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Growth Characteristics of Cytomegalovirus in Human Fibroblasts with Demonstration of Protein Synthesis Early in Viral Replication

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In high-multiplicity infection of human fibroblasts, human cytomegalovirus of WI-38 human diploid cells produced early cell rounding 6 to 24 h after inoculation. This early cell rounding was caused only by inoculation with infectious virions. Inhibitors of protein synthesis, but not DNA inhibitors, prevented this cytopathic effect. Apparently, a new protein is synthesized in infected fibroblasts from about 2 h postinoculation. Infectivity of cell-associated and supernatant infectious virus reached maximal levels at 5 to 7 and 10 days postinoculation, respectively. Synthesis of DNA, infectious virus, complement-fixing antigen, and precipitin antigen all began between 24 and 48 h, with the bulk of synthesis occurring 48 to 96 h postinoculation.

Although human cytomegalovirus (CMV) is related to *Herpesvirus hominis* (12), it differs from the latter in its restricted host range, the close association of the virus with the host cell, and the lability of the infectious virus after cell release (6). These characteristics made high-titer CMV preparation difficult to obtain until recently (3, 21). We applied a high-yield CMV system to the study of one-step virus curves of infectious virus and viral antigens in human fibroblasts in which we noted that cell rounding occurred long before there was any evidence of viral replication. In this paper, we describe some characteristics of this early cytopathic effect (CPE) and show that it is correlated with protein synthesis early in the course of infection.

MATERIALS AND METHODS

Virus. The CMV strain used in this paper was isolated in our laboratory from an infant (Town) with congenital CMV infection. This agent was identified as a strain of CMV by its typical CPE with intranuclear inclusions in tissue culture, by serological studies, and also by the limited spectrum of cell types in which it would grow.

Tissue culture. WI-38 human diploid cells obtained from L. Hayflick were used between the 20th and 30th passage levels. The cells were grown in Eagle minimal essential medium (MEM) supplemented for growth and maintenance with 10% or 2% fetal-calf serum, respectively.

Chemicals. The ^{3}H-thymidine and ^{14}C-L-amino acid mixture was purchased from New England Nuclear Corp. (Boston, Mass.) actinomycin D was from Sigma Chemical Co. (St. Louis, Mo.), and cycloheximide was from Nutritional Biochemicals Corp. (Cleveland, Ohio).

Preparation of nuclear and cytoplasmic fractions. Trypsinized cells were washed in phosphate-buffered saline (PBS) and suspended in 0.5% sodium citrate containing magnesium (1 mM) and calcium (1 mM). After the cells were disrupted in a tight-fitting Dounce homogenizer, the homogenate was centrifuged at 2,000 rpm for 10 min to sediment nuclei. Homogenization was monitored by microscopy observation to insure that all cells have been ruptured.

Labeling of virus. MEM supplemented with 2% fetal-calf serum containing 0.1 μCi of ^{3}H-thymidine per ml was added after a 1-h period for virus adsorption, and the cultures were maintained until the time of harvest.

Procedure for assaying radioactivity. Trichloracetic acid (10%) was added to samples, and the precipitate was filtered on membrane filters (450 μm pore size). After drying the membrane filter, toluene-ethanol-based scintillation fluid was added. The samples were counted in a scintillation spectrometer.

Infectivity assay. Tube cultures of WI-38 cells were used for infectivity assays. Inoculated cultures were incubated stationary at 37 C. The end point of infectivity was read 14 days after infection, and the tissue culture infective dose ($TCID_{50}$) titer was calculated by the Reed and Muench method (15).

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Complement-fixing activity. The complement-fixing (CF) antigen titer was determined by using the microtiter technique (20), four units of standard antisera, and two units of complement.

Precipitin antigen. Infected cells were removed by 0.25% trypsin and 0.1% EDTA. The cell suspension was centrifuged at 1,000 rpm and resuspended in phosphate-buffered saline (pH 7.2) to a concentration of 5%. This suspension was then sonicated for 2 min. The resultant material served as the precipitin antigen. The titer of antigen was expressed as the highest dilution necessary to give a precipitin band against a standard serum.

Cytological study. To observe cytopathology, cells were grown in petri dishes containing cover slips. After cells were fixed on the cover slips, they were stained with May-Grünwald Giemsa.

