Cell Cycle-dependent Chronic Infection of Human Cytomegalovirus in Human Osteogenic Sarcoma Cells

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SUMMARY

Human osteogenic sarcoma cells transformed by murine sarcoma virus (R970) showed restricted growth of human cytomegalovirus (HCMV). Virus was attached to the same extent as in human fibroblasts. HCMV growth was blocked at early stages after virus penetration. Splitting of infected R970 cultures resulted in infection of all cells. In experiments using synchronized R970 cells it was found that factor(s) associated with the S-phase of the cell cycle might be necessary for establishment of the infection.

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the herpes group, is known to cause latent, persistent infection in vivo (Wright, 1973). Epithelial cells are most frequently infected in vivo, but HCMV is believed to be both host- and tissue-specific in vitro. However, it was originally reported by Rowe et al. (1956) that no c.p.e. was produced in the KB and HeLa lines widely used as human epithelial cells. Although several reports have appeared showing that HCMV replicates to a limited degree in human epithelial cells and a primate line (Waner & Weller, 1974; Michelson-Fiske et al. 1975; Knowlers, 1976; Vonka et al. 1976), human fibroblasts have been the only efficient cells for HCMV growth.

It has been speculated that growth of HCMV in epithelial cells was inefficient because the epithelial cells may have the inhibitors for HCMV growth as reported by St Jeor & Rapp (1973), who were able to grow HCMV in human embryo kidney cells after pre-treatment of the cells with 5 iodo-2'-deoxyuridine. In this communication, we describe the interaction of HCMV with R970 cells, an epitheloid line of human osteogenic sarcoma cells transformed by murine sarcoma virus which carries the transforming gene (Rhim et al. 1975).

METHODS

Viruses. The Towne strain of HCMV was used throughout these experiments. It has been continuously propagated in WI-38 and MRC-5 cells in our laboratory. The history of this strain was described elsewhere (Furukawa et al. 1973). Stock pools of the Indiana strain of vesicular stomatitis virus (VSV), kindly provided by Dr H. Fred Clark, of The Wistar Institute, were prepared on MRC-5 cells.

Cell cultures. MRC-5 human diploid fibroblasts were obtained from the Medical Research Council in the United Kingdom and R970 cells from Dr Chern's Laboratory, The Wistar Institute. Cell lines were continuously propagated in our laboratory in Eagle's minimal essential medium (MEM) with 7.5% foetal calf serum (FCS) and maintained with 2% FCS.

Infectivity assay. At various intervals p.i., the infectivity of HCMV was measured after cells were disrupted by sonication (Branson Sonifier) for 30 s. Infectivity in the supernatant fluid was also assayed after sedimentation of the cells by centrifuging at 2000 rev/min for
The infectious virus titre was determined on MRC-5 cells using the plaque assay described by Wentworth & French (1970).

**Interferon assay.** MRC-5 monolayers in 5 cm Petri dishes were treated overnight at 37 °C with 2 ml of serial dilution of pre-treated supernatant with acid, according to the method of Barron (1969). The monolayers were then challenged with approx. 50 p.f.u. of VSV. A 50% reduction in VSV plaque number as compared with the control was considered an indication of interferon activity.

**Cell synchronization.** R970 cells were synchronized according to the method of Mueller & Kajiwara (1969). Cells were seeded on Petri dishes (3 cm diam.) at a density of $1 \times 10^6$/dish. After 24 h, the culture medium was replaced with medium containing 2 mM-thymidine in 1 ml. After another 24 h, cells were transferred into medium without thymidine for 15 h. A second thymidine treatment was given for 24 h. As soon as the cells were returned to culture medium, the length of each phase of the cell cycle was measured by thymidine uptake and mitotic indices.

**Cloning of R970 cells.** R970 cells dispersed by 0.25% trypsin plus 0.1% EDTA were plated in Petri dishes (10 cm diam.) and cultivated in a CO2 incubator for 2 weeks. Well isolated colonies were picked up with a piece of filter paper soaked in 0.25% trypsin and transferred to Petri dishes.

**Immunofluorescent antibody (FA) technique.** Assays for cells containing virus antigen were carried out with demonstration of late virus antigen (Furukawa et al. 1975a) by immunofluorescent staining. Cells cultured on coverslips were fixed for 10 min at $-10$ °C with acetone and stored at 4 °C until stained. The indirect FA technique was used with a convalescent human serum that contained complement-fixing and FA antibody titres of 1:64 and 1:320 respectively. Serum was diluted 1:40 in phosphate-buffered saline (PBS) for FA staining.

