Replication of Human Cytomegalovirus at Supra-optimal Temperatures is Dependent on the Virus Strain, Multiplicity of Infection and Phase of Virus Replication

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SUMMARY

The kinetics of replication of five strains of human cytomegalovirus (CMV) were studied to determine the influence of (i) temperature, (ii) virus strain, (iii) m.o.i. and (iv) cell type. Relative to growth at 37 °C (m.o.i. = 3 to 9) eclipse periods were extended from 24 to 48 h at 33 °C and to 72 h at 40.5 °C. Yields were reduced at 33 °C and almost eliminated at 40.5 °C. No replication occurred in most instances at 40.5 °C and with 0.05 p.f.u./cell. Temperature shift studies (40.5 to 37 °C) indicated that the block to replication at 40.5 °C occurred about 12 to 16 h p.i. resulting in little synthesis of CMV DNA or late antigens. The degree of inhibition of late functions at 40.5 °C is virus strain and m.o.i. dependent, but is not dependent on the type of fibroblastic cell used. These data suggest that persistent CMV infections are favoured at 40.5 °C.

INTRODUCTION

It has been reported previously (Gönczöl et al. 1975) that several common laboratory strains of human cytomegalovirus (CMV) did not replicate at a supra-optimal temperature of 40 °C. In another report (Michelson-Fiske et al. 1977) using a different strain of CMV, synthesis of infectious virus was observed at 40 °C, but productive replication was dependent on the cultural passage level of the human embryo lung fibroblasts used as cultural substrate in the study. In our attempts to form persistent infections using supra-optimal temperatures, CMV replication was repeatedly observed in a variety of cells at 40.5 °C (Li & Albrecht, 1979). This study was undertaken to help to resolve the general confusion and conflicting data available about the effects of virus strains, cell types, m.o.i. and temperature on the growth pattern of CMV. In this report, we have examined the kinetics of replication and the extent of expression of five laboratory strains of CMV at 33, 37 and 40.5 °C in human embryo cells derived from the lungs, skin and underlying muscles, and the thyroid glands.
METHODS

Cell cultures. Human embryonic cell cultures were prepared as previously described (Albrecht & Weller, 1980) using γ-irradiated trypsin (γ-trypsin) (Flow Laboratories, Rockville, Md., U.S.A.). Cells utilized in these studies were derived from the thyroid glands (THY), skin and pectoral muscles (SM) and lungs (LU) and were used between the 5th and 20th cultural passage. Although primary cultures of THY were predominantly epithelial in nature (Albrecht & Weller, 1980), at the passage levels employed in these studies they consisted predominantly of fibroblastic cells. Cells derived from SM or LU were similarly fibroblastic in character at these passage levels. Cell cultures were initiated with Eagle’s MEM with Earle’s salts (Grand Island Biological, Grand Island, N.Y., U.S.A.) supplemented with 10% γ-irradiated foetal calf serum (γ-FCS; Flow Laboratories), 0.075% NaHCO₃, 100 units/ml penicillin and 100 μg/ml streptomycin. When cells became confluent, the cultures were maintained with Eagle’s MEM with Earle’s salts supplemented with 2% γ-FCS (LU) or 5% γ-FCS (SM, THY) and 0.15% NaHCO₃. Antibiotics were not included in the maintenance medium. For subculture, cells were dissociated with 0.25% γ-trypsin, washed once with initiating medium and inoculated into 32 oz glass prescription bottles. γ-FCS and γ-trypsin were used to minimize the risk of contamination with mycoplasma. Cell cultures and virus stocks were examined for mycoplasma as previously described (Albrecht & Weller, 1980) and no evidence of contamination was observed.

Virus isolates. The strains of CMV studied in this report were: AD169 (Rowe et al. 1956), C-87 (Benyesh-Melnick et al. 1964), Davis (Weller et al. 1957), Espaillat (Esp.; Weller et al. 1957) and Kerr (Weller et al. 1957). Strain AD169 was provided by Dr W. Rowe at the time of isolation from an inapparent infection of a child undergoing adenoidectomy. C-87 was recovered by Dr M. Benyesh-Melnick from an infant who died following surgery for congenital heart disease (Benyesh-Melnick et al. 1964) and was provided by Dr F. Rapp. The Davis, Esp. and Kerr strains were isolated from the urine or liver tissue of infants with severe cytomegalic inclusion disease.