Preparation and analysis of sucrose and CsCl gradients. Linear sucrose gradients (15 to 60%) in 0.01 M Tris-hydrochloride buffer (pH 7.2) were prepared by using a Buchler gradient maker. Fractions of 1 ml were collected and analyzed for infectivity, CF activity, and radioactivity as described above.

Linear cesium chloride gradients from 1.17 to 1.30 g/ml in Tris-hydrochloride buffer were also prepared in the same manner as the sucrose gradient.

RESULTS

Growth curve of CMV. WI-38 cells were infected at a virus-to-cell multiplicity of 50. After 1 h of adsorption, the cells were washed twice with PBS. At various intervals, supernatant infectious virus, cell-associated infectious virus, cell-associated complement-fixing antigen, and cell-associated precipitin antigen were assayed. The results are shown in Fig. 1.

Viral replication began about 48 h postinfection. Cell-associated virus reached a peak 5 days postinfection, whereas supernatant virus titers attained high levels 7 to 9 days postinfection when 10^6 to 10^8 $TCID_{50}$/ml were present. CF and precipitin antigens first appeared in the cells at 2 and 3 days postinfection, respectively. Both antigens reached their highest titers at 5 days postinfection. Inclusion bodies were not seen until 3 days postinfection, even with high multiplicity of infection. However, an early CPE, characterized by rounded cells without inclusion bodies, was noted beginning at 6 to 12 h postinfection. Despite the cell rounding, there were no inclusion bodies, and new virus was not detected at this stage. We call this effect "early CPE" and describe it in more detail below.

Comparison of virus level in the nucleus and cytoplasm. At various times postinfection, separated fractions of nucleus and cytoplasm were assayed for infectivity. The amounts of infectious virus recovered from nuclei and cytoplasm were similar for 48 h postinfection. A dissociation of infectivity was noticed at 72 h when the nuclear infectivity reached a plateau, and cytoplasmic infectivity continued to rise (Fig. 2).

Because douncing may not provide completely "clean" nuclei, we interpret these data only to show the relationship between virus closely associated to the nucleus and virus easily recovered from the cytoplasm.

DNA and protein synthesis in infected cells. At various times postinfection, the maintenance media were replaced with media containing ^{3}H-thymidine or a mixture of ^{14}C-labeled L-amino acids. After incubation with the label for 1 h the cells were washed, scraped, resuspended in PBS, and sonicated for 2 min. The protein concentrations and precipitated radioactivity were determined. The data suggest that DNA synthesis did not begin until just before 48 h postinfection (Fig. 3). A different pattern was seen for amino acid incorporation which increased 12 h postinfection and remained elevated until 48 h postinfection.

Development of CF and precipitin antigens. In another experiment, virus was

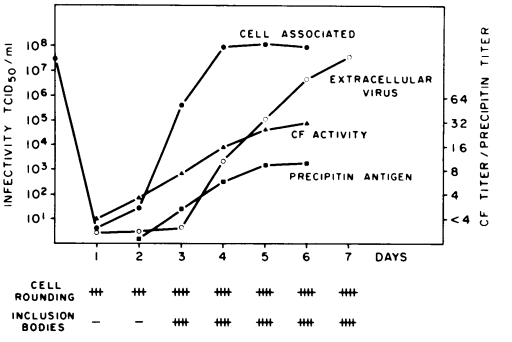

FIG. 1. *Development of infectious, cell-associated virus, early-cell-rounding type of cytopathic effect and inclusion bodies in WI-38 cells inoculated with cytomegalovirus at a multiplicity of infection of 50:1.*

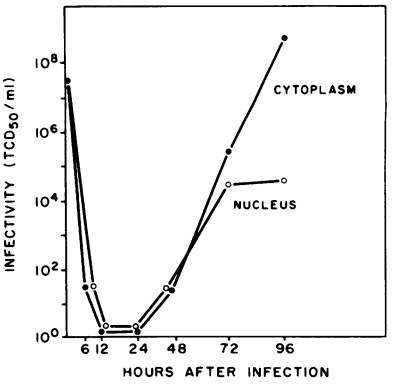

FIG. 2. *Virus recovery from nucleus and cytoplasm. The titers represent the infectivity recovered from 10^6 cells.*

labeled with ^{3}H-thymidine (see Materials and Methods) and harvested at the peak of antigen content, 6 days postinfection. Cells packed by low-speed centrifugation were sonicated in the same manner as for preparation of precipitin antigen. The sonicated virus was layered on preformed 15 to 60% linear sucrose gradients. Centrifugation was carried out for 90 min at 24,000 rpm.

The infectivity peak coincided with that of radioactivity (Fig. 4). Both CF activity and precipitin activity were recovered in two zones, one coinciding with the band of infectious virus and the second localized at the top of the gradient. An incidental fact developed in these experiments was that the sucrose fractions containing the highest peaks of infectivity and radioactive thymidine label had a density of 1.22 g/ml^3 when centrifugation was carried to equilibrium (6 h at 35,000 rpm) in cesium chloride gradients.

Early CPE. May-Grünwald Giemsa staining of the cells 6 to 48 h postinfection revealed only rounded cells with increased basophilia. The percentage of rounded cells was directly dependent on the input multiplicity of the infectious virus. Multiplicities lower than one virus to one cell failed to induce early CPE. Figures 5, 6, and 7 show normal-looking WI-38 cells at the time of inoculation, rounded WI-38 cells 12 h postinfection, and inclusion-body-bearing WI-38 cells 72 h postinfection.

Because it was clear that neither synthesis of viral DNA, infectious virus, CF antigen, nor precipitin antigen could account for the early CPE, we sought to characterize this phenomenon further. The CMV virus inoculum was centrifuged at 22,000 rpm for 1 h. The supernatant fraction and the resuspended pellet were then tested for their ability to produce early CPE. Only the cells inoculated with the pelleted virus showed an early CPE. Incubation of virus inocula at 56 C for 30 min, at 4 C for 48 h, or with equal volumes of ether for 15 min each destroyed CMV infectivity and also eliminated the early CPE. Incubation of the virus inoculum with immunoserum to CMV for 1 h at 37 C eliminated the CPE, but when the same antiserum was applied after virus adsorption, the CPE was not prevented. Although inactivation of the virus inoculum to a level of $10^{3.5}$ $TCID_{50}$ by UV irradiation did not prevent early CPE, dilution of the virus inoculum to the same low dose eliminated it (Table 1).

These results indicated that the early CPE was induced only by the virion itself.

Effect of metabolic inhibitors on early CPE. Three inhibitors of DNA synthesis were tested: 5-fluorodeoxyuridine (FUdR), cytosine arabinoside, and bromodeoxyuridine (BUdR). The cells were treated with either 20 μg of FUdR, 20 μg of cytosine arabinoside, or 100 μg of BUdR, respectively, 24 h before infection, and then they were maintained for 4 days postinfection in the presence of the inhibitors. The same early CPE observed in control cultures occurred in the cultures treated with inhibitors, although synthesis of infectious virus was almost completely inhibited (Table 2). Synthesis of complement-fixing and precipitin antigens was unimpeded in the BUdR-treated cells, but was limited or absent in FUdR- or

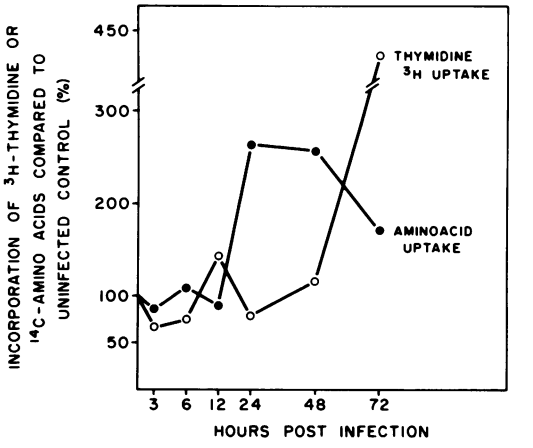

FIG. 3. *Incorporation of labeled amino acid into protein and incorporation of thymidine into DNA at various times after infection with cytomegalovirus. At the points indicated, the media were replaced with Eagle basal minimal essential medium containing one-fifth the usual amount of amino acids plus 0.5 μCi of ^{14}C-L-amino acid mixture or 0.3 μCi of ^{3}H-thymidine per ml. One hour later, the cells were harvested, and the amount of the incorporated label was determined in relation to uninfected controls.*

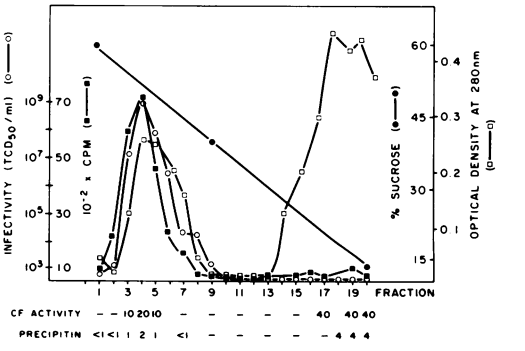

FIG. 4. *Sucrose density gradient centrifugation of ^{3}H-thymidine-labeled cytomegalovirus showing localization of infectivity, complement-fixing, and precipitin activities. The conditions were: 25 ml of 15 to 60% sucrose density gradient, 24,000 rpm, and 90 min in a Spinco SW25.1 rotor.*

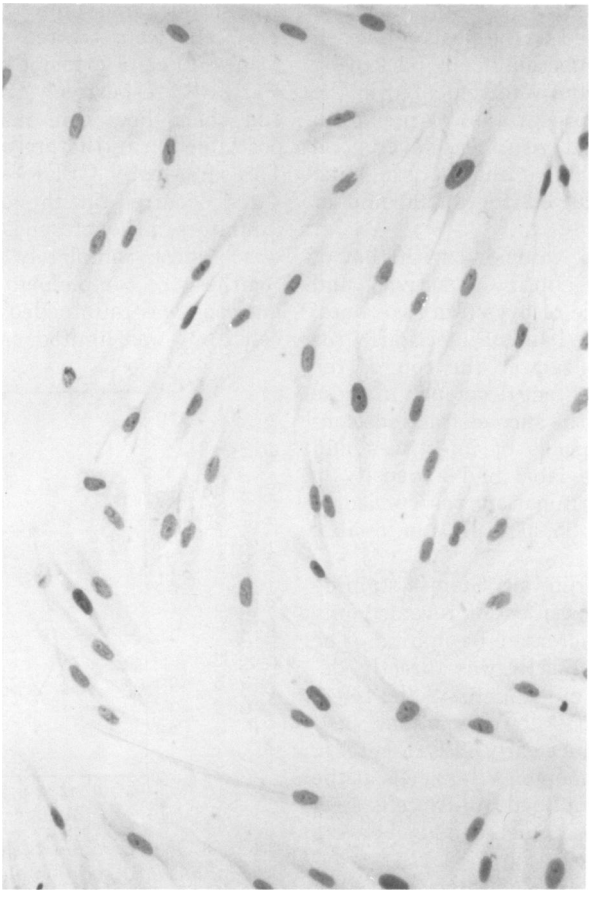

FIG. 5. *Normal WI-38 cells at time of inoculation with cytomegalovirus.*

cytosine arabinoside-treated cells. It was interesting that when FUdR and cytosine arabinoside were removed by changing the maintenance media, infectivity and viral antigens rapidly proceeded to normal levels.

In contrast to the failure of DNA inhibitors to prevent the early CPE, both actinomycin D and cycloheximide, when added before or within 2 h of virus inoculation, completely inhibited it. However, addition of these inhibitors later than 2 h postinfection failed to inhibit the early CPE (Table 3).

Exposure of WI-38 cell cultures to 30 s of UV irradiation resulted in death of the cells as was demonstrated by vital staining 24 h later. Nevertheless, inoculation of these cells with CMV resulted within 12 h postinfection in the same, early CPE as in unirradiated cells.

Immunofluorescence studies. At various intervals postinfection, cells on several cover slips were fixed with cold acetone and then were stained by indirect fluorescent antibody (FA) technique by using human convalescent sera. At 3 h postinfection a granular form of fluorescence was seen in the cytoplasm. At 6 h postinfection, nuclear and perinuclear fluorescence was seen which continued to be present for the rest of the growth cycle. At 12 h postinfection, the cells still fluoresced, but the location of fluorescence was difficult to evaluate because of cell rounding.

To test the specificity of this staining, similar experiments were done in which it was shown that addition of actinomycin D and cycloheximide to infected cultures eliminated early FA activity, whereas FUdR did not.

