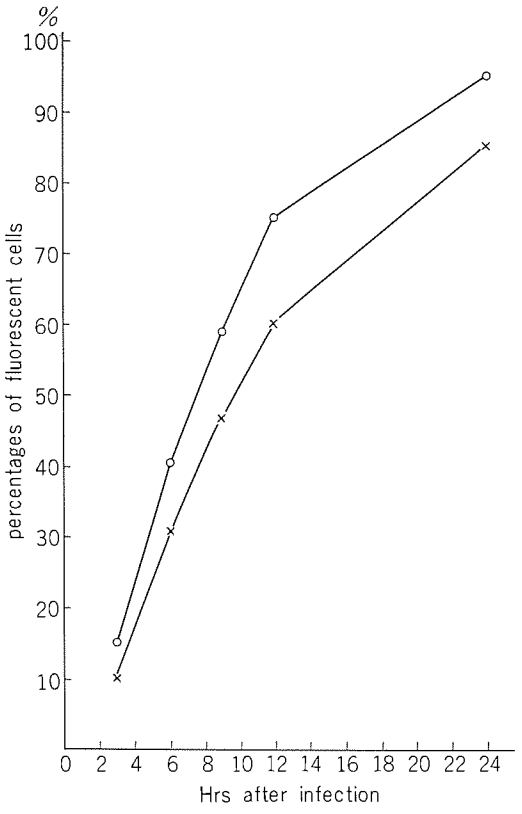

FIGURE 4. *Effect of E-Tdr upon fluorescent cell formation in FL cells infected with CPV. FL cells were infected with CPV (moi 8) after treatment with 8 mM E-Tdr for 20 hr.*
in the absence of E-Tdr after treatment: ○———○
in the presence of 8 mM E-Tdr after treatment: ×———×.

cultures were not exposed to E-Tdr and served as controls. In group D, E-Tdr was added to the culture during virus adsorption. The CPV growth curves of these groups were compared (Fig. 5b). The results are summarized as follows.

1) Virus growth in group B was identical with that in group D. Thus virus growth was not influenced by the presence of E-Tdr before virus inoculation.

2) Virus growth in group C was identical with that in group D, so the presence of E-Tdr during virus adsorption did not influence virus growth.

3. Virus growth was lowest in group A. Thus the presence of E-Tdr after the adsorption period influences virus growth.

4. *Growth of CPV in FL cells which were resistant to E-Tdr*

To avoid the complication of the inhibitory influence of E-Tdr on nuclear DNA synthesis during studies on virus growth, FL cells which were resistant to E-Tdr (FL-r cells) were used.

The growth curves of FL-r cells and FL cells, serving as controls, in the presence and absence of 2.5 mM E-Tdr were examined by estimation of the cell numbers in the culture bottles (Fig. 6). FL-r cells grown in the presence of 2.5 mM E-Tdr and FL cells grown in GM only were removed by EDTA treatment and cell suspensions were diluted appropriately with GM and 2.5 mM E-Tdr in GM. These four kinds of cell suspensions were dispersed to small bottles and incubated at 37 C. One day later the medium was replaced by fresh medium and maintained at 37 C. At appropriate times, 3 bottles for each series were taken for counting cells. The medium in these bottles was replaced by EDTA solution and after 10 min incubation at 37 C the cells were suspended and counted in a haemocytometer. The points indicate the average number of cells in the 3 bottles.

FL-r cells grew at the same rate in the presence and absence of E-Tdr as FL cells in the absence of E-Tdr, while FL cells could not grow in the presence of E-Tdr.

The effects of E-Tdr upon cellular DNA synthesis of FL cells and FL-r cells were compared using autoradiography with ^{3}H-thymidine. As shown in Table 1 and Fig. 7, 4 experimental groups (A, B, C, D) were prepared.

A) FL cells were treated with 2.5 mM E-Tdr for 20 hr. Then 6 hr after removal of E-Tdr, they were pulse labeled with 2 μc/ml ^{3}H-Tdr for 1 hr at 37 C. The autoradiogram showed that 87% of these cells were in the S phase. These cells were well synchronized by E-Tdr treatment.

