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Nonproductive Infection of Guinea Pig Cells with Human Cytomegalovirus

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Human cytomegalovirus was capable of adsorbing to and penetrating guinea pig cells, but was unable to replicate new virus. Cultures infected with virus inoculum of high titer showed a cytopathic effect (CPE) characterized by cell rounding. This CPE depended upon the presence of infectious virus, and its extent was directly related to the multiplicity of infection. Staining by indirect immunofluorescence by using human convalescent sera was positive as early as 4 h postinfection. Maximal fluorescence was observed 24 h postinfection when 50% of the cells contained fluorescent antigens both in nuclei and cytoplasm. No evidence for viral replication was found, and no defective particles were detected by electron microscopy. Treatment with actinomycin D or with cycloheximide strongly inhibited both the fluorescent antigens and the CPE, whereas 5-fluorodeoxyuridine and bromodeoxyuridine were ineffective.

Cytomegaloviruses (CMV) are reported to have a marked specificity with regard to susceptible hosts in contrast to the broad host range of the herpes simplex type viruses. Exceptions to the host specificity of these viruses have been reported in a few instances, such as the growth of murine CMV in cell lines obtained from various species (3, 8), the growth of vervet CMV in human fibroblasts (7), and the adaptation of a human CMV to growth in monkey cells (5).

This communication reports the infection of guinea pig cells with a high-titer inoculum of human CMV and presents evidence that guinea pig cells take up human CMV and sustain a cytopathic effect, but the viruses are unable to replicate in the cells. This is the first abortive infection recorded for human cytomegalovirus, and it provides a system for studying early events in CMV infection.

MATERIAL AND METHODS

Cells. Guinea pig (GP) cells were obtained by trypsinization of skin and muscle from 4-week-old embryos and were used between the first and seventeenth passage level. Monolayers consisted of fibroblasts and a few epithelial cells. WI-38 human diploid cells were obtained from L. Hayflick (Stanford University) and from Flow Laboratories.

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Medium. Eagle minimal essential medium (MEM) supplemented with penicillin (100 μg/ml), gentamicin (50 μg/ml), and amphotericin B (20 μg/ml), and fetal calf serum (FCS), at 10% for growth or 2% for maintenance, was used for all cells. For solid overlays, a medium containing 4× MEM vitamins and amino acids, 2× salts, and 4% FCS was mixed in equal volume with 0.8% agarose in water.

Virus. Town, a strain of human CMV, was isolated in our laboratory from an infant with congenital CMV disease. Viral stock was prepared in WI-38 cells by harvesting the cells 4 to 5 days after infection. Infected cells suspended in a small amount of medium were treated with ultrasonic vibrations (Raytheon Sonifier) for 2 min and then were centrifuged for 10 min at 2,000 rpm.

Assays of virus. Viral infectivity titers were determined in culture tubes of WI-38 cells, with cytopathic effect (CPE) used as the index of infection. The 50% end points were calculated by the method of Reed and Muench (9).

Plaque titration. Plaque assays were performed by inoculating virus onto WI-38 cell monolayers in 35-mm petri dishes. The virus was allowed to adsorb for 1 h at 37 C before the agarose overlay was added. A second agarose overlay was applied on day 5. Plaques were counted on day 12 with the aid of a light microscope.

Sera. Sera from congenitally infected infants and sera obtained from rabbits immunized with Town virus were used for the detection of various antigens.

To prepare antiserum, rabbits were inoculated once a week for 3 weeks. The antigen consisted of both cell-associated virus prepared by sonic disruption of infected cells and of cell-free virus from infected

medium. Cell-associated virus (1 ml) was injected intramuscularly, and cell-free virus (5 ml) was injected intravenously. Rabbits were also inoculated with uninfected cells and supernatant fluids. The rabbits were bled 10 days after the last injection.

Indirect immunofluorescent staining. Infected cultures on cover slips were washed twice with phosphate-buffered saline (PBS) and then were fixed with acetone for 10 min. After fixation, cultures were air dried, washed once with PBS, and treated with human convalescent serum for 30 min at 37 C. Cultures then were washed twