Induction of Cellular DNA Synthesis and Increased Mitotic Activity in Syrian Hamster Embryo Cells Abortively Infected with Human Cytomegalovirus

By T. ALBRECHT,* M. NACHTIGAL,† S. C. ST JEOR AND F. RAPP‡

The Department of Microbiology, The Milton S. Hershey Medical Center, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033, U.S.A.

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SUMMARY

The effect of human cytomegalovirus (CMV) on cell DNA synthesis and mitotic activity in hamster embryo fibroblasts was examined. The results indicated that CMV infected cells had increased rates of cell DNA replication and mitotic activity. Detection of the effect of CMV on these two parameters necessitated arrest of cells prior to infection with low serum concentrations. This lowered the background levels of DNA synthesis and cell division so that the effect of virus infection could be detected. The data indicate that cells arrested prior to infection demonstrate increased susceptibility to virus infection. It was also observed that the effect of CMV on both DNA replication and mitotic activity could be enhanced by irradiation with ultraviolet light of the virus prior to infection.

INTRODUCTION

It has been well established that cytomegaloviruses (CMV) abortively infect a number of cell types (Kim & Carp, 1971; Fioretti et al. 1973; Waner & Weller, 1974). In a recent study involving transformation of hamster embryo fibroblast (HEF) cells by human CMV, it was observed that CMV is capable of both cell transformation and the induction of cytopathology in HEF cells (Albrecht & Rapp, 1973). Other evidence obtained in this same investigation indicated that human CMV would not replicate in HEF cells. Considering the ability of CMV to transform HEF cells, as well as the reported stimulatory effect of CMV on cell DNA synthesis (St Jeor & Rapp, 1973b, St Jeor et al. 1974), we report the results of our investigations on the apparent abortive infection of CMV in HEF cells.

METHODS

Cells. The methods used for preparation of HEF and human embryonic lung (HEL) cells were, in general, those described previously (St Jeor & Rapp, 1973b; Albrecht & Rapp, 1973). Eagle's medium supplemented with 10% foetal calf serum, 0.075% sodium
bicarbonate, 100 units of penicillin and 100 µg of streptomycin/ml was used for cell propagation. Secondary cultures of HEF and HEL cells between passages 5 to 15 were used in these studies.

**Arrest of cell growth.** For experiments requiring cells with minimal DNA synthesis, primary HEF were trypsinized and passed at a 1:3 ratio (based on surface area) to glass prescription bottles or glass coverslips using Eagle's medium supplemented with 0.5% foetal calf serum. Seventy-two to 96 h later these cells had formed about a 90% confluent monolayer with few, if any, mitotic figures. At this time the medium was poured off and reserved, and the cells were either virus or sham inoculated. After 1 h adsorption the inocula were removed and the cells washed twice with tris-buffered saline. The reserved medium was then added back to the cell cultures.

HEF cells were arrested in the following manner to examine the effect of CMV on the mitotic index: the cells were dissociated with trypsin and passed at a 1:3 ratio (based on surface area) to 35 mm plastic tissue culture dishes containing 18 mm glass coverslips. Eagle's medium supplemented with 5% foetal calf serum was employed for these experiments. Forty-eight h after seeding, the cells had formed a confluent monolayer. The growth medium was then removed and reserved. The cells were sham or virus inoculated and after 1 h adsorption, the inocula were decanted. The cells were washed twice with tris-buffered saline and a portion (1.5 ml) of the reserved growth medium was restored to each dish.

**Virus and infectivity assay.** The C-87 strain (Benyesh-Melnick, Rosenberg & Watson, 1964) of human CMV was used exclusively throughout these studies. The methods used for virus propagation and assay have been published previously (Albrecht & Rapp, 1973).

**Ultraviolet (u.v.) irradiation.** Virus stocks were irradiated in 1.5 ml portions with a GE-G8T5 bulb at a dose rate of either 40 or 80 ergs/s/mm². The dose rate was determined with a Blak-ray ultraviolet meter (UltraViolet Products, Inc., San Gabriel, California).