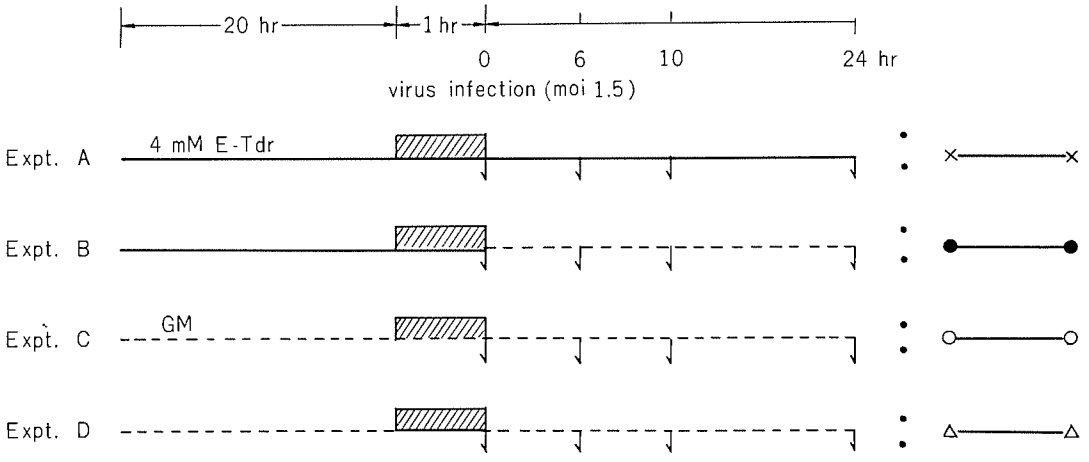

FIGURE 5a. *Scheme of experiment on E-Tdr treatment and virus infection in Fig. 5b.*

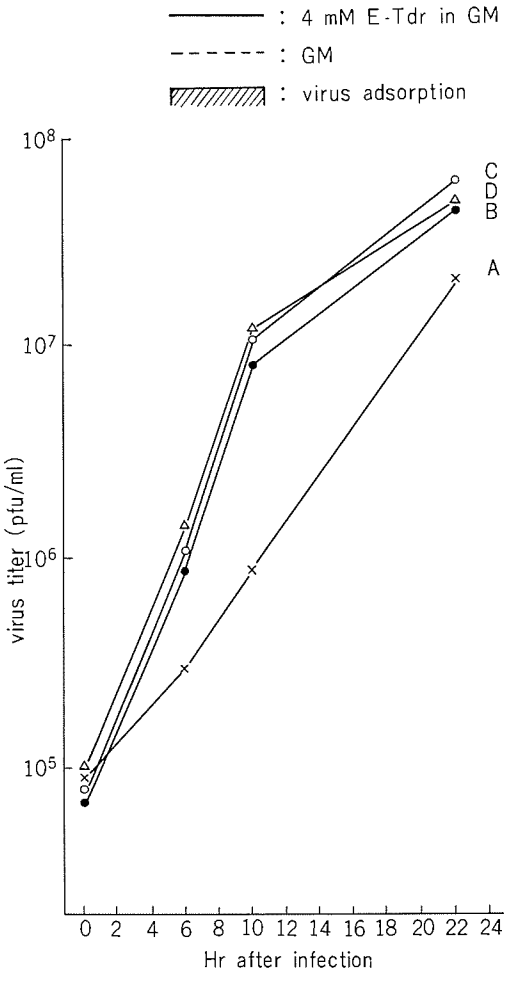

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FIGURE 5b. *Effect of E-Tdr during the adsorption period upon CPV growth.*

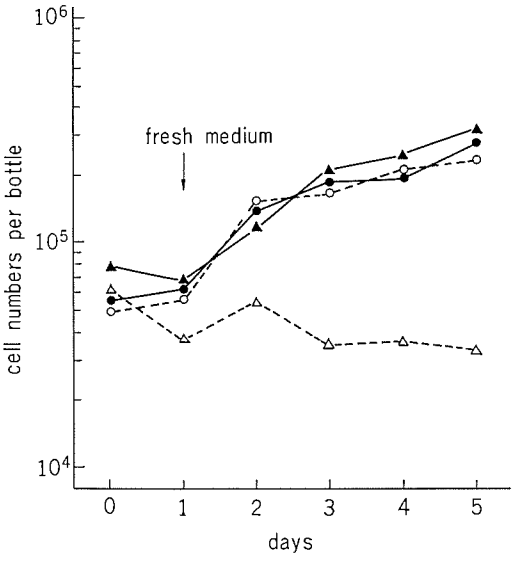


FIGURE 6. *Growth of FL and FL-r cells in the presence and absence of 2.5 mM E-Tdr.*