Stocks of CMV were prepared by inoculating confluent 32 oz bottle cultures of LU, SM or THY with a 1:6 dilution of a previous stock propagated in the same cell type. Such infections provided an m.o.i. of about 0.067 as previously reported in detail (Albrecht & Weller, 1980). Virus stocks had infectivities of about $2 \times 10^6$ to $8 \times 10^6$ p.f.u./ml estimated from plaque assays under agarose in fibroblastic monolayers of SM.

Kinetics of CMV replication. The rate of CMV replication was studied using confluent Leighton tube cultures of LU, SM and THY cells, prepared by inoculating 1 ml of the appropriate cell suspension in initiating medium without antibiotics. When the cells attained confluency, the initiating medium was poured off and the cells were inoculated at an m.o.i. ranging from 0.05 to about 9, depending on the design of the experiment. The cell cultures were incubated for 1 h at 37°C, washed twice with phosphate-buffered saline (PBS, pH 7.2) and inoculated with 1 ml of maintenance medium (0 h p.i.). Infected and control cultures were incubated at 33, 37 or 40.5°C as described in Results. Cultures were maintained at the desired temperature in Wedco model E2-17MM incubators (Wedco, Silver Spring, Md., U.S.A.). Temperatures were monitored with Rustrak model 1400A recorders fitted with P4133 temperature adaptors (30 to 45°C) (Gulton Industries, Manchester, N.H., U.S.A.) and thermistor probes (model 405, Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The recording thermometers were calibrated weekly with a certified ASTM thermometer. At the indicated times, duplicate cultures for each datum point were quickly frozen at $-85$ °C. Before infectivity assay, cultures were rapidly thawed, refrozen, rethawed and then sonicated for 15 s in a Branson model ATH610-8s bath sonicator (Branson Ultrasonics, Shelton, Conn., U.S.A.) and assayed for virus infectivity as described below.
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Plaque assay. The agarose overlay plaque technique of Wentworth & French (1970) as modified (Albrecht & Rapp, 1973) and currently used has been previously described in detail (Albrecht & Weller, 1980). Plaques were counted using a stereomicroscope at a magnification of about 20 diam.

Virus cytopathology. The cytopathogenic effects at 37°C of the CMV strains employed in this report have been previously described (Albrecht et al. 1980). Using similar procedures, the development and progression of CMV cytopathologies were examined at supra-optimal temperatures in SM cells. Photographs were obtained with a Carl Zeiss Photomicroscope III equipped with apochromatically corrected objectives and an aplanatically-achromatically corrected condenser (numerical aperture = 1.4) and Panatomic-X film (Eastman Kodak, Rochester, N.Y., U.S.A.).

Immunofluorescence test. CMV antigens were detected by the indirect immunofluorescence test (Albrecht & Rapp, 1973) using human convalescent sera with and without CMV reactivity and fluorescein-conjugated anti-human globulin goat globulin (H and L chain-specific) (Hyland, Costa Mesa, Calif., U.S.A.). Infected and control cells grown on coverslips were washed three times in PBS, air dried and fixed at appropriate times after infection for 10 min in acetone at 0°C. Human convalescent sera were reacted with premoistened cells for 30 min at ambient temperature and the unreacted material was washed off with PBS. The fluorescein conjugates were reacted with cells for an additional 30 min and the excess was removed as before. After drying, the cells were mounted in glycerol-PBS and examined microscopically with a u.v. light source.

Isolation and analysis of virus DNA. LU or SM cells were grown to confluency in 25 cm² plastic flasks. Following infection of the cells with about 1 to 4 p.f.u./cell, the cultures were transferred to maintenance medium. Virus and cell DNA were labelled with methyl-³H-thymidine (New England Nuclear, Boston, Mass., U.S.A.: 52-91 Ci/mmol) for 24 h periods as previously described (Albrecht et al. 1976) using 10 μCi/ml. Cell and virus DNA were released by the method of Crouch & Rapp (1972a) using an overnight incubation of the cell lysate in 0.1% Sarkosyl NL30 (Chemical Additives Co., Farmingville, N.Y., U.S.A.), 0.02 M-EDTA and 0.1% preincubated Pronase (Calbiochem, B grade, La Jolla, Calif., U.S.A.). Virus and cell DNA were separated in CsCl gradients (St. Jeor et al. 1974) using a 40-3 rotor (Beckman Instruments, Palo Alto, Calif., U.S.A.) at 30,000 rev/min for 66 h. Fractions were collected from the bottom of gradients and acid-insoluble radioactivity was measured on paper filters in toluene containing 0.25% Omnifluor (New England Nuclear) with a Beckman LS-9000 liquid scintillation spectrophotometer.