DISCUSSION

These studies were made possible by the high titers of CMV obtained by serial passage of virus in WI-38 cells. High multiplicity of infection permitted us to do one-step growth curves, in which a delay of 48 h occurred between the addition of the inoculum and the appearance of

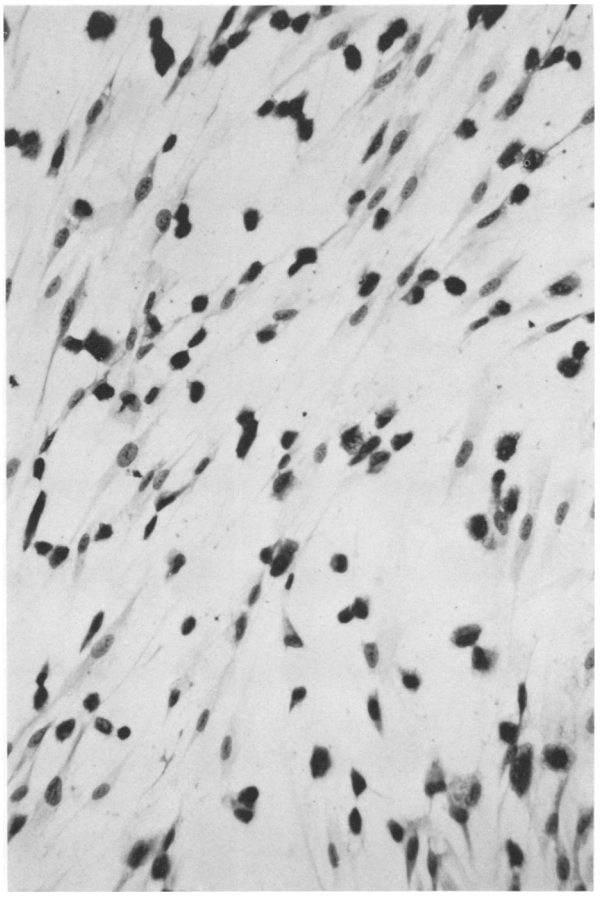

FIG. 6. *WI-38 cells 12 h postinfection showing cell rounding.*

significant amounts of new infectious virus. Virus synthesis then proceeded until 96 h postinfection. Peak extracellular viral yields occurred 3 to 4 days after fullblown cytopathogenicity.

On the basis of a complementary electron microscope study to be published separately (8) and previous reports from other laboratories (11, 18), it can be said that intranuclear particles are not observed until 48 h postinfection and that particles produced in the nucleus soon migrate into the cytoplasm. The long delay between virus entrance and virus synthesis is in contrast to the rapid synthesis of herpes simplex virus in infected cells, which takes place over a period of 18 h (17, 19). Plummer et al. (14) also reported a long eclipse period of 55 h for human CMV.

In our study, the density of whole CMV virus was 1.22 g/ml[3]. This value was identical to that reported by Kim and Carp for murine CMV (10), but differs from the density of 1.18 g/ml[3] reported for human CMV by Chambers et al. (3) based on sucrose gradients.

The finding of greatest interest was that rounding in human fibroblasts infected with high multiplicity of CMV occurs many hours before viral replication takes place. Only the infectious virion induced this early CPE. The fact that inhibitors of protein synthesis, but not inhibitors of DNA synthesis, prevented this early CPE suggests that it is mediated through synthesis of a protein. At this point we do not know whether the protein is eventually incorporated into the virion or is a nonvirion antigen.

The early-cell-rounding type of CPE is apparently also found in nonpermissive CMV infection. Kim and Carp (9) reported that murine CMV induced CPE on nonpermissive human cells, without the later development of infectivity. In our laboratory, guinea pig cells exposed to high-titer human CMV also showed early cell rounding which could be prevented by actinomycin D and cyclohexamide, despite the

