Cell DNA Replication as a Function in the Synthesis of Human Cytomegalovirus

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SUMMARY

The rate of virus and cell DNA synthesis was studied in human embryonic lung cells pre-treated with 5-iodo-2'-deoxyuridine (IdUrd) and exposed to cytomegalovirus (CMV) or medium. Analysis of DNA in CMV-infected cells following sequential 4 h pulses with 3H-thymidine indicated that a temporal relationship existed in the pattern of virus and cell DNA synthesis. The pattern of DNA replication in infected cells resembled that of a typical cell cycle, whereas the rate of cell DNA synthesis in uninfected cells remained low throughout the study. Increased rates of cell and virus DNA synthesis began concomitantly at 16 h post-infection and reached a maximum at 36 h post-infection. The rate of DNA synthesis then declined and remained at lower levels until 48 h post-infection. This was subsequently followed by a second increase in the rate of cell and virus DNA synthesis. The rates of cell and virus DNA replication were similar throughout the study in that increased and decreased rates of synthesis occurred simultaneously. It was of interest to note that CMV induced cell DNA replication in IdUrd arrested cells; in contrast, addition of fresh serum did not induce a similar increase in the rate of DNA synthesis in IdUrd arrested, but uninfected, cells.

INTRODUCTION

Herpesviruses exhibit wide variation in the length of time required for replication. Typical of the more rapidly replicating members of the group are herpes simplex virus types 1 and 2. Herpes simplex viruses (HSV) have an eclipse period of at least 4 h with a latent period of approx. 2 to 3 h; in addition, early mRNA is made within 3 h after infection and virus DNA replication is initiated 6 to 9 h after infection (Ben-Porat & Kaplan, 1973; Darlington & Granoff, 1973). Conversely, human cytomegalovirus (CMV) is a relatively slow replicating virus with a latent period of 48 h, detectable virus DNA synthesis occurring between 24 and 48 h after infection, and early protein synthesis initiated at 8 h post-infection (Furukawa, Fioretti & Plotkin, 1973; Huang, Chen & Pagano, 1973; St Jeor & Rapp, 1973a). In addition, HSV will form detectable plaques in monolayers of cells within 48 to 72 h whereas CMV requires 14 days for plaque formation (Darlington & Moss, 1968; Wentworth & French, 1970). The reasons for the relatively slow rate of CMV replication are unknown.

Reports from this laboratory indicated that CMV induces cell DNA replication following
infection (St Jeor & Rapp, 1973b; St Jeor et al., 1974). Virus induction of cell DNA replication occurred during both productive and abortive infections and was not dependent upon virus DNA replication. These findings have been confirmed by several independent investigators (Furukawa, Tanaka & Plotkin, 1975a; DeMarchi & Kaplan, 1976). We recently reported a correlation between CMV and cell DNA synthesis (St Jeor, Hutt & Rapp, 1975). Furukawa, Tanaka & Plotkin (1975b) reported a dependence on host cell function for CMV replication. These investigators reported that CMV would not replicate in cells exposed to u.v. light prior to infection whereas HSV would replicate in these cells. It has recently been suggested (DeMarchi & Kaplan, 1976) that CMV DNA synthesis can occur independently of cell DNA synthesis and that virus and cell DNA are synthesized in separate cell populations. The data to be reported in this manuscript indicate a temporal relationship between cell and virus DNA synthesis.

METHODS

Cell culture and medium. Human embryonic lung cells (Flow Laboratories, Rockville, Maryland) were used throughout these investigations for virus propagation and assay as well as for DNA studies. Stock cells were propagated in roller bottle cultures and transferred to the appropriate containers for experimental studies. All cell cultures employed Dulbecco medium supplemented with 15% foetal calf serum (FCS), 0.075% sodium bicarbonate, and 100 units of penicillin and 100 μg of streptomycin per ml.

Virus propagation and assay. The AD-169 strain of human CMV, obtained from Dr Paul Feorino (Center for Disease Control, Atlanta, Georgia), was used exclusively in these investigations. Virus was propagated in confluent monolayers of HEL cells in roller bottles. The cells were infected at a virus to cell ratio of 0.5 to 1.0. Following infection the virus was harvested at 6 to 14 days post-infection (p.i.) or when maximum cytopathology had developed. Harvesting was accomplished by freeze-thawing the cultures, sonicating for 2 min in a Branson Sonifier (Cole-Palmer Instrument and Equipment Co.) and clarifying the fluids by centrifuging at 600 g for 10 min. Virus preparations were stored at −65 °C for later use.