RESULTS

Kinetics of CMV multiplication at 37°C

The relative rates of CMV replication were determined at 37°C at moderately high m.o.i. (3 to 8) as described in Methods. Infectivities obtained in cultures of LU, SM and THY cells at 37°C were plotted as a function of time. Only one representative of these kinetic plots (SM) is shown (Fig. 1), since the rates of replication were essentially the same in the three cell types. Input levels of infectivity were recovered about 48 h p.i. and the eclipse period apparently ended about 24 h p.i. regardless of the virus strain or cell type. Maximum levels of infectivity were usually reached about 96 h p.i. and from this time through 192 h p.i. the infectivities of the five CMV strains remained relatively constant. In these studies at 37°C, strains AD169 and C-87 replicated to the highest relative levels of infectivity, about 1 × 10⁷ to 5 × 10⁷ p.f.u./ml. The Davis and Kerr strains typically reached maximum levels of infectivity about 10- to 20-fold less. Maximum levels of infectivity attained were 100-fold or more above input.
The rate of replication of CMV at 33 °C was examined in the same cell types as before. The kinetic curves at 33 °C were similar in the three cell types as they had been at 37 °C. Data from a representative experiment in THY cells are plotted in Fig. 2. In contrast to the data seen at 37 °C, the eclipse period was extended to 48 h p.i. and maximum infectivities fell off gradually after 96 h p.i. Maximum virus yields were lower relative to 37 °C, achieving a minimum of a 10-fold increase over input inocula. Relative increases of infectivity over input were much less for strains AD169 and C-87 relative to Davis or Kerr.

**Kinetics of CMV replication at supra-optimal temperature**

Repeated experiments at 40·5 °C in LU, SM and THY at moderate m.o.i. (0·1 to 1·0) gave variable results. Typically, strains AD169, Davis and Esp. failed to replicate. Replication of C-87 was observed in four of six experiments, while replication of strain Kerr consistently occurred. It was determined that the remaining variability did not correlate with the passage level of the host cells as Michelson-Fiske et al. (1977) had observed with one strain of CMV, but with the input m.o.i. of the CMV inoculum as demonstrated in the following experiments.

SM or THY cells were infected with either undiluted virus resulting in a moderately high m.o.i. (about 3 to 9) or with an m.o.i. of 0·05. The results of one experiment using this protocol with THY cells are shown (Fig. 3). At the higher m.o.i. all five CMV strains replicated (Fig. 3a). However, the initiation of virus replication was substantially retarded when compared to replication at 33 or 37 °C, excepting strain Kerr. In Kerr-infected cells, the eclipse period apparently ended shortly after 48 h p.i. when an exponential phase of increase in virus infectivity began. Eclipse of virus persisted for about 24 h longer in cells infected with the other four strains. The rate of virus replication in Davis-infected cells was slower than that of the other strains. Maximum levels of infectivity attained at 40·5 °C with moderately high m.o.i. were reduced to about 60- to 2800-fold relative to those observed at 37 °C (Table 1).
Fig. 3. The kinetics of replication at 40.5 °C of human CMV at (a) moderately high m.o.i. using strains AD169 (●—●, 5 p.f.u./cell), C-87 (○—○, 6.2 p.f.u./cell), Davis (□—□, 6.5 p.f.u./cell), Esp. (△—△, 7.3 p.f.u./cell) and Kerr (▲—▲, 8.2 p.f.u./cell) or at (b) lower m.o.i. [0.05 p.f.u./cell: symbols for CMV strains as for (a)] in human embryo thyroid cells.