●——● *FL-r cell in GM*
○------○ *FL-r cell in GM with E-Tdr (2.5 mM)*
▲——▲ *FL cell in GM*
△------△ *FL cell in GM with E-Tdr (2.5 mM)*

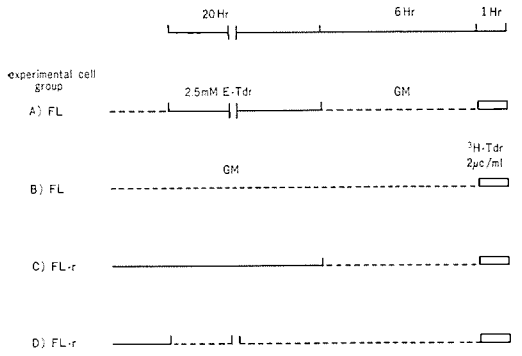


FIGURE 7. *Scheme of experiment on E-Tdr treatment of FL-r cells in Table 1*

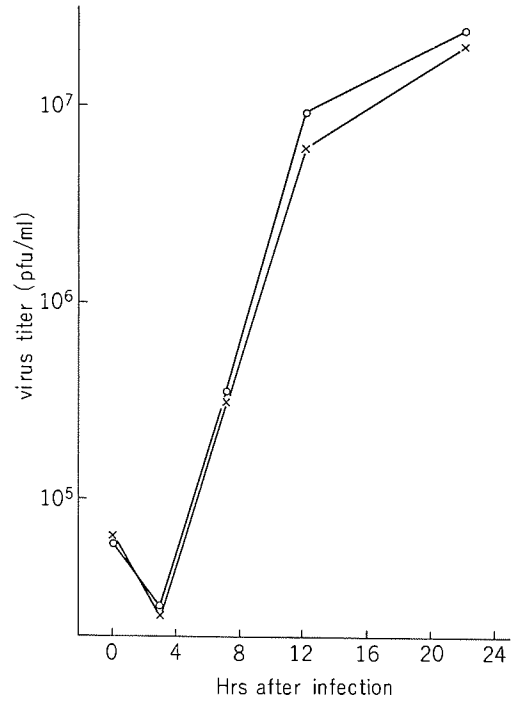


FIGURE 8. *Growth curves of CPV in FL-r cells resistant to 2.5 mM E-Tdr.*

FL-r cells were grown in GM containing 2.5 mM E-Tdr for 4 months. These FL-r cells were infected with CPV (moi 2). After the adsorption period, the same concentration of E-Tdr in GM was added (×———×), *or GM only was added* (○———○).

TABLE 1. *Percentages of FL and FL-r cells in S phase of the cell cycle*

cell	percentages of labeled cells
A) FL	87 %
B) FL	23 %
C) FL-r	26 %
D) FL-r	25 %