Infectious virus was assayed as described by Wentworth & French (1970). Confluent monolayers of cells, grown in Falcon 15 mm × 60 mm plastic tissue culture dishes, were infected with varying concentrations of virus. After a 1 h virus adsorption, the cells were overlaid with Dulbecco medium supplemented with 0.25% agarose, 0.225% sodium bicarbonate, 5% FCS and the antibiotics mentioned previously. Cells were then placed at 37 °C in a 5% CO2 atmosphere for one week, at which time a second overlay of Dulbecco medium supplemented with agarose was placed on the cells. Two weeks after infection the cells were fixed with formalin, stained with methylene blue and the plaques counted.

DNA labelling and analysis. The method used for DNA labelling and analysis has been described (St Jeor & Rapp, 1973). The following general procedures were used in these studies. Cells were either infected with CMV at a virus to cell ratio of 3 or mock-infected with growth medium. Cultures for labelling were exposed to 10 μCi/ml of methyl tritiated thymidine (3H-dThd, 20 Ci/mmol, New England Nuclear) for the appropriate labelling period. DNA was extracted using sarcosyl, pronase and EDTA. Analysis of DNA consisted of isopycnic centrifugation of the sample in the presence of a 14C-cell DNA marker in a neutral CsCl gradient (initial density 1.716 g/ml, 34000 rev/min, 60 h, Beckman 40·3 rotor, 20 °C). The gradients were collected on filter paper discs by bottom puncture and the DNA precipitated with 5% trichloroacetic acid. The samples were placed in a toluene omnifluor cocktail and counted in a liquid scintillation counter. The technique for preparing 14C-DNA markers was described in a previous publication (St Jeor et al., 1974).
Radioactive counts were recorded and analysed using a Hewlett Packard 9820A calculator equipped with a 9863 tape reader and a 9862A plotter.

**Treatment of cells with 5-iodo-2'-deoxyuridine.** The method of pre-treating cells with 5-iodo-2'-deoxyuridine (IdUrd) was described earlier (St Jeor & Rapp, 1973a). It consisted of growing HEL cells in the presence of 100 µg per ml of 5-iodo-2'-deoxyuridine for at least 72 h. This pre-treatment greatly reduced both the rate of cell DNA replication and cell division.

**RESULTS**

Earlier studies in this laboratory indicated that to detect virus-induced cell DNA replication, background DNA synthesis must be lowered either by pre-treatment of cells with IdUrd or by starvation in medium containing low serum concentrations (0.2%; St Jeor et al. 1974). This observation is in agreement with other studies of polyoma or SV40 induced cell DNA replication (Henry et al. 1966; Fried & Pitts, 1968). Consequently, IdUrd pre-treatment was used in these studies as the method of arresting cell DNA replication. Cultures in Falcon 25 cm² plastic tissue culture flasks consisting of 4 x 10⁵ cells were treated with 100 µg/ml of IdUrd. At this cell concentration the cell numbers are such that the monolayers are approx. 30% confluent. The cells were maintained in the presence of IdUrd for 72 h, at which time cell replication and DNA synthesis was arrested; the cells remained approx. 60% confluent. It is important that the concentrations of IdUrd incorporated are high enough to completely inhibit cell proliferation and thus inhibit cell DNA synthesis. To insure that enough IdUrd has been incorporated to inhibit cell DNA synthesis, IdUrd was removed after 72 to 96 h, the cells rinsed twice with PBS and fresh growth medium containing 15% foetal calf serum added to the cultures. The cells were then examined 24 h later and observed to determine whether additional cell growth had occurred. Only cultures which demonstrated complete inhibition of cell proliferation were used in these studies. The cultures were then rinsed twice with PBS and infected with CMV or mock-infected with medium containing 5% fresh FCS. Following infection the cultures were labelled with 16 sequential 4 h pulses of ³H-dThd. After each labelling period the DNA was extracted and analysed by isopycnic centrifugation in neutral CsCl. The gradient was fractionated by bottom puncture and the radioactivity in each fraction determined (see Methods). The measurement of total number of counts incorporated into virus and cell DNA was facilitated using a Hewlett Packard calculator, plotter and tape reader. Density determinations for the caesium chloride gradients were determined initially using a ¹⁴C-cell DNA marker and by determining the refractive index of selected samples in the gradient. A ¹⁴C-cell DNA marker was then added to all gradients prior to centrifugation. A typical gradient observed in the CMV-infected cells late in infection (Fig. 1, 36 to 40 h) contains three peaks of radioactivity. We have demonstrated in an earlier publication (St Jeor et al. 1974) that the peak of greatest density (1.747 g/ml) near the bottom of the gradient (fractions 5 to 20) represents cell DNA with an increased density due to the substitution of IdUrd for thymidine in the DNA molecule. If a sample of the DNA placed on the neutral gradient (Fig. 1) is centrifuged in alkaline caesium chloride, the duplex DNA separates into separate strands. Since the ³H-thymidine was added during virus infection and after the IdUrd was removed, the strand induced by the virus infection to replicate (containing ³H-dThd) separates from the DNA strand substituted with IdUrd and consequently bands with normal cell DNA (St Jeor et al. 1974). This earlier study indicated that the DNA synthesis induced by the virus was semi-conservative DNA synthesis and not repair DNA synthesis (St Jeor et al. 1974). The radioactive peak present in the middle of the gradient (fractions 23 to 33, density 1.716 g/ml) is CMV DNA. This
Fig. 1. Isopycnic centrifugation of DNA from cytomegalovirus infected and IdUrd pre-treated human embryonic lung cells. The cells were pre-treated with IdUrd and infected with the AD-169 strain of CMV. Following infection, cells were labelled from 36 to 40 h p.i. with \(^{3}\text{H}-\text{dThd}\) (10 \(\mu\text{Ci}/\text{ml}\) of methyl-/tritiated thymidine, sp. act. 20 Ci/mmol). After the labelling period, cells were digested with sarcosyl and pronase and the DNA centrifuged to equilibrium in CsCl (initial density 1.716 g/ml. Beckman 40-3 rotor, 34,000 rev/min, 60 h at 20 °C). The gradients were collected by bottom puncture, the DNA precipitated in 5 % trichloroacetic acid and counts determined in a scintillation counter. The data were analysed and plotted with a Hewlett-Packard calculator, plotter and tape reader. A cell DNA marker was included for density determinations. In addition, representative samples were used for refractive index readings for density determinations.

Density for CMV DNA has been observed in numerous laboratories (Crawford & Lee, 1964; Plummer et al. 1969). The lightest peak of radioactivity (fractions 34 to 48) is at a density of 1.695 g/ml and is normal diploid human fibroblast cell DNA.

Fig. 2 represents the results obtained from the isopycnic centrifugation of CMV infected and control cultures for the following labelling periods: (a) 0 to 4 h uninfected; (b) 0 to 4 h infected; (c) 20 to 24 h infected; (d) 28 to 32 h infected. The arrow in each panel indicates the position of the cell DNA marker. Analysis of the data indicated that DNA replication was comparable and at low levels in both infected and control cultures during the 0 to 4 h labelling period. The uptake of \(^{3}\text{H}-\text{dThd}\) did not change in uninfected cells during the entire study. Since the 0 to 4 h labelling period (Fig. 2a) was typical of gradients obtained for the other labelling periods it is the only period representing the uninfected cells; however, the total counts for all periods for the uninfected cultures are presented in Fig. 4. Approximately 20 to 24 h p.i. the rate of DNA replication increased in the infected cultures but remained at a low level in the control, uninfected cultures. The gradient represented in Fig. 2 (c) is quite rough because a relatively small amount of \(^{3}\text{H}-\text{dThd}\) was incorporated due to the fact that the rate of DNA synthesis is still low. When this gradient is compared to the control Fig. 2 (a), there are increased counts incorporated in fractions 10 to 18 (cell DNA substituted with IdUrd), fractions 21 to 30 (density 1.716 g/ml CMV DNA) and fractions 34 to 39 (density 1.695 g/ml normal HEL cell DNA). The control gradient for the 20 to 24 h labelling...
Relationship between CMV and cell DNA replication

Fig. 2. Isopycnic centrifugation of DNA from cytomegalovirus infected or sham-infected human embryonic lung cells. Human embryonic lung cells were arrested by pre-treatment with IdUrd and infected with CMV or mock-infected with growth medium as described in Fig. 1. Following infection, the cells were labelled with sequential 4 h pulses of H-dThd as described in Fig. 1. The DNA was then extracted, centrifuged in CsCl and the amount of radioactivity incorporated determined as described in Fig. 1. The arrow in each panel indicates the position of the H-dThd cell DNA marker. (a) 0 to 4 h post mock infection, uninfected; (b) 0 to 4 h p.i.; (c) 20 to 24 h p.i.; (d) 28 to 32 h p.i.

period (not shown), had a single peak of radioactivity banding at the same density as uninfected normal HEL cell DNA (1.696 g/ml).