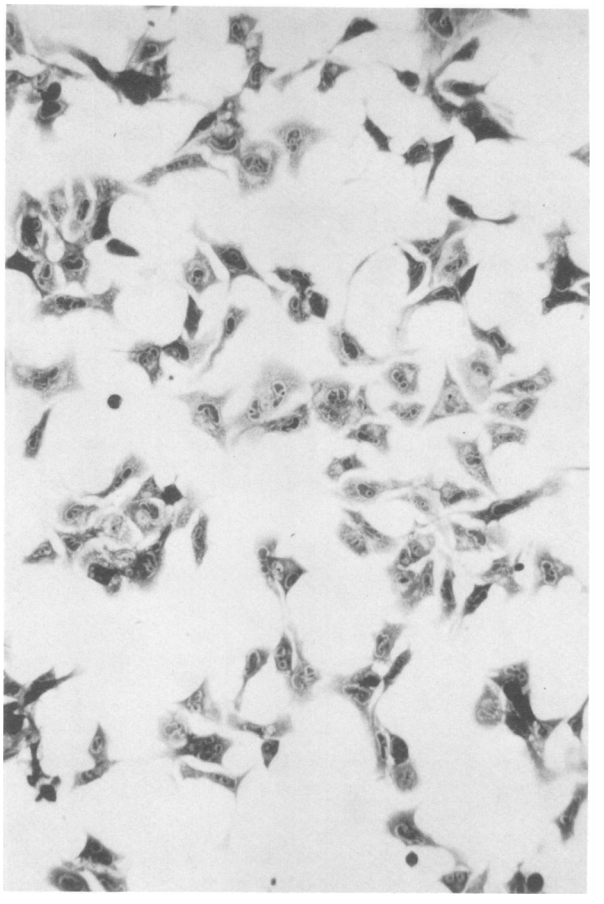

FIG. 7. *WI-38 cells 72 h postinfection showing inclusion bodies.*

TABLE 1. *Effects of various treatments of inoculum on early cytopathic effect (CPE) and virus yield*

Treatment	Early CPE	Infectivity $TCID_{50}$/ml
None	+	$10^{7.5}$
Heat (56 C, 30 min)	–	$<10^{2.0}$
Ether (15 min)	–	$<10^{2.0}$
Ultracentrifugation: pellet	+	$10^{7.5}$
Ultracentrifugation: supernatant fraction	–	$10^{2.5}$
Inactivation at 4 C (2 days)	–	$<10^{2.0}$
Neutralization with antiserum	–	$<10^{2.0}$
UV irradiation (15 min)	+	$10^{3.5}$

TABLE 2. *Effect of BUdR, cytosine arabinoside, and FUdR on early cytopathic effect of CMV*

Addition to medium	Early CPE	Infectivity[b] ($TCID_{50}$)	CFA	Precipitin
None	+	$10^{6.5}$	1:8	+
BUdR (100 μg/ml)	+	$<10^{1.5}$	1:8	+
Cytosine arabinoside (20 μg/ml)	+	$<10^{1.5}$	1:1	–
FUdR (20 μg/ml)	+	$<10^{1.5}$	1:1	–

[a] BUdR, Bromodeoxyuridine; FUdR, 5-fluorodeoxyuridine; CPE, cytopathic effect; CMV, cytomegalovirus; CFA, complement-fixing antigen.

[b] Cells were harvested at 4 days postinfection and sonicated in a 10% suspension.

failure of the cells to produce infectious virus (5).

The early CPE is reminiscent of the effect of adenovirus toxin: (4), but in the case of reference 4, the CPE was caused by a nonvirion substance. Closer to our results is the demonstration by Bablanian et al. (1, 2) that the CPE caused by poliovirus does not occur without prior synthesis of protein which is probably viral coat.

Herpes simplex virus-infected LLC-MK_2 cells show early CPE and cytoplasmic and nuclear fluorescence on staining with convalescent sera

TABLE 3. *Effect of actinomycin D and cycloheximide on early cytopathic effect of cytomegalovirus*

Added agent	Time of addition[a]	CPE[b]	Infectivity ($TCID_{50}$/ml[c])
Actinomycin D (0.2 μg/ml)	−1	−	$<10^{2.0}$
	+1	−	$<10^{2.0}$
	2	+	ND[d]
	6	++	$<10^{2.0}$
	12	+++	$<10^{2.0}$
Cycloheximide (20 μg/ml)	−1	−	$<10^{2.0}$
	+1	−	$<10^{2.0}$
	2	+	ND[d]
	6	+++	$<10^{2.0}$
	12	+++	$<10^{2.0}$

[a] Hours pre- or postinoculation of virus.

[b] CPE, Cytopathic effect; −, no cell rounding; +, 25% cell rounding; ++, 50% cell rounding; +++, ≥75% cell rounding. In the control, the CPE was ≥75% and infectivity was $10^{5.5}$.

[c] Cells were harvested at 3 days postinfection and sonicated in a 10% suspension.