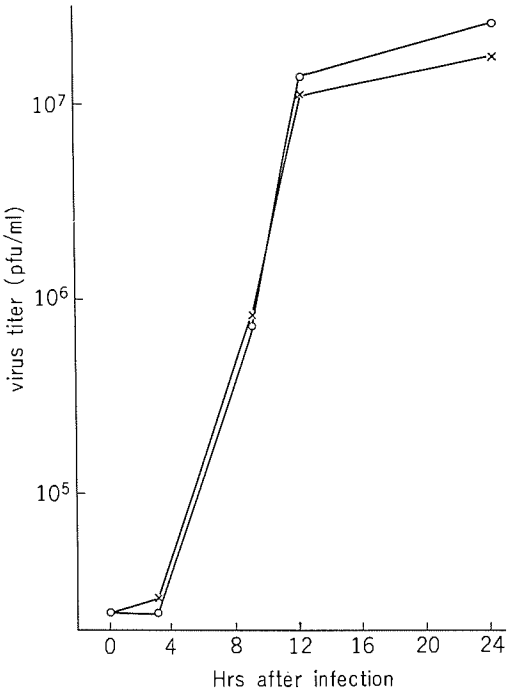


FIGURE 9. *Growth curves of CPV in FL-r cells resistant to 8 mM E-Tdr. FL-r cells were grown in GM containing 8 mM E-Tdr for 4 weeks after 4 months in 2.5 mM E-Tdr. These FL-r cells were infected with CPV (moi 1). After adsorption period, 8 mM E-Tdr in GM* (×———×), *or GM only was added* (○———○).

B) FL cells in randomly growing cultures were pulse labeled. Twenty-three per cent of the cells were in the S phase.

C) FL-r cells grown in the presence of E-Tdr were pulse labeled with ^{3}H-Tdr for 1 hr,

6 hr after removal of E-Tdr. Twenty-six per cent of the cells were in the S phase.

D) FL-r cells grown in the presence of E-Tdr were pulse labeled 26 hr after removal of E-Tdr. Twenty-five per cent of the cells were in the S phase.

These results indicate that FL-r cells were not synchronized by E-Tdr and treatment of FL-r cells with E-Tdr had no effect on the percentage of cells in the S phase.

The growth curves of CPV in FL-r cells in the presence and absence of 2.5 mM E-Tdr were compared. As shown in Fig. 8, the two curves are nearly identical and E-Tdr caused neither lag, nor decrease of viral yield. Similar results were obtained using 8 mM E-Tdr (Fig. 9).

From these results two possibilities may be considered. One is that the prolongation of the eclipse period and the decrease of viral yield observed in Figs. 1 and 2 might be related with the inhibition of cellular DNA synthesis caused by E-Tdr. The other is that growth of CPV sensitive to E-Tdr may be inhibited and some CPV which is resistant to E-Tdr may multiply selectively in the presence of E-Tdr.

5. *Harvest of CPV propagated in the presence of E-Tdr and growth of CPV in FL cells in the presence and absence of E-Tdr*

To determine whether the CPV harvested from FL-r cells in the presence of E-Tdr was resistant to E-Tdr, CPV was harvested from FL-r cell cultures 24 hr after infection with CPV in the presence of 8 mM E-Tdr which was designated CPV (E-Tdr). Two series of FL cells were grown for 15 hr in the presence of E-Tdr and both series were infected with CPV (E-Tdr) of moi 1.5. One series of infected FL cells was then cultured in the presence of E-Tdr, while the other was cultured in the absence of E-Tdr. As shown in Fig. 10, virus titers of CPV (E-Tdr) growing in the presence of E-Tdr increased after a lag period and reached a maximum titer at 24 hr which was a little lower than that of CPV (E-Tdr) grown in the absence of E-Tdr. The difference between these two growth curves of CPV (E-Tdr) is nearly identical tc the difference between those with CPV shown in Figs. 1 and 2. Thus growth of CPV (E-Tdr) was also influenced by the presence of E-Tdr. These results suggest that the prolongation of the eclipse period and the decrease of viral yield are not due to the inhibition of growth of CPV