Table 1. Inhibition of CMV yield at 40.5 °C

<table>
<thead>
<tr>
<th>CMV strain</th>
<th>Cells</th>
<th>Yield* (p.f.u./ml) at 37 °C</th>
<th>Yield* (p.f.u./ml) at 40.5 °C</th>
<th>Fold inhibition of CMV yield at 40.5 °C relative to 37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD169</td>
<td>SM</td>
<td>1.2 x 10^7</td>
<td>4.3 x 10^3</td>
<td>2791</td>
</tr>
<tr>
<td>C-87</td>
<td>SM</td>
<td>1.5 x 10^6</td>
<td>1.5 x 10^4</td>
<td>1000</td>
</tr>
<tr>
<td>Davis</td>
<td>SM</td>
<td>4.1 x 10^6</td>
<td>4.6 x 10^3</td>
<td>891</td>
</tr>
<tr>
<td>Esp.</td>
<td>SM</td>
<td>3.4 x 10^6</td>
<td>1.5 x 10^4</td>
<td>227</td>
</tr>
<tr>
<td>Kerr</td>
<td>SM</td>
<td>6.7 x 10^5</td>
<td>1.1 x 10^4</td>
<td>61</td>
</tr>
<tr>
<td>AD169</td>
<td>THY</td>
<td>3.1 x 10^7</td>
<td>1.9 x 10^4</td>
<td>1632</td>
</tr>
<tr>
<td>C-87</td>
<td>THY</td>
<td>4.7 x 10^6</td>
<td>2.6 x 10^4</td>
<td>181</td>
</tr>
<tr>
<td>Davis</td>
<td>THY</td>
<td>6.2 x 10^6</td>
<td>6.4 x 10^3</td>
<td>970</td>
</tr>
<tr>
<td>Esp.</td>
<td>THY</td>
<td>7.4 x 10^6</td>
<td>6.3 x 10^4</td>
<td>117</td>
</tr>
<tr>
<td>Kerr</td>
<td>THY</td>
<td>2.3 x 10^5</td>
<td>8.5 x 10^3</td>
<td>270</td>
</tr>
</tbody>
</table>

* Determined 144 h p.i. using initial m.o.i. of 3 to 8.

The results obtained in cultures infected with a low m.o.i. (0.05) and maintained at 40.5 °C (Fig. 3b) were strikingly different from those observed at higher m.o.i. (Fig. 3a). With the exception of cultures infected with strain Kerr, no evidence of virus replication was observed. On the contrary, a family of curves illustrating the loss of infectivity of input virus was obtained, which was quite similar for the five CMV strains. Newly replicated infectious Kerr was not detected until 144 h p.i. Replication of strain Kerr was a consistent finding in four experiments at this low m.o.i., whereas replication of other strains (C-87, AD169) was variable, but always at low levels when evident. Esp. and Davis did not replicate at 40.5 °C when infected at a low m.o.i. Thus, CMV replication at this supra-optimal temperature was dependent on both the virus strain and the m.o.i.

**Sensitivity of the replication of CMV to 40.5 °C**

The effect of elevated temperature on the replication of CMV at 37 °C was measured by shifting infected (3 to 8 p.f.u./cell) and control cultures at various times after infection from 37 to 40.5 °C and then measuring virus infectivities after appropriate intervals. The
data from one experiment using this protocol and strain Davis in SM cells are plotted in Fig. 4(a). The rate of virus replication was substantially affected by shifts from 37 to 40.5°C at any time after infection. Shifts at 0 or 5 h p.i. caused similar delays in the beginning of the exponential phase of virus replication. No delay in the initiation of the exponential phase was observed when cultures were shifted at 24 or 48 h p.i. but maximum levels of infectivity were only 0.023% and 0.57% respectively, of those attained at 37°C. Thereafter, the level of infectivity declined in these cultures. An exponential loss of virus infectivity was observed following shifts to 40.5°C at 72 or 96 h p.i.

The type of host cell used did not affect this phenomenon as the same results were obtained using LU or THY cells. However, some distinctive features were observed by substituting the several strains of CMV. Four features of the families of kinetic curves such as those shown (Fig. 4) seem to be dependent on the CMV strain: (i) the length of the eclipse period following shifts at 0, 5, 24 or 48 h p.i.; (ii) the period of exponential increase in virus infectivity; (iii) the maximum levels of infectivity attained following shifts at 0, 5, 24 or 48 h p.i.; and (iv) the rate of decline of virus infectivity following shifts at 72 or 96 h p.i.