[d] ND, Not done.

at a time that precedes synthesis of new virus (13). New cell surface antigens also have been described in herpes infection (7), at least one of which is formed despite inhibition of DNA synthesis. Several proteins functioning as enzymes appear in herpes-infected cells during the first 6 h postinfection, including thymidine kinase and DNA polymerase (16).

Thus far we have not identified the function of the proteins synthesized early in the replication of CMV.

If it is related to the change in the cell surface that leads to rounding, then it may be a new cell surface antigen analogous to that of herpes simplex virus.

In view of the long eclipse period of CMV, it is important to characterize the new proteins in relation to later viral replication.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Bablanian, R., H. J. Eggers, and I. Tamm. 1965. Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. Virology **26**:100–113.
2. Bablanian, R., H. J. Eggers, and I. Tamm. 1965. Studies on the mechanism of poliovirus-induced cell damage. II. The relation between poliovirus growth and virus-induced morphological changes in cells. Virology **26**:114–121.
3. Chambers, W. R., A. J. Rose, A. S. Rabson, E. H. Bond, and W. T. Hall. 1971. Propagation and purification of high-titer human cytomegalovirus. Appl. Microbiol. **22**:914–918.
4. Everett, S. F., and H. S. Ginsberg. 1958. A toxinlike material separable from type 5 adenovirus particles. Virology **6**:770–771.
5. Fioretti, A., T. Furukawa, D. Santoli, and S. A. Plotkin. Nonproductive infection of guinea pig cells with human cytomegalovirus. J. Virol. **11**:998–1003.
6. Hanshaw, J. B. 1968. Cytomegaloviruses. *In* S. Gard et al. (ed.), Virology monographs, v. 3. Springer-Verlag, Vienna.
7. Ito, M., and A. L. Barron. 1972. Surface antigen produced by herpes simplex virus (HSV). J. Immunol. **108**:711–718.
8. Iwasaki, Y., T. Furukawa, S. Plotkin, and H. Koprowski. 1973. Ultrastructural study on the sequence of human cytomegalovirus infection in human diploid cells. Arch. Gesamte Virusforsch. **40**:311–324.
9. Kim, K. S., and R. I. Carp. 1972. Abortive infection of human diploid cells by murine cytomegalovirus. Infect. Immunity **6**:793–797.
10. Kim, K. S., and R. I. Carp. 1971. Growth of murine cytomegalovirus in various cell lines. J. Virol. **7**:720–725.
11. McGavran, M. H., and M. G. Smith. 1965. Ultrastructural, cytochemical, and microchemical observations on cytomegalovirus (salivary gland virus) infection of human cells in tissue culture. Exp. Mol. Pathol. **4**:1–10.
12. Melnick, J. L. 1971. Classification of nomenclature of animal viruses. Progr. Med. Virol. **13**:462–484.
13. Nii, S. 1972. Abortive infection of LLC-MK_2 cells by herpes simplex virus. Biken J. **15**:43–47.
14. Plummer, G., C. R. Goodheart, D. Henson, and C. P. Bowling. 1969. A comparative study of the DNA density and behavior in tissue cultures of fourteen different herpesviruses. Virology **39**:134–137.
15. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. Amer. J. Hyg. **27**:493–497.
16. Roizman, B. 1969. Herpesviruses, p. 415–482. *In* H. B. Levy (ed.), The biochemistry of viruses. Marcel Dekker, New York.
17. Roizman, B., and P. R. Roane, Jr. 1964. The multiplication of herpes simplex virus. II. The relation between protein synthesis and the duplication of viral DNA in infected HEP-2 cells. Virology **22**:262–269.
18. Ruebner, B. H., T. Hirano, R. J. Slusser, and D. N. Medearis, Jr. 1965. Human cytomegalovirus infection. Electron microscopic and histochemical changes in cultures of human fibroblasts. Amer. J. Pathol. **46**:477–496.
19. Russell, W. C., E. Gold, H. M. Keir, H. Omura, D. H. Watson, and P. Wildy. 1964. The growth of herpes simplex virus and its nucleic acid. Virology **22**:103–110.
20. Sever, J. L. 1962. Application of a microtechnique to viral serological investigations. J. Immunol. **88**:320–329.
21. Wentworth, B. B., and L. French. 1970. Plaque assay of cytomegalovirus strains of human origin. Proc. Soc. Exp. Biol. Med. **135**:253–258.