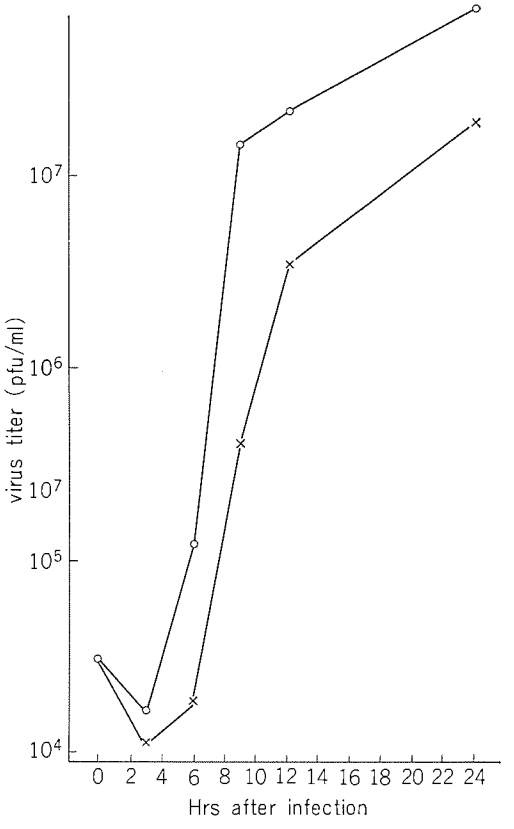


FIGURE 10. *Growth curves of CPV harvested from FL-r cells grown in the presence of 8 mM E-Tdr.*

FL cells were treated with 8 mM E-Tdr for 15 hr. The growth curves of CPV were determined in FL cells. FL cells were infected with CPV harvested from FL-r cells grown in the presence of 8 mM E-Tdr 24 hr after infection (moi 1.5). The growth curves were similar to those in Fig. 1 or Fig 2.

GM was added after infection (○———○).

GM with 8 mM E-Tdr was added after infection (×———×).

which is sensitive to E-Tdr and that the difference may be related with conditions in the host cells in which DNA synthesis was inhibited by E-Tdr.

6. *Growth of ChV in the presence of E-Tdr*

Four series of BHK21 cell cultures were infected with ChV of moi 2. After adsorption of virus for 1 hr, the 1st series of infected cells was kept in the presence of 8 mM E-Tdr, the 2nd in the presence of 3 μg/ml ActMS and the 3rd in the presence of both 8 mM E-Tdr and 3μg/ml ActMS. The 4th series of cells was kept in GM only. ActMS inhibits cellular RNA synthesis, but not the RNA synthesis of

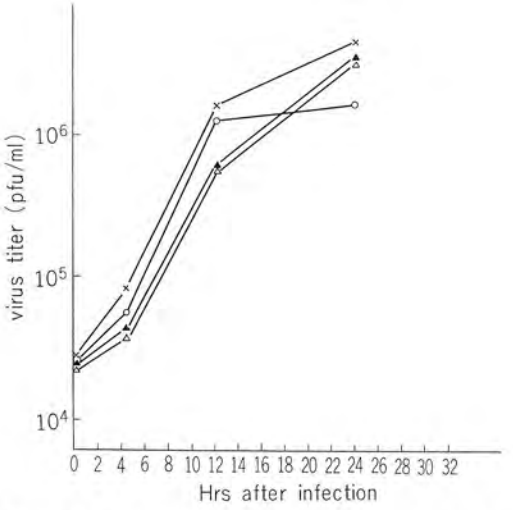
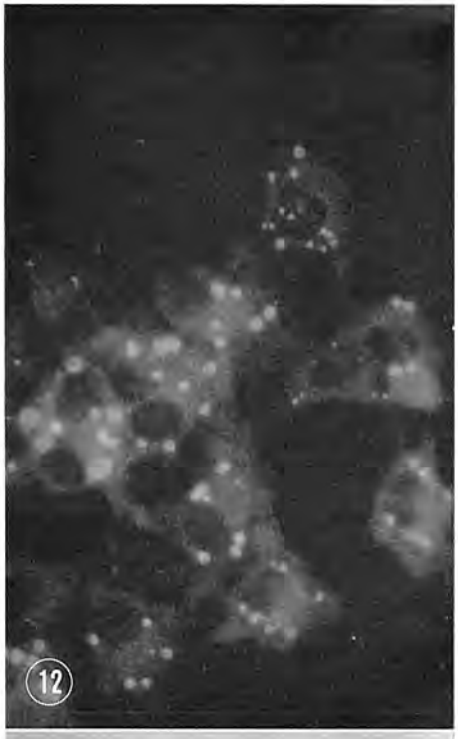


FIGURE 11. *Growth curve of ChV in the presence of 8 mM E-Tdr BHK21 monolayer cells were infected with ChV (moi 2).*

After adsorption period, 5 ml of GM with the following additions were added. GM only: ●——●, 8 mM E-Tdr: ▲——▲, 3 ug/ml ActMS: △——△, 8 mM E-Tdr and 3 ug/ml ActMS: ×——×.