**Fluorescent antibody assay.** The indirect immunofluorescent (IF) assay was used to detect CMV antigens. Human convalescent sera reactive with either CMV [reactive with varicella-zoster (VZ), non-reactive with herpes simplex virus (HSV)] or with VZ (non-reactive with either CMV or HSV) were used in these tests. Rabbit anti-human gamma globulin (H and L chain specific) labelled with fluorescein isothiocyanate was purchased from Hyland, Costa Mesa, California. The conditions employed for these assays have been reported (Albrecht & Rapp, 1973).

**Virus growth kinetics.** Confluent monolayers of cells were prepared in 25 cm² plastic tissue culture flasks. After growth medium was removed, the monolayers were washed once with tris-buffered saline. Virus (0.5 ml) diluted to obtain the stated multiplicity of infection was adsorbed to the cells for 1 h at 37 °C with occasional rotation. The monolayers were then washed three times with tris-buffered saline and maintenance medium (Eagle's supplemented with 2% foetal calf serum) was added to each flask (5 ml/flask). All flasks were incubated at 37 °C. At appropriate times, individual flasks were removed, quick frozen in ethanol-dry ice, and stored at 76 °C. The cells were twice frozen and thawed, sonicated for 30 s with a Bronson model D-50 bath sonicator, and the debris pelleted by centrifuging at 200 g for 10 min. The infectivity of the various samples was assayed as previously described.

**Isotopic labelling and DNA analysis.** The methods used for labelling and analysis of DNA are described in earlier reports (Crouch & Rapp, 1972). Isotope concentrations of 10 µCi/ml of [3H]-methyl thymidine ([3H]-TdR, sp. act. 17 Ci/mmol) and 20 µCi/ml of [8-3H]-hypoxanthine (sp. act. 19.8 Ci/mmol) were used. All isotopes were obtained from Schwarz-Mann. Samples labelled with [3H]-hypoxanthine after DNA extraction were treated with 0.3 M-KOH at 37 °C to hydrolyse labelled RNA.
Abortive infection with human CMV

Fig. 1. Photomicrograph of non-arrested hamster embryo fibroblasts, 24 h p.i. with human cytomegalovirus (3 p.f.u./cell). Stained with haematoxylin and eosin.

**Measurement of the mitotic index.** The mitotic index of infected and control cultures was determined using partially arrested cells (previously described). The cells were infected with u.v.-irradiated or non-irradiated virus. Control cultures were inoculated with medium containing the same percentage of serum as the virus-infected cell lysate. After a 1 h adsorption, the inoculum was removed and the monolayers were washed three times with tris-buffered saline. The reserved culture fluids were then dispensed to each culture dish. At various intervals post infection (p.i.), paired coverslips of infected and control cells were removed and washed twice in tris-buffered saline. The cells were fixed with absolute methanol and stained with 2 % Giemsa solution. Randomly chosen microscopic fields were examined with oil immersion objective and the number of resting cells and mitotic figures was recorded. The mitotic index was expressed as the ratio between cells in different stages of mitosis and cells in interphase.

**RESULTS**

*Cytopathology in arrested and non-arrested HEF cells*

Earlier studies reported from this laboratory indicated that when cells were arrested by either low serum concentrations or 5-iodo-2-deoxyuridine (IUdR) there was an increase in cellular DNA synthesis when CMV infected cells were compared to control cells (St Jeor et al. 1974). A preliminary study was initiated to determine what effect the arrest of HEF cells with low serum concentrations would have on the development of virus cytopathology (c.p.e.). HEF cells, either arrested as described in the Methods section or non-arrested, were infected with CMV at an input multiplicity of 3 p.f.u./cell. The cells were then examined
for the development of both c.p.e. and CMV antigens. At 24 h p.i., c.p.e. was observed in only about 1% of the infected non-arrested cells. The c.p.e. consisted of a rather homogeneous cytoplasmic inclusion and an eccentric nucleus (Fig. 1). A few small rounded cells were also observed at this time. The number of cells with c.p.e. increased only slightly 24 to 48 h after infection.

In contrast, the c.p.e. observed in infected, arrested cell cultures was extensive 24 h after infection. Greater than 40% of the cells were lost from the surface of the coverslips. This was more pronounced than loss (10 to 15%) in arrested, non-infected cells. About 40% of those cells remaining had cytoplasmic inclusions and an eccentric nucleus (Fig. 2). Some of the cells with inclusions were round, but most were fibroblastoid and many of the cells (about 80%) were refractile. By 48 h p.i., less than 10% of the infected arrested cells remained attached to the glass and extensive c.p.e. was evident in nearly all of these cells.