**Labelling of cells with radioisotope and analysis of DNA.** At various intervals p.i. a small vol. of radioisotope was added to the incubation medium at the final concentration described in each experiment. For the separation and assay of virus and cellular DNA, cells were washed in buffer (0.01 M-tris, pH 7.4, 0.01 M-KCl and 0.0013 M-MgCl2) and incubated in the same buffer containing 1% sarkosyl 97 for 15 min at 60 °C. Pronase (final concentration 5 mg/ml) was added to the lysed cell suspension which was then incubated for a further 2 h at 37 °C. The treated cell lysates were mixed with CsCl (4.55 g/3.5 ml sample). Isopycnic centrifugation was carried out in an SW 50.1 rotor at 35000 rev/min for 64 h. The fractions were precipitated with 5% trichloroacetic acid (TCA); precipitates were filtered and counted.

**Labelling of virus.** Monolayers of MRC-5 cells were infected with HCMV at an input multiplicity of 0.1. One h after adsorption, cells were re-fed with medium containing 3H-thymidine at a concentration of $1 \mu$Ci/ml. At 5 to 6 days p.i. the supernatant was clarified by centrifugation for 30 min at 10000 g. Clarified supernatant was centrifuged again in an SW 25.1 rotor for 90 min at 22000 rev/min and the pelletted virus on a 50% sucrose cushion was resuspended in tris buffer, pH 7.4.

**RESULTS**

**Growth of HCMV on R970 MRC-5 cells**

R970 and MRC-5 cells were infected with an input multiplicity of 5. At various intervals p.i., the infectivity of cell-associated virus was assayed. In MRC-5 cells, infectivity began to increase at 48 h.p.i. and reached a peak of $4 \times 10^6$ p.f.u./ml at 3 or 4 days p.i. In contrast, HCMV showed restricted growth on R970 cells, reaching a maximum titre of $7 \times 10^3$ p.f.u./ml at 3 and 4 days p.i. (Fig. 1).
Fig. 1. MRC-5 and R970 cells were infected with CMV at a m.o.i. of 5. At various times, two samples of each cell type were assayed; trypsinized cells and supernatant were combined and sonicated for 20 s as described in the Methods. After centrifugation at 2000 g for 10 min, the supernatant was assayed for total virus product. ○—○, Infectivity on MRC-5; ▲—▲, infectivity on R970.

Fig. 2. Adsorption of HCMV to MRC-5 (●—●) and R970 (○—○) cells.

Table 1. Infectivity and virus antigen of HCMV-infected R970 and MRC-5 cells at 4 days p.i.*

<table>
<thead>
<tr>
<th>Cells</th>
<th>Number of cells</th>
<th>Number of cells containing virus antigen</th>
<th>Infectivity (total)</th>
<th>p.f.u./virus antigen positive cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>R970</td>
<td>$9.8 \times 10^6$</td>
<td>$1.2 \times 10^6$</td>
<td>$7.0 \times 10^6$</td>
<td>0.06</td>
</tr>
<tr>
<td>MRC-5</td>
<td>$6.6 \times 10^6$</td>
<td>$6.5 \times 10^6$</td>
<td>$4.0 \times 10^6$</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* Monolayer cultures were infected at a m.o.i. of 5. Virus antigen and total virus production were assayed as described in Methods.

Histologically, R970 cells did not show the early c.p.e. (Furukawa et al. 1973) or the production of early antigen (Reynolds, 1978) seen in MRC-5 cells. Only a few nuclear inclusion bodies were demonstrated in R970 cells at 4 days by both FA and haematoxylin eosin staining and only 10 to 14% of R970 cells had infectious centres. In contrast, virtually 100% of HCMV-infected MRC-5 cells produced infectious centres as detected by FA staining. In addition, the amount of virus yielded per cell was at least 100 times lower in R970 cells than in MRC-5 cells (Table 1).

**Adsorption rate of HCMV in R970 and MRC-5 cells**

Monolayers of R970 and MRC-5 cells grown in plastic dishes ($8 \text{ to } 10 \times 10^6 \text{ cells/dish}$) were washed with PBS, pH 7.2, and exposed to 0.4 ml of partially purified $^3$H-HCMV (containing $8 \times 10^7 \text{ p.f.u., } 20000 \text{ ctf/min/ml}$). The cultures were incubated at 37 °C in a CO₂ incubator. At various intervals the inoculum was removed from each of two Petri dishes and pooled for assay of residual acid-precipitable radioactivity in the supernatant. The results, summarized in Fig. 2, indicate that the rate of adsorption of virus to R970 and MRC-5 cells was similar.
Fig. 3. Cells infected with HCMV were incubated at 2 days p.i. for 48 h with medium containing \(^{3}H\)-thymidine (5 μCi/ml). DNA from infected cells was extracted and analysed by isopycnic centrifugation. 〇--〇, MRC-5; ●--●, R970; △--△, density.