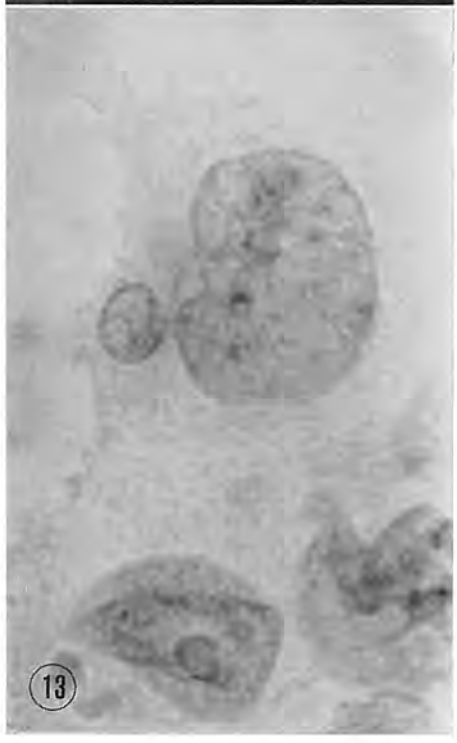
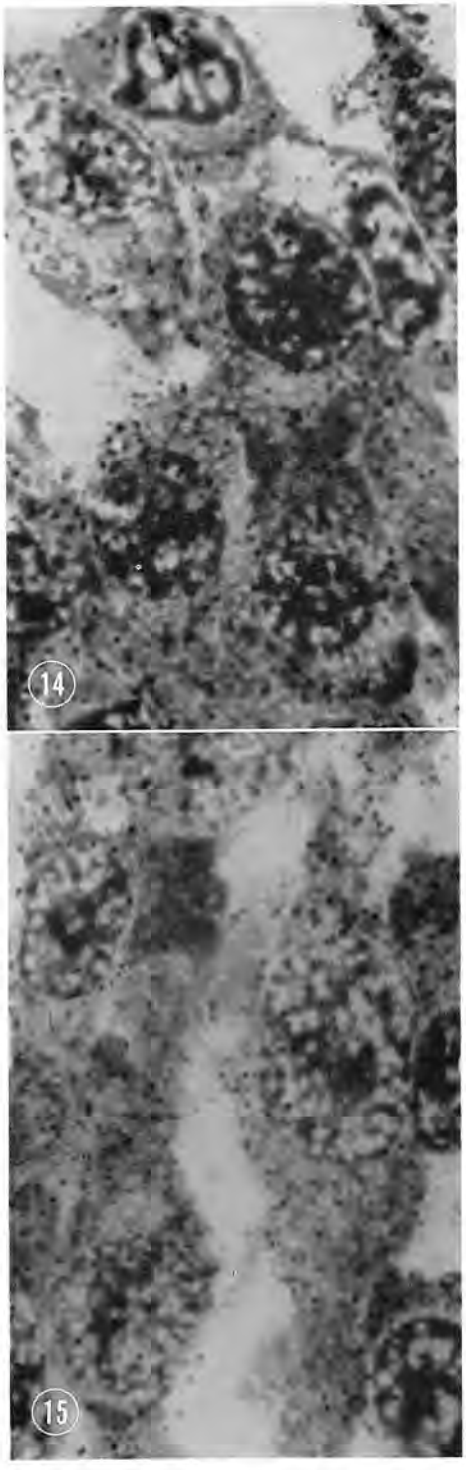


FIGURE 12. *FL cells infected with CPV in the presence of 8 mM E-Tdr, stained with fluorescein-isothiocyanate coupled with anti-CPV rabbit gamma-globulin. Infected cells were taken 24 hr after infection. Fluorescence was seen in the cytoplasm.*

FIGURE 13. *FL cells infected with SFV in the presence of 8 mM E-Tdr, stained by the Feulgen reaction. Inclusion bodies of SFV were seen in the cytoplasm.*



ChV (Mantani et al. 1967). ActMS was used to see the effect of ActMS together with E-Tdr upon virus growth. The growth curves of ChV in these four series of infected cells were compared. As shown in Fig. 11, there was no essential difference between them.