Detection of CMV antigens in arrested and non-arrested HEF

Preparations of infected and control arrested and non-arrested HEF cells were examined for the presence of CMV antigens using the indirect IF test. When human convalescent serum reactive with CMV was used in this test with infected non-arrested cells, a diffuse dim cytoplasmic fluorescence was observed in about 1% of the cells. However, when infected arrested cells were examined, a very bright fluorescence was observed in about 90% of the cells (Fig. 3). The nucleus, cytoplasmic inclusions, and cytoplasm all fluoresced, although not with equal intensity. Fluorescence was not observed with control arrested and non-
Abortive infection with human CMV

Fig. 3. Fluorescence photomicrograph of hamster embryo fibroblasts arrested by serum starvation, 24 h p.i. with human cytomegalovirus (3 p.f.u./cell). These cells were fixed with cold acetone for 10 min and stained with human convalescent serum reactive with cytomegalovirus and with fluorescein isothiocyanate conjugated rabbit anti-human gamma globulin.

Table 1. Assay for cytomegalovirus antigens in arrested and non-arrested hamster embryo fibroblasts*

<table>
<thead>
<tr>
<th>Condition of cells</th>
<th>Virus inoculated</th>
<th>Specificity of antisera†</th>
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<tbody>
<tr>
<td>Arrested</td>
<td>+</td>
<td>CMV‡ + (90 %)‖ VZ§ —</td>
</tr>
<tr>
<td>Non-arrested</td>
<td>+</td>
<td>—</td>
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* Cells were fixed in cold acetone for 10 min.
† Human convalescent.
‡ Reactive with CMV and VZ but not HSV.
§ Reactive with VZ but not HSV or CMV.
‖ Percentage of cells positive in the IF test.

arrested HEF cells and CMV reactive serum, nor with infected and control, arrested or non-arrested HEF cells and VZ reactive serum (Table 1).

Pre-treatment of HEF with 100 µg/ml of IUdR (St Jeor & Rapp, 1973a) yielded results similar to those observed in serum arrested cells. However, the cytopathology was not as extensive and the fluorescence with anti-CMV serum was not as bright as that observed in serum arrested cells.

Adsorption and rate of inactivation of CMV in arrested and non-arrested cells

A possible explanation for the difference observed between arrested and non-arrested cells infected with CMV might be that the virus either adsorbed more rapidly to the arrested
cells or was inactivated more rapidly in the non-arrested cells. To test this hypothesis the following study was initiated. Cultures of arrested and non-arrested HEF cells were prepared in 1 oz glass prescription bottles and inoculated with CMV at an input multiplicity of 2 p.f.u./cell. Following a 1 h adsorption period, the infected cells were washed 3 times with tris-buffered saline. The amount of virus infectivity present in the washes from the cells was determined by a plaque assay to determine differences in the efficiency of virus adsorption in arrested and non-arrested cells. The results of this study indicated that > 90% of the virus in both the arrested and non-arrested cells remained adsorbed to the cells throughout the washings. The rate of inactivation of the virus in arrested and non-arrested cells was then determined by assaying cultures for infectious virus at various periods following infection.

The inoculum decay curves (Fig. 4) in arrested and non-arrested cells were similar for about 0 to 48 h following infection. Thereafter, the infectivity was lost more rapidly from the arrested cell cultures than from the non-arrested cell cultures. At no time was an increase in virus infectivity above input levels observed.

**Effect of CMV on HEF cell DNA synthesis**

Earlier reports from this laboratory indicated that CMV induced increased cell DNA synthesis in both productive and abortive infections in a variety of human cell types (St Jeor & Rapp, 1973a, b; St Jeor et al. 1974). In order to determine the effect of CMV on DNA synthesis in HEF cells, the following study was initiated. HEF cells, either arrested
Abortive infection with human CMV

Fig. 5. Kinetics of cellular DNA synthesis in non-arrested hamster embryo fibroblasts. Cell cultures were inoculated with non-irradiated human cytomegalovirus (■—■), ultraviolet irradiated (24000 ergs/mm²) cytomegalovirus (▲—▲), or with maintenance medium (□—□) containing the same concentration of foetal calf serum as the virus inoculum. The cell cultures were pulse-labelled with [3H]-TdR as described. Each datum point represents the total radioactivity incorporated into cellular DNA during a 24 h interval and is indicated at the midpoint of the pulse period. DNA was analysed as described in the text.