Fig. 4. Effect of m.o.i. on number of cells containing virus antigen demonstrable by immuno-fluorescence. △--△, MRC-5 cells; ●--●, R970 cells.

**Synthesis of HCMV DNA in R970 and MRC-5 cells**

In order to determine whether HCMV DNA is synthesized in R970 cells, MRC-5 and R970 cells were infected with HCMV at an input multiplicity of 1. The infected cells were incubated with \(^{3}H\)-thymidine from 48 to 96 h p.i. DNA from infected cells was extracted as described in Methods. Cellular DNA was differentiated from virus DNA by isopycnic centrifugation in CsCl and the amount of \(^{3}H\)-thymidine incorporated into cellular or virus DNA was determined. As shown in Fig. 3, the high peak (density 1.716 g/ml) corresponds to the density of virus DNA (Plummer *et al.* 1969). The lower peak corresponds to the density of DNA extracted from uninfected cells (density 1.698 g/ml). It is clear that the infected R970 cells do not form appreciable quantities of virus DNA.

**Effect of m.o.i. on number of cells containing virus antigen**

In order to determine whether a high m.o.i. would overcome the restriction of HCMV growth on R970 cells, monolayer cultures of R970 and MRC-5 cells were infected with HCMV at various m.o.i. The number of cells containing virus antigen at 4 days p.i., as determined by FA staining, was considered evidence of virus infection. As shown in Fig. 4, at a multiplicity of over 100, virtually all R970 cells became infected and the number of infected cells decreased as the m.o.i. decreased. In contrast, 100% of the MRC-5 cells became infected even at a multiplicity of 1.

Cultures infected with a high multiplicity showed failure of cell growth and multiplication. These observations suggest that R970 cells required a higher m.o.i. to become infected than did MRC-5 cells and also that immunity should be overcome by infecting the cell at high m.o.i.

**Ability to carry HCMV in R970 and MRC-5 cells**

The above experiments studied the growth of HCMV in R970 cells over a relatively short period. To investigate the fate of infected cultures, R970 and MRC-5 cells were infected with various multiplicities. Infected cultures were then split once a week at a ratio of 1:4. Infected cultures stopped growing at different splitting levels depending on the m.o.i.
Cultures infected with multiplicities of 1, 0.1 or 0.01 ceased to grow at 3, 5 and 7 weeks, respectively. In MRC-5 cultures, cells stopped growing after the first split even with a low m.o.i. When R970 cultures ceased growing, virtually all of the cells showed typical inclusion bodies in the nucleus (Fig. 5).

**Infection of synchronized R970 cells with HCMV**

Synchronized R970 cells were obtained with the double thymidine block procedure described in Methods. When cultures were released from the second thymidine block cells were synchronized in the S period, as demonstrated by the increased uptake of thymidine
Fig. 6. Synchronization of R970 cells. The cells were synchronized as described in Methods. 
$^3$H-thymidine (1 μCi/ml) was added at various intervals after a second thymidine treatment. After a 
1 h labelling period, cultures were washed three times with cold PBS. Cells were solubilized with a 
1 % of solution of SDS. Cold TCA was then added to a final concentration of 5 %. The precipitates 
were collected on Milipore filter disks and radioactivity was assayed. The mitotic indices were 
also counted by fixing cultures and staining them with Giemsa solution. Arrows indicate the 
time of infection. ●—●, $^3$H-thymidine uptake; ○ -- ○, mitotic index.

Table 2. Effect of time of infection on development of virus antigen in R970 cells*

<table>
<thead>
<tr>
<th>Time of infection after</th>
<th>% of cells containing virus antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>release of thymidine</td>
<td>m.o.i. = 20</td>
</tr>
<tr>
<td>(h)</td>
<td></td>
</tr>
<tr>
<td>0–1</td>
<td>46.0</td>
</tr>
<tr>
<td>2–3</td>
<td>69.5</td>
</tr>
<tr>
<td>7–8</td>
<td>77.0</td>
</tr>
<tr>
<td>11–12</td>
<td>14.0</td>
</tr>
<tr>
<td>non-synchronized cells</td>
<td>28.0</td>
</tr>
</tbody>
</table>

* Synchronized R970 cells were infected with HCMV at the time indicated after release of thymidine. 
One h after adsorption, cells were washed and placed in maintenance media. At 3 days p.i. infectious centres 
were determined by FA staining.

between 2 and 8 h (Fig. 6). The number of cells in mitosis reached a peak (26%) 14 h after 
thymidine release.