To study the morphology of growth of ChV at a cellular level in the presence of E-Tdr, autoradiography of ^{3}H-uridine was carried out as reported by Mantani et al. (1967). Nine hr after infection, a part of each series of cell cultures was pulse labeled with 2 μc/ml of ^{3}H-uridine for 1 hr at 37 C. Then autoradiography was carried out. As shown in Figs. 14 and 15, RNA synthesis of ChV was as active in the presence of both ActMS and E-Tdr as in the presence of only ActMS.

These results indicate that E-Tdr does not affect growth of ChV.

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FIGURE 14. *Autoradiogram of ^{3}H-uridine incorporated into FL cells 10 hr after infection with chikungunya virus in the presence of actinomycin S (3 μg/ml). Stained with Giemsa solution. Cells with many silver grains in the cytoplasm were seen.*

FIGURE 15. *Autoradiogram of ^{3}H-uridine incorporated into FL cells 10 hr after infection with chikungunya virus in the presence of E-Tdr (8 mM) and actinomycin S (3 μg/ml). Cells with many silver grains in the cytoplasm were seen.*

DISCUSSION

Studies on the relations between synchronized mammalian cells and virus growth have provided valuable information on the nucleocytoplasmic interaction in virus infection (Groyon and Kniazeff, 1967, Mantani et al. 1968). The most common method used to synchronize mammalian cells is E-Tdr treatment (Xeros 1962; Puck, 1964; Bootsma et al. 1964). This method is simple and highly efficient in synchronizing a large population of cells.

With regard to the effects of E-Tdr upon cellular metabolism, Morris and Fischer (1963) thought that E-Tdr acted by inhibiting cellular DNA synthesis and cells which were in the G_2 phase, the M phase or the G_1 phase should proceed normally through these phases of the cell cycle to the beginning of the S phase. Studzinski and Lambert (1968) showed that the concentration of E-Tdr commonly employed to produce synchronized cells did not arrest cells at the G_1-S boundary, but allowed slow progress through the S phase in respect to DNA synthesis, and near normal progress toward G_2 as regards RNA and protein accumulation and cell enlargement.

Before these experiments, we expected that viral DNA synthesis of poxviruses would also be inhibited by E-Tdr and synchronized by removal of E-Tdr. However, we found that CPV and SFV multiplied well in FL cells in the presence of E-Tdr. Thus viral DNA synthesis is not inhibited so much by E-Tdr as cellular DNA synthesis. Various mechanisms of inhibition of cellular DNA synthesis by E-Tdr have been reported (Cleaver, 1967). Further investigations are required on the different effects of E-Tdr on DNA synthesis in poxviruses and cells. It is known that growth of poxviruses is possible in vivo in cells in which nuclear DNA synthesis has been suppressed by some regulatory mechanism (Kato et al. 1963; Hara et al. 1964). However, these cells have the potential ability to induce nuclear DNA synthesis. Some RNA viruses, such as paramyxoviruses and polio virus have been reported to grow in anucleated fragments of cells (Cheyne and White 1969; Crocker et al. 1964). Dissociation between cellular and viral DNA synthesis induced by mitomycin C was reported (Magee et al. 1962; Kato et al. 1961). Our results provide another example of dissociation between cellular and viral DNA synthesis. However, the problem of whether viral DNA synthesis of poxviruses can occur in cells which cannot synthesize cellular DNA or in anucleated cells remains unsolved.

Poxvirus can multiply in FL cells in the presence of E-Tdr, but it was found that their growth was delayed and slightly suppressed by E-Tdr. This is in contrast to the growth of ChV, an RNA virus, which is not influenced by E-Tdr. It seems that growth of poxvirus in cells is rather difficult in the presence of E-Tdr. Our experiments show that growth of poxvirus is not directly influenced by E-Tdr, since virus growth in FL-r cells was not influenced by E-Tdr. And the possibility that virus sensitive to E-Tdr caused the inhibitory influence on virus growth was eliminated, since the growth of virus grown in the presence of E-Tdr is still influenced by E-Tdr. Thus growth of poxvirus was influenced by conditions in the host cells caused by E-Tdr. Whether the conditions are related with cellular DNA metabolism remains unknown.

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