For example, strain Esp.-infected cultures (Fig. 4b) when shifted at 5, 24 or 48 h p.i. to 40.5°C, attained about 10% of the maximum infectivity of cultures maintained at 37°C (about 18- to 400-fold more than that observed with Davis). The delay in the initiation of virus synthesis when Esp. was shifted at 5 h p.i. to 40.5°C was about 24 h shorter and virus synthesis continued for 24 h longer at an exponential rate than in Davis-inoculated cultures. The level of infectivity in Esp.-infected cultures remained relatively constant after shifts to 40.5°C at 72 or 96 h p.i., in contrast to Davis-infected cultures where a rapid decay in infectivity was observed.
CMV replication at supra-optimal temperatures

Fig. 5. The kinetics of replication of human CMV strains (a) Davis (8 p.f.u./cell) or (b) Esp. (3 p.f.u./cell) in human embryo skin muscle cells at 40.5 °C (●——●) and after shifting to 37 °C at various times p.i. (arrows; ●——●, 0 h; ○——○, 5 h; △——△, 24 h; ▲——▲, 48 h; △——△, 72 h; ○——○, 96 h). In order to obtain a compression of scale, the abscissa is measured in h after shift of temperature. The unshifted curve at 40.5 °C (●——●) and the curve obtained by temperature shift at 0 h p.i. (●——●) to 37 °C in (a) and (b) are derived from the same data as those of Fig. 4 with which these parts of the experiments were done in concert.

Table 2. Insensitivity of early events in CMV replication to supra-optimal temperature

<table>
<thead>
<tr>
<th>CMV strain</th>
<th>5 h p.i.</th>
<th>24 h p.i.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD169</td>
<td>4.8</td>
<td>12.8</td>
</tr>
<tr>
<td>C-87</td>
<td>3.6</td>
<td>13.9</td>
</tr>
<tr>
<td>Davis</td>
<td>4.1*</td>
<td>13.6*</td>
</tr>
<tr>
<td>Esp.</td>
<td>3.1*</td>
<td>14.3*</td>
</tr>
<tr>
<td>Kerr</td>
<td>5.2</td>
<td>15.6</td>
</tr>
</tbody>
</table>

* Average of two experiments.

Persistence of the replicative potential of CMV at 40.5 °C

To determine the relative amount of CMV gene expression and replication at 40.5 °C, the rates of recovery of infectious virus were measured following the initial incubation of CMV-infected cultures at 40.5 °C and later shifted to 37 °C. Data from representative experiments using strains Davis and Esp. at moderately high m.o.i. are plotted (Fig. 5). Several observations can be made from these plots. Cultures inoculated with strain Davis and shifted at 5 or 24 h p.i. had somewhat shorter eclipse periods after down shift and attained the same maximum level of infectivity as cultures incubated entirely at 37 °C. In cultures shifted at 48, 72 or 96 h p.i. the maximum level of infectivity reached was less than 10% of that seen in cultures shifted at or before 24 h p.i.

Kinetic curves obtained with other strains of CMV were similar with regard to the shortened eclipse periods observed following shifts down to 37 °C at 5 or 24 h p.i. (Table 2). These data suggest that a block in CMV replication occurred about 12 to 16 h p.i. and that
early events in CMV replication were relatively insensitive to this temperature. The maximum level of virus infectivity attained following shifts at 48, 72 or 96 h p.i. varied with the infecting strain. For example, Esp.-infected cultures (Fig. 5b) shifted at 48 or 72 h p.i. reached maximum levels of infectivity similar to those observed in cultures maintained at 37 °C. The Esp.-infected cultures shifted at 96 h only attained about 25% of the maximum infectivity observed in control cultures.

**Development of CMV cytopathology at supra-optimal temperature**

The effects of elevated temperature on the replication of CMV in individual cells were investigated by examining the development of virus cytopathologies in coverslip cultures of SM prepared as described in Methods. At moderately high m.o.i. (3 to 8) and 40.5 °C, extensive cytopathology developed in greater than 95% of the cells infected with strains AD169, C-87 or Esp. and in about two-thirds of the cells inoculated with strains Davis or Kerr. As suggested by the previous observations (Table 2), early c.p.e. such as cell rounding and cytoplasmic inclusions developed with kinetics similar to those observed at 37 °C (Albrecht et al. 1980). Yet by 96 to 120 h p.i., when the cells were synthesizing virus, nuclear inclusions had developed in only about 60% of the infected cells with other cytopathologies. These eosinophilic nuclear inclusions were considerably smaller in cells maintained at 40.5 °C (Fig. 6a) than in those incubated at 37 °C (Fig. 6b).