Synchronized cells were infected with HCMV at the following stages of their cycle: 
G₁ (0 to 2 h), S (2 to 8 h) and M (8 to 12 h). One h after virus adsorption, cells were washed 
and placed into maintenance medium. At 3 days p.i. infectious centres were determined by 
FA staining. The results are summarized in Table 2. The highest number of infected cells 
was obtained when cells were infected during the S phase. The rate of virus adsorption 
during different phases of the cell cycle was also determined, as described in the Methods. 
No significant differences were observed between different phases of the cell cycle, or between 
synchronized and non-synchronized cells (data not shown).

Defective virus and interferon production

Interferon production, involvement of defective virus and heterogenicity of cell population 
were also studied as possible causes of restricted virus growth. Supernatant from both 
infected MRC-5 and R970 cells were harvested and assayed for interferon, as described in 
Methods. No significant plaque reduction was demonstrated, even at a 1:4 dilution of 
supernatant in either cell system (data not shown). The possible interference of defective
virus with standard virus was determined as follows. MRC-5 cells infected with HCMV grown in R97o cells was challenged with standard HCMV. After 4 days of incubation, infectivity in the cultures was compared to that in cultures infected with standard virus only. No interference was observed. Eighteen clones from R97o cells were established as described in the Methods. Those clones were infected with HCMV and infectious centres were determined. No significant difference of susceptibility to the infection was observed among the clones.

**DISCUSSION**

The results of the present study indicate that HCMV showed restricted growth in R97o cells and that the mechanism involved could be an inefficient infective process and/or a block of virus replication in early stages of the infectious process. This conclusion is supported by experiments showing that: (i) the early c.p.e. resulting from the synthesis of a variety of early proteins (Michelson-Fiske et al. 1977; Stinski, 1978), was not observed in infected R97o cells; (ii) the adsorption of input virus to R97o cells was not found to be reduced in comparison with fully permissive MRC-5 cells, although our data does not indicate whether HCMV is successfully uncoating; (iii) restriction of infection was overcome when R97o cells were infected at a multiplicity of over 100 p.f.u./cell; (iv) the infected R97o cells do not form appreciable quantities of HCMV DNA.

Reports on the abortive infection of various cells by HCMV have been made, but none has found the limiting factor to be the inability of the virus to attach to the plasma membrane (Fiorietti et al. 1973; Waner & Weller, 1974). These abortive infections resulted in partial expression of the virus genome without replication of HCMV (Fiorietti et al. 1973). However, R97o cells differed from abortive cells in two aspects: (i) the restriction was overcome with a high m.o.i., and (ii) when infected cultures were split all cells became infected and eventually ceased growing.

The experiments using synchronized cells and the increased number of infected cells resulting from cell splitting suggest that establishment of infection might be more efficient in the S phase of the cell cycle. There are two possible explanations for the efficient establishment of infection in the S phase. One is that the rate of virus adsorption differs during different phases of the cell cycle and the other is that the host factor(s) responsible for virus growth change during the cell cycle. Our data on adsorption rate during the cell cycle rule out the first possibility. This suggests that the development of antigen-positive cells depends upon cell cycle-related changes in the host that might influence the expression of the virus genome. Several reports have appeared showing that virus replication depends upon cell cycle and host enzymes, such as RNA polymerase which is apparently necessary for the initial transcription of early herpes simplex virus (Tennant & Hand, 1970; Eremenko et al. 1972; Yanagi et al. 1977).

Although it has been suggested by us and others (Furukawa et al. 1975b; St Jeor & Hutt, 1977) that HCMV growth is dependent on host cell factors, experiments to confirm this suggestion have been hampered by the difficulty of synchronizing human fibroblasts and the slow growth of HCMV (eclipse period 36 to 48 h).

A variety of alterations might be produced during the cell cycle including ones that affect macromolecular synthesis and the cell surface. In this communication, we could not determine the factor(s) involved in the restriction of HCMV growth in R97o cells. It will be of interest to determine the transcription of HCMV DNA and any of the virus protein in different cell phase.

Since the recent report indicates that HCMV induces the factors responsible for activation of infected cell chromatin which presumably is necessary for virus replication (Kamata et al. 1978), those studies will provide more information regarding the relationship between
host cell function and the fate of the virus genome. The interaction between R970 cells and HCMV also provides a model of HCMV growth in the latent and/or carrier state in vivo.

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REFERENCES


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