Fig. 6. Kinetics of cellular DNA synthesis in arrested hamster embryo fibroblasts. Total radioactivity incorporated into cellular DNA in cells infected with non-irradiated cytomegalovirus (hatched bars), ultraviolet irradiated cytomegalovirus (solid bars), or control cultures (open bars) inoculated with maintenance medium containing the same concentration of foetal calf serum as the virus inoculum was measured. The conditions employed in analysing the DNA are identical to those described in Fig. 5.

Significant differences in the rate of cell DNA synthesis between non-arrested infected and uninfected cells were not detected (Fig. 5); however, differences were observed in the uptake of [3H]-methyl thymidine into cellular DNA in arrested cells (Fig. 6). From 0 to 24 h p.i., about three times as many counts were incorporated into cellular DNA in infected cell cultures than in control cell cultures. From 24 to 48 h p.i., the level of isotope incorporated in virus infected cell cultures fell to the level of control cultures; however, the incorporation of labelled thymidine in irradiated virus infected cell cultures was about 4.5 times greater than the level in control cultures.

Since there is the possibility that CMV infection may alter the relative proportion of endogenously synthesized and exogenous thymidine available for DNA synthesis, these experiments were extended using [3H]-hypoxanthine and serum-arrested cells. The results of this experiment for the 24 to 48 h p.i. period are summarized in Fig. 7. As in the previous experiments, the level of DNA synthesis in CMV infected cultures at 24 to 48 h was less than that observed in control cultures. However, cell cultures infected with virus irradiated with 24000 ergs/mm² of u.v. light had about a threefold greater rate of DNA synthesis during this period than that observed in control cultures. Cell cultures inoculated with
virus exposed to greater doses of irradiation demonstrated decreased levels of isotope incorporated into cellular DNA when compared with cells inoculated with virus irradiated with 24,000 ergs/mm². With doses equal to or greater than 96,000 ergs/mm², the stimulating activity of the virus inoculum was lost.

Mitotic activity of HEF following infection with CMV

Stimulation of cell DNA synthesis by CMV represents new semi-conservative DNA synthesis (St Jeor et al. 1974). In order to determine whether the increased DNA synthetic activity would be succeeded by increased mitotic activity, the following study was initiated. The methodology employed in reducing the background of cellular DNA synthesis to levels where stimulation of cellular DNA synthesis might be detected could prohibit cells from subsequently entering mitosis. Therefore, a protocol (previously described) was used which allowed cells to enter mitosis following virus infection. Employing these cell cultures and virus stock treated with various doses of u.v. light, increased mitotic activity was demonstrated following CMV infection (Fig. 8). The greatest stimulation of mitotic activity (up to sixfold) in cell cultures infected with u.v.-irradiated stock (24,000 ergs/mm²) was observed in the initial 24 h p.i. period. Other doses of irradiation of the virus inoculum gave lower stimulatory effects. By 48 h p.i., the level of mitotic activity in cell cultures inoculated with irradiated virus was at the level of the control. In contrast, little increase in mitotic activity was observed in cell cultures inoculated with non-irradiated virus.
Abortive infection with human CMV

Fig. 8. Mitotic index of hamster embryo fibroblasts inoculated with non-irradiated human cytomegalovirus (●—●); with ultraviolet-light-irradiated cytomegalovirus: 12,000 ergs/mm² (▲—▲), 24,000 ergs/mm² (▲—▲), 48,000 ergs/mm² (■—■), 144,000 ergs/mm² (■—■); or with maintenance medium (○—○) containing the same concentration of foetal calf serum as the virus inoculum. The cells were fixed with absolute methanol and stained with Giemsa. Cells were counted from 400 microscopic fields to determine each datum point.