**CMV DNA synthesis at 40.5 °C**

The lack of virus nuclear inclusions in some cells and their small size when evident in cells at 40.5 °C suggested that virus DNA synthesis might be considerably reduced at this temperature. To test this hypothesis, SM or LU cells were infected with each of the
five CMV strains at 3 to 7 p.f.u./cell and labelled with methyl-\textsuperscript{3}H-thymidine at a time (72 to 96 h p.i.) at which we have previously shown maximum virus DNA synthesis (Albrecht, 1973). The results of this experiment are summarized in Table 3. It is evident that virus DNA synthesis was reduced by a factor of 4- to 24-fold at 40.5 °C for the five strains. Since it seemed possible that CMV DNA synthesis was delayed at 40.5 °C, as was virus replication, the rate of CMV DNA synthesis at 37 and 40.5 °C was compared using cultures labelled for consecutive 24 h intervals through 144 h p.i. In most instances, synthesis of virus DNA occurred late and the overall amount of radioactivity incorporated was much reduced relative to 37 °C (Fig. 7). Occasionally, virus DNA synthesis was not detected through 144 h p.i. with some strains (particularly Davis).

**Synthesis of CMV antigens at supra-optimal temperature**

Taken together these results suggest that at 40.5 °C, expression of early CMV functions takes place at rates similar to or faster than those observed at 37 °C for the first 12 to 16 h p.i., but thereafter, late virus events are selectively inhibited at the supra-optimal temperature. To test this hypothesis further, the synthesis of CMV antigens was
Table 4. Detection of CMV antigens* in human embryo skin muscle cells at supra-optimal temperature

<table>
<thead>
<tr>
<th>Time p.i. (h)</th>
<th>0-05 p.f.u./cell†</th>
<th>3 p.f.u./cell†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37 °C</td>
<td>40-5 °C</td>
</tr>
<tr>
<td>5</td>
<td>&lt; 0-04</td>
<td>&lt; 0-04</td>
</tr>
<tr>
<td>24</td>
<td>1-6</td>
<td>3-1</td>
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<tr>
<td>48</td>
<td>2-1</td>
<td>11-2</td>
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<tr>
<td>96</td>
<td>4-2</td>
<td>7-2</td>
</tr>
<tr>
<td>144</td>
<td>10-9</td>
<td>6-2</td>
</tr>
</tbody>
</table>

* CMV antigens were detected by the indirect immunofluorescence test with human convalescent serum and fluorescein-conjugated anti-human globulin (H and L chain-specific) goat globulin.
† Human embryo skin muscle cells were infected with CMV strain Davis.

compared at 40-5 and 37 °C. These studies were also performed to determine whether virus expression at 40-5 °C was limited to those cells displaying virus cytopathology. Strain Davis was chosen for these studies since virus cytopathology failed to develop in about one-third of the cells infected with strains Davis or Kerr at 40-5 °C with virus multiplicities which produced cytopathology in greater than 95% of similar cells at 37 °C. CMV antigen synthesis was observed sooner in a larger portion of the cells at 40-5 than at 37 °C (Table 4) at either high or low m.o.i. Although the fraction of cells synthesizing virus antigens at 40-5 and 37 °C was found to be similar with high m.o.i. from 24 to 144 h p.i. (Table 4), the quality of the staining was quite dependent on the temperature of incubation.

During the first 24 h p.i., the quality of the fluorescence was similar in Davis-infected cells maintained at either 37 or 40-5 °C (Fig. 8a, b). After 48 h incubation at 37 °C, the intensity of fluorescence was much greater than it had been at 24 h p.i. and consisted in part of large, brightly fluorescent nuclear and cytoplasmic inclusions. By contrast, infected cells maintained at 40-5 °C were stained with an intensity that had not increased beyond that observed after 24 h incubation at either temperature. Nuclear inclusions were only infrequently encountered at 40-5 °C in cells infected with 3 p.f.u./cell and the size of these inclusions was significantly reduced. Nuclear inclusions were rarely encountered, if at all, in cells exposed to 1 p.f.u./cell or less.