From 20 h p.i. the rate of both cell and virus DNA replication increased in the infected cultures, reaching a maximum at 32 to 36 h. Fig. 2(d) illustrates the results obtained with infected cells during the 28 to 32 h labelling period. There are three obvious peaks of radioactivity in (d). Fraction 14 is cell DNA substituted with IdUrd, fraction 31 is CMV DNA (density 1.716 g/ml) and fraction 42 to 43 is normal HEL cell DNA (density 1.695 g/ml). Again the rate of DNA replication in the uninfected control cultures remained at low levels.

In order to clarify the relationship between cell and virus DNA synthesis the total counts incorporated into both cell and virus DNA were determined for each labelling period. These data are presented in Fig. 3. An examination of the results indicates that both virus and infected cell DNA replication increased to a maximum at 32 to 36 h p.i. There was a subsequent decrease in the rate of both cell and virus DNA synthesis until approx. 48 h p.i., at which time there was an increase in the rate of both virus and cell DNA replication to a second peak at 60 to 64 h p.i.

To determine the relative amount of cell DNA replicated from both light (unsubstituted) and heavy (IdUrd substituted) cell DNA in infected and uninfected cells, the total amount of H-dThd incorporated into cell DNA at the heavy and light positions was determined. These
Fig. 3. The relationship between CMV and cell DNA replication. Human embryonic lung cells were pre-treated with IdUrd and infected with CMV. Following infection, cells were labelled with sequential 4 h pulses of $^3$H-dThd. After the labelling period, the DNA was extracted and centrifuged to equilibrium in CsCl gradients as described in Fig. 1. The total ct/min of $^3$H-dThd incorporated into cell and virus DNA were determined from plots of the gradient profiles. 
•—•, Cell DNA; □—□, virus DNA.

Fig. 4. Relative amount of DNA synthesized from IdUrd substituted or unsubstituted cell DNA in infected or uninfected HEL cells. HEL cells, pre-treated with IdUrd, were treated as described in Fig. 1. The total counts of $^3$H-dThd incorporated into DNA at the light and heavy positions of the CsCl gradient were determined with the aid of a Hewlett-Packard calculator, tape reader and plotter. •—•, CMV infected light cell DNA; □—□, CMV infected heavy cell DNA; △—△, uninfected light cell DNA; ▲—▲, uninfected heavy cell DNA.
results are presented in Fig. 4. Examination of the data indicates that in infected cells virus-induced DNA synthesis occurred from both unsubstituted (light) and IdUrd substituted (heavy) cell DNA. DNA replication in the uninfected cells, although low, occurred principally from a light (unsubstituted), rather than from a heavy (IdUrd substituted) parental DNA template. As is evident in this study, the addition of fresh medium containing 5% foetal calf serum at zero time did not induce cell DNA synthesis in the IdUrd-treated uninfected control cells. In addition, as mentioned earlier in this manuscript, the addition of medium containing 15% foetal calf serum will not induce cell proliferation in cultures treated with IdUrd.

**DISCUSSION**

The results of this study support earlier observations that CMV induces replication of cell DNA during the virus replicative cycle. Furthermore, they indicate a correlation between the time sequence of virus and cell DNA synthesis. Examination of the rate of both virus and cell DNA replication (Fig. 3) suggests a resemblance to DNA synthesis during the cell cycle in an *in vitro* mammalian cell population. The data obtained reveal an obvious increased rate of cell and virus DNA synthesis at approx. 24 h p.i., with a maximum rate during the 32 to 36 h labelling period. This was followed by a subsequent reduction and a re-initiation of cell and virus DNA replication at 48 h p.i. The second increase probably does not represent secondary virus infection made possible by low input since every cell exhibited virus c.p.e. within 12 h p.i. In addition virus is not released from CMV-infected cells until 48 to 72 h p.i. (Furukawa *et al.* 1973). Consequently it is doubtful that release of virus could explain the secondary initiation of virus DNA synthesis.

The data presented in this manuscript only indicate a temporal correlation between virus and cell DNA synthesis, which does not in any way mean the two events are dependent upon each other. However, when these results are considered in relation to work published by Furukawa *et al.* (1975b) which indicates that HSV will replicate but CMV will not replicate in cells which have been exposed to u.v. light, there appears to be a dependence on host cell function for CMV replication.