Fig. 9. Mitotic index of hamster embryo fibroblasts in the presence of colcemide (1 μg/ml). Cell cultures were inoculated with non-irradiated human cytomegalovirus (●—●); with ultraviolet-light-irradiated cytomegalovirus: 12,000 ergs/mm² (▲—▲), 48,000 ergs/mm² (□—□), 144,000 ergs/mm² (■—■); or with maintenance medium (○—○) containing the same concentration of foetal calf serum as the virus inoculum. The cells were fixed with absolute methanol and stained with Giemsa. Cells were counted from 200 microscopic fields to determine each datum point.

The increase in mitotic figures observed at 48 h p.i. in the cultures inoculated with non-irradiated CMV could represent a release of contact inhibition for cellular division by massive destruction in the confluent monolayer created by virus-induced cell death with the subsequent arrest of cells in mitosis following virus infection. Therefore, the experiments were repeated in the presence of colcemide (1 μg/ml). The results of this experiment are graphed in Fig. 9. Stimulation of mitotic activity was observed in all cell cultures inoculated with irradiated virus. However, the greatest mitotic activity was observed in cultures inoculated with virus irradiated with 12,000 ergs/mm² of u.v. light. Increasing doses of u.v. irradiation progressively diminished the stimulating effect upon mitotic activity. The mitotic index of cultures inoculated with non-irradiated virus never exceeded that of the controls.
DISCUSSION

Qualitatively, the cytopathology of CMV infected HEF is very similar to that reported by Waner & Weller (1974) in bovine cells and by Fioretti et al. (1973) in guinea-pig cells. The cytopathology observed in these cell types (all of which are abortively infected) appears early (5 to 24 h p.i.), and consists of refractile cells, cytoplasmic inclusions and eccentric nuclei. Nuclear inclusions, as observed in productively infected HEL, and prominent multinucleated giant cells, as observed in CMV inoculated Vero cells (Waner & Weller, 1974), were absent from CMV inoculated HEF.

Quantitatively, however, CMV infection of HEF cells appears to be different from the other cell types (bovine, guinea pig) sustaining abortive infections. Waner & Weller (1974) found cytopathology in about 40% of CMV infected bovine cells and CMV antigens in a similar percentage of inoculated cells. Similar results were obtained by Fioretti et al. (1973) in guinea-pig cells. In contrast, cytopathology and virus antigens were observed in only about 1% of CMV infected non-arrested hamster cells. Furthermore, while both Fioretti et al. (1973) and Waner & Weller (1974) found nuclear and cytoplasmic antigens in their respective cell types, only cytoplasmic antigens were detected in infected non-arrested hamster cells. However, if the hamster cells were arrested by serum starvation, then cytopathology was observed in the vast majority of inoculated cells and virus antigens were present in the nucleus and cytoplasm.

Cellular DNA synthesis was also affected by CMV infection. The degree of stimulation observed was about 50% of that previously reported in other cell types (HEL, Vero) by St Jeor et al. (1974) which sustain productive and inefficient infections, respectively (Waner & Weller, 1974).

Since stimulation of cell DNA synthesis was observed in these cells, it is not surprising to find an increased level of mitotic activity. Lang, Montagnier & Latarjet (1974) also observed increased mitotic activity following CMV infection. The observed increases in the mitotic index do not appear to be the result of cells arrested in mitosis, since there is an accumulation of mitotic cells in the presence of colcemide. It is interesting to note that at least a fivefold greater dose of u.v. light to the virus stock is required to eliminate the induction of DNA synthesis and mitosis, than to eliminate cytopathology. Apparently, the test for mitosis (on individual cells) is somewhat more sensitive than stimulation of DNA (on cell populations) as a measure of CMV activity.

These data indicate that CMV is capable of inducing both DNA synthesis and mitosis in hamster embryo cells. Earlier studies (St Jeor & Rapp, 1973a, b; St Jeor et al. 1974) indicated that CMV can induce cell DNA synthesis in a variety of cell types. Studies in this laboratory (S. C. St Jeor & J. J. M. Walboomers, unpublished experiments) indicate that CMV, rather than coding for a virus distinct thymidine kinase, induces higher levels of cellular thymidine kinase. It is therefore possible that the replication of CMV is dependent upon the cell cycle.

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Abortive infection with human CMV

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