After 96 or 144 h p.i. the contrast between CMV antigen synthesis at 37 and 40-5 °C was much greater than it had been at 48 h p.i. The fluorescence in cells maintained at 37 °C was much brighter than at 48 h p.i. (Fig. 8c). In cells incubated at 40-5 °C for 96 h p.i. fluorescence was substantially diminished (Fig. 8d), except for small nuclear bodies and a very dim matrix of fluorescence observed in about 60% of the nuclei. At the decreased film exposure necessary to view the fluorescence in Fig. 8(c), the much weaker fluorescence in Fig. 8(d) hardly shows up at all. The small nuclear bodies evident in Fig. 8(d) were stained more intensely and were somewhat larger at 96 h p.i. than they had been at 24 (Fig. 8a, b) or 48 h p.i. Additional studies using cytosine arabinoside (20 µg/ml) at either 37 or 40-5 °C suggest that the antigen forming these small nuclear bodies is an early CMV protein which continues to be synthesized after virus DNA synthesis would normally begin.

DISCUSSION

The results obtained at 37 °C show that virus eclipse ends and the exponential phase of virus replication begins apparently much earlier than in a number of previous reports (McAllister et al. 1963; Rapp et al. 1963; Smith & De Harven, 1973, 1978; St. Jeor &
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Rapp, 1973). The kinetic curves obtained in this study are, however, in good agreement with several recent studies (Iwasaki et al. 1973; Figueroa et al. 1978) of the replication of strains Towne and AD169 in cells derived from human embryo lungs. They are also consistent with recent studies of the kinetics of virus DNA synthesis (Albrecht, 1973;
St. Jeor et al. 1974; Stinski, 1978) and the development of virus cytopathology (Albrecht et al. 1980). The slower kinetics of CMV replication observed in the earlier reports are possibly related to differing culture conditions or the lower m.o.i. resulting from early CMV stocks which had lower infectivity than those currently available. The type of fibroblastic cell does not influence these results at this temperature or the other temperatures studied.

Replication of CMV at a supra-optimal temperature of 40.5°C was dependent on the m.o.i. and virus strain, and in contrast to the general slowing of virus replication seen at 33°C, shows a block in replication of CMV about 12 to 16 h p.i. (Table 2). At m.o.i. of 3 to 8, all five CMV strains used in this study consistently replicated to moderate levels of infectivity (5 × 10^3 to 4 × 10^5 p.f.u./ml) at 40.5°C (Fig. 3a). With lower multiplicities (0.05 p.f.u./cell), the replication of CMV was strain dependent (Fig. 3b), with only strain Kerr consistently replicating. The dependence of the multiplication of CMV at supra-optimal temperatures on both the virus strain and the m.o.i. may account for some of the contrasting observations in previous reports (Gönczöl et al. 1975; Michelson-Fiske et al. 1977).

Decay of infectivity at 40.5°C was similar for the five CMV strains and was similar to earlier studies (Gönczöl et al. 1975). The limited CMV replication observed at high m.o.i. and the lack of replication at low m.o.i. does not appear to result from lability of CMV virions at 40.5°C (Gönczöl et al. 1975). However, the decreased number of cells with nuclear inclusions and the substantial decrease in the size of these inclusions at 40.5 relative to 37°C suggests an explanation for the reduced virus yields at 40.5°C. These observations imply that virus DNA synthesis is much reduced at 40.5°C and may be entirely blocked in some cells. The reduction of virus DNA synthesis by 74 to 96% in cultures at 40.5°C confirms this supposition. A similar block in virus DNA synthesis was observed by Stevens (1966) with bovine rhinotracheitis virus (BRV), but not by Crouch & Rapp (1972a) in hamster embryo fibroblasts which were non-permissive for the replication of herpes simplex virus type 2 (HSV-2) at 39°C. Apparently, different phases of virus replication may be affected in the study of HSV-2 and that of CMV or BRV. The host cell (e.g. epithelial, fibroblast) may be an important consideration in these studies, since Crouch & Rapp (1972a) obtained contrasting results in rabbit kidney cells.

