Inhibition of a Complete Replication Cycle of Human Cytomegalovirus in Actinomycin Pre-treated Cells

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SUMMARY

The study of human cytomegalovirus (HCMV) in cultures of human embryo lung fibroblasts, pre-treated with actinomycin D, has shown that under these conditions the virus infection does not proceed beyond the 'early' events of the virus replication cycle.

In the same experimental conditions the growth of poliovirus type 1, vaccinia virus and herpes simplex type 1 virus, was completely unaffected.

These results suggest that the complete HCMV replication cycle requires some cellular function(s) between early transcription of the input virus genome and virus DNA synthesis.

The human cytomegalovirus (HCMV) exhibits the strict species and cell type dependency of the other mammalian cytomegaloviruses (Benyesh-Melnick, 1969) and can be grown only in cultures of human fibroblasts, where its replication cycle is characterized by an exceptionally long eclipse period (Plummer et al. 1969). In contrast to what happens in cells infected by other cytocidal herpesviruses, the host cell macromolecular syntheses are not inhibited.

Furthermore, cells infected by HCMV present an increased RNA (Tanaka et al. 1975) and DNA synthesis (St Jeor et al. 1974) and a stimulation of DNA polymerase (Huang, 1975) and thymidine kinase activities (Estes & Huang, 1977), suggesting a possible involvement of cellular functions in virus replication. However, the experimental evidence available on this point is still scanty and controversial.

Plotkin and colleagues (Tanaka et al. 1975) have observed that HCMV stimulates the total RNA synthesis of the host cell, beginning at 24 h p.i. and have obtained some evidence that this stimulation is linked to protein synthesis in the early stages of infection.

Rapp and colleagues (St Jeor et al. 1974; St Jeor & Hutt, 1977) have shown, that in HCMV-infected cells, cellular DNA synthesis is stimulated and suggest that HCMV replication is dependent upon cell DNA synthesis and cell cycle. In contrast, DeMarchi & Kaplan (1976, 1977) claim that the replication of HCMV is independent of cellular DNA synthesis and that the two events are mutually exclusive.

The research reported in this paper demonstrates that in human fibroblasts pre-treated with actinomycin D and subsequently infected with HCMV, only the early events of the replication cycle are expressed, which strongly suggests the necessity of some cellular function(s) for productive virus replication.

Human embryo lung fibroblasts (HEL) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% foetal calf serum and antibiotics, and used between the 15th and 26th passage.

The AD 169 strain of HCMV was employed in all the experiments. The virus was propagated in confluent HEL monolayers. When maximum c.p.e. was present (between 10
and 14 days p.i.) the virus was harvested by freezing and thawing the cultures. The suspension was sonicated for 40 s at a setting of 3 in a Branson B-12 Sonifier and then clarified by centrifugation at 1000 rev/min, for 10 min.

In all the experiments HEL monolayers were infected at a multiplicity of infection (m.o.i.) of 5 to 10 p.f.u./cell. After 2 h adsorption at 36°C the inoculum was removed and replaced with MEM containing 2% (v/v) serum. The virus titre was calculated either by plaque assay (Wentworth & French, 1970) or by determination of the TCID₉₀ (Reed & Muench, 1938).

As a control the following viruses were employed: herpes simplex type I strain M.T. 11, vaccinia virus strain IHD and poliovirus type 1 (Brunhilde).

Actinomycin D pre-treatment of the cell cultures was performed as follows: confluent HEL monolayers were re-fed with MEM, without serum, containing 0.5 µg/ml of actinomycin D (Merck, Sharp & Dohme) and maintained overnight at 36°C. The cells were then washed several times with MEM supplemented with 2% (v/v) serum and left for 2 h at 36°C with the same medium: this last procedure was repeated three times before virus inoculation.

Actinomycin D pre-treated and control HEL cell cultures, both infected and mock-infected, were checked for RNA and DNA synthesis as follows: to label RNA, two Falcon flasks (25 cm²) of cell cultures were pulse-labelled with 3 µCi/ml of 5-³H-uridine (sp. act. 5 Ci/mmol) for 2 h. After labelling, the cultures were washed three times with cold PBS and then lysed with 1% (w/v) sodium dodecyl sulphate in PBS.

The sample was precipitated with 10% (w/v) trichloracetic acid (TCA), collected on Whatman GF/C filters washed with 5% (w/v) TCA, dried and counted in Unisolve using a Beckman 3150 T liquid scintillation counter. To label DNA two Falcon flasks were pulse labelled with 3µCi/ml of methyl-³H-thymidine (sp. act. 22 Ci/mmol) for 2 h. After labelling the cultures were washed three times with cold PBS, lysed with 0.1 M-sodium hydroxide, and counted as previously described.

At various times after infection duplicate HEL cultures grown on glass coverslips in Leighton tubes were fixed in situ with 2.5% (v/v) glutaraldehyde in 0.1 M-phosphate buffer, pH 7.3, at 4°C, postfixed in 1% (w/v) OsO₄ in veronal buffer dehydrated and embedded as previously described (Laschi & Rizzoli, 1968). Ultrathin sections, double stained with uranyl acetate and lead citrate, were examined in a Jeol Jem 100b electron microscope.

Immediate early antigens (IEA; Michelson-Fiske et al. 1977), early antigens (EA; The et al. 1974) and late antigens (LA; Rapp et al. 1963) were detected by indirect immunofluorescence. Nuclear antigens (NA; Geder, 1976) were studied by anti-complement immunofluorescence staining.