A recent publication by DeMarchi & Kaplan (1976) indicates that virus DNA synthesis can occur in cells which have not undergone cell DNA synthesis. The criterion for assuming a cell had undergone DNA synthesis was based on autoradiography. If cell DNA synthesis had occurred the autoradiograph could be developed within 24 h. However, cells undergoing virus DNA synthesis had to be developed a greater length of time and demonstrated a much lower amount of $^3$H-dThd uptake. In arriving at this conclusion, these authors are assuming that the entire genome of the cell is induced to replicate. If only a small fraction of the cell DNA was induced to replicate by the virus infection then those cells undergoing cell DNA synthesis would have reduced $^3$H-dThd uptake and consequently would not appear as cells in which the entire genome is replicated. In addition Shackney, Ford & Wittig (1973) reported that the onset and termination of DNA synthesis does not occur abruptly during the cell cycle, and that increased emulsion exposure times in autoradiography studies of uninfected cells indicate a greater number of cells synthesizing DNA but at a lower rate. Thus, the criteria used by DeMarchi & Kaplan (1976) for determining the number of cells undergoing cell DNA synthesis are questionable. We feel that only a small percentage of the cell DNA is induced by the virus to replicate for the following reasons. If cells which are arrested by IdUrd are infected with CMV and the newly synthesized cell and virus DNA examined for size by velocity sedimentation gradients, the cell DNA which is induced to replicate represents relatively small pieces of DNA and not the entire cell
genome (our unpublished data). Consequently, the observation by DeMarchi & Kaplan (1976) could be in error in that CMV would replicate in cells in which only a small portion of the cell genome was replicated. Thus, a longer emulsion exposure time would be required to detect cell DNA synthesis.

An unusual observation concerning virus induction of cell DNA replication is that DNA replication occurs in IdUrd arrested cells. Normally, when HEL cells are arrested with high concentrations of IdUrd, DNA synthesis is greatly reduced (St Jeor et al. 1974). In fact, cell DNA synthesis cannot be re-induced with factors which normally initiate DNA replication, such as high serum concentrations. However, following infection with CMV, replication occurs from both parental DNA strands (substituted and unsubstituted with IdUrd). Earlier evidence from this laboratory indicated that virus-induced DNA replication which occurred in the IdUrd substituted cells was normal semi-conservative DNA synthesis (St Jeor et al. 1974). The reason for the low rate of DNA synthesis in the control cells as compared to the virus infected cells is unknown. It could be postulated that the DNA polymerase in the control cells is unable to copy from an IdUrd substituted DNA template. The cell polymerase can replicate new DNA from unsubstituted DNA and insert IdUrd in place of thymidine but is unable to copy from a hybrid molecule substituted with IdUrd. It was recently reported by Huang (1975) that CMV both induces increased levels of cell DNA polymerase and codes for its own unique polymerase. This observation would support the possibility that a new polymerase might be able to copy a DNA molecule substituted with IdUrd more efficiently. Another explanation is that the polymerase in the control cultures has a short half life and must be continually synthesized. Because of the IdUrd substitution, the cell is unable to make functional mRNA to code for more DNA polymerase. There is substantial evidence in the literature that treatment of cells with thymidine analogues, such as IdUrd or 5-bromo-deoxyuridine, causes the production of faulty cell proteins (Holmes, Gilson & Deinhardt, 1964; Stockdale et al. 1964). The reason for the increased rate of DNA synthesis in the infected cells may be that CMV is coding for a DNA polymerase which will copy from a DNA template substituted with IdUrd.

If, as it appears from the data presented in this manuscript, CMV replication is dependent upon cell DNA synthesis, and consequently the cell cycle, then a theory for the slow rate of replication of CMV can be postulated. CMV DNA replication and synthesis of new virus is dependent upon cell DNA synthesis; consequently, whenever a cell becomes infected with CMV, it will not synthesize new virus DNA until the cell goes through the synthetic or S phase of the cell cycle. Once a single cell is infected that cell will have to pass through the S phase of the cell cycle before DNA and consequently infectious virus can be replicated. When virus is released to the surrounding cells, each of these cells will have to enter the S phase of the cell cycle before new virus DNA is produced.

A possible explanation for the species and type cell dependency of CMV would be that the cytomegaloviruses have evolved a method of replication which depends on the cell cycle. After penetration of the virion into a susceptible cell, a function of early virus mRNA and consequently virus protein synthesis would be to induce cellular macromolecular synthesis and cell enzymes involved in DNA replication. Each CMV type peculiar to a particular species would have evolved to enable it to coordinate its own replicative requirements with the specific cell of the animal species with which it has evolved.

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Relationship between CMV and cell DNA replication

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