The timing of the appearance of CMV antigens and the results of the temperature shift experiments indicate that expression of early, in contrast to late, genes occurred with good efficiency at 40.5°C. If this hypothesis were true, then infectious CMV should be produced quickly and efficiently after a shift from 40.5 to 37°C. This in fact was observed (Table 2; Fig. 5). Further support for the contrasting sensitivity of early and late events in CMV replication to supra-optimal temperature comes from examination of the development of virus cytopathology. Early events, such as cell rounding and cytoplasmic inclusions (Iwasaki et al. 1973; Smith & De Harven, 1973; Albrecht et al. 1980), were observed at 40.5°C in cell numbers predictable from the m.o.i. By contrast, CMV nuclear inclusions, which are a late event (McAllister et al. 1963; Goodheart et al. 1964), are much delayed in their appearance and very much reduced in size even at moderately high m.o.i. As the m.o.i. is decreased, synthesis of virus DNA, late virus antigens and infectious virus are both reduced and delayed and eventually appear to be completely blocked.

These observations, however, provide neither an explanation nor an understanding as to why CMV replication occurs to a limited extent at 40.5°C at an m.o.i. of 5, but not at an m.o.i. of 0.05. If the problem is that late genes are expressed very inefficiently at 40.5°C, then the greater the number of copies of each gene present, the more efficient expression will be (gene dosage effect) as was observed in this study. If the above were the case, one might expect that the real critical function blocked at 40.5°C is a control
function responsible for turning on late gene expression. The contrasting observations of late gene products after shifts to 40.5°C at or shortly after CMV inoculation and after virus DNA synthesis has begun (Fig. 4; Fig. 8) may support this hypothesis. This critical function might be either of cell or virus origin. Stimulation of cellular DNA (St. Jeor et al. 1974), RNA (Tanaka et al. 1975) and protein synthesis (Stinski, 1977) appears to play an important role in CMV replication. The observations of De Marchi & Kaplan (1977a) that when this stimulation is blocked, virus yields are adversely affected, may provide additional support for this concept.

Alternatively, perhaps not all of the CMV genomes are identical. It has been previously shown that CMV stocks are composed of a preponderance of defective particles (Smith & Rasmussen, 1963; Ramirez et al. 1979; Stinski et al. 1979) which have biological activity (Albrecht et al. 1974, 1976; De Marchi & Kaplan, 1977b). Some genomes may lack a gene whose function is more important at 40.5 than at 37°C. At the higher m.o.i., every cell would be infected with a virion carrying the necessary gene. However, stocks enriched for defective particles by serial undiluted passage of virus result in restriction of CMV replication and formation of persistent infections (Stinski et al. 1979; Mocarski & Stinski, 1979; Li & Albrecht, 1979 and unpublished data). Since these cells are also infected at high m.o.i. it would seem that an abundance of defective particles might inhibit CMV expression at 40.5°C. Additional studies will be necessary to determine whether a critical function (perhaps cellular) or defective particles are responsible for the influence of m.o.i. on these results.

In any case, supra-optimal temperatures appear to result in circumstances favouring the formation of persistent CMV infections (Li & Albrecht, 1979 and unpublished data). As the temperature is raised, late functions are selectively inhibited resulting in reduced virus yields. One would predict that similar conditions (fever) in vivo could lead to decreasing m.o.i. at the periphery of an infected tissue and then to restriction of the productive replication of CMV while early CMV genes continue to be expressed. Other factors such as antibody, interferon or defective CMV particles might easily contribute to such a phenomenon in vivo.

Ratcliffe (1971) and Crouch & Rapp (1972b) observed a differential sensitivity of the replication of HSV-1 and HSV-2 to supra-optimal temperature. Although such type-associated observations were not made in the present study with CMV, variation in the biological potential of CMV strains (Fig. 3, 4 and 5; Albrecht & Weller, 1980; Albrecht et al. 1980) may be an important aspect in the formation and characteristics of persistent CMV infections. However, the insensitivity of early CMV gene expression to supra-optimal temperature common among all strains tested suggests strong evolutionary selection for maintenance of this characteristic and should facilitate further studies on the mechanisms of persistent CMV infections.

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