Selected human sera with high titre of antibodies against the different HCMV antigens were used in the various tests.

The actinomycin D treatment inhibited about 60% of cellular RNA synthesis up to at least 72 h after the removal of the drug. In these conditions the growth of poliovirus type 1, vaccinia virus and herpes simplex type 1 virus was completely unaffected (data not shown).

The electron microscope appearance of virion morphogenesis was identical in actinomycin D pre-treated and in mock-treated cell cultures with all the viruses which were used as a control. The results of the observations in herpes simplex virus-infected cells, at 36 h.p.i., are shown in Fig. 1. Moreover, the total virus yields in actinomycin D pre-treated cells, examined at 48 h.p.i. (poliovirus type 1) and at 72 h.p.i. (vaccinia virus and herpes simplex virus) were exactly the same as in mock-treated cell cultures (data not shown). In addition, regular growth of herpes simplex virus is obtained in cells pre-treated with actinomycin D 36 h before infection.
Fig. 1. Herpes simplex type 1 and human cytomegalovirus infected HEL fibroblasts examined in the electron microscope 36 h and 7 days p.i. respectively. (a) Herpes simplex virus nucleocapsids in the nuclei of untreated control HEL cells. (b) Herpes simplex nucleocapsids in the nuclei of actinomycin D pre-treated HEL cells. (c) Cytomegalovirus nucleocapsids in the nuclei of untreated control HEL cells. (d) Absence of virus structures in the nuclei of actinomycin D pre-treated HEL cells infected with HCMV. Magnification × 24 000.
Table 1. *HCMV*-specific events, virus yield and RNA and DNA synthesis in cell cultures pre-treated with actinomycin D

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Cell rounding</th>
<th>Immediate early</th>
<th>Nuclear</th>
<th>Early</th>
<th>Late</th>
<th>Virus yield*</th>
<th>²H-uridine incorporation (% of control cells) h p.i.</th>
<th>²H-thymidine incorporation (% of control cells) h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D pre-treated cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>0†</td>
<td>38 41 45</td>
<td>45 50 75</td>
</tr>
<tr>
<td>Mock pre-treated cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>10⁶-²</td>
<td>40 395 405</td>
<td>55 240 1350</td>
</tr>
</tbody>
</table>

*TCID₉₀/0·2 ml at 6 days p.i.
† < 10⁴ TCID₉₀/0·2 ml.
The results obtained with HCMV were consistently different. No infectious virus was recovered from the actinomycin D pre-treated cell cultures harvested at 6 days p.i. while in mock-treated cells the virus yield was $10^{6.2}$ TCID_{50}/0.2 ml (Table I) and no virus structures were observed in actinomycin D pre-treated cells examined in the electron microscope 7 days p.i. (Fig. 1). Furthermore, the increase in RNA and DNA synthesis demonstrable in the HCMV-infected mock-treated cell cultures, was completely suppressed in actinomycin D pre-treated cells (Table I).

In spite of these results, however, a number of HCMV-specific events could be observed in actinomycin D pre-treated cell cultures at various times p.i. Cell rounding, which is known to occur long before any evidence of virus replication (Furukawa et al. 1973) was observed, beginning at 8 h p.i. and progressing throughout the entire observation period, involving almost all the cells present in the cultures. Indirect immunofluorescence and anti-complement immunofluorescence studies with human sera reactive with the various HCMV-specific antigens, revealed the presence of 'immediate early antigens' 1 h p.i. of 'nuclear antigens' 8 h p.i. and of 'early antigens' 32 h p.i. without any appreciable, difference between actinomycin D pre-treated and mock-treated cell cultures (Table I).

No 'late antigens' which were detected in mock treated cell cultures beginning at 72 h p.i., were observed in actinomycin D pre-treated cells examined up to 5 days after infection.

The results obtained here demonstrate that in actinomycin D pre-treated cells HCMV infection cannot proceed beyond the early events of the virus replication cycle. In the same conditions, the normal growth of viruses used as control (namely poliovirus type I, vaccinia virus and herpes simplex type I virus) rules out the possible intervention of aspecific cellular damage and/or of unbound actinomycin D in the results obtained with the HCMV.

We believe, therefore, that our results indicate that the complete HCMV replication cycle depends upon some cellular function(s), which is required between early transcription of the input virus genome and virus DNA synthesis, and which is severely impaired even by the incomplete inhibition of cellular RNA synthesis obtained under our experimental conditions.

It is possible that the inhibition of HCMV replication by rifampin reported by Furukawa et al. (1975) could be interpreted as a consequence of the same phenomenon, as it has been shown that rifamycin derivatives are not without effect on eukaryotic transcribing enzymes (Riva et al. 1972; Lancini & Zanichelli, 1977).

The cell function(s) required by HCMV and suppressed in actinomycin D pre-treated cells cannot be identified at the moment. Certainly the results we have obtained cannot be interpreted as a consequence of an impaired synthesis of cellular RNA polymerase B which has been shown to transcribe the genome of other herpesviruses (Ben-Zeev & Becker, 1977; Costanzo et al. 1977) both because at least some 'early' transcription of HCMV genome must have occurred in actinomycin D pre-treated cells, and because of the unaffected growth of herpes simplex virus under the same conditions.

The demonstration of the cell-dependence of the HCMV replication can offer a useful model for an experimental approach to the study of the mechanism of the species and cell type specificity of this group of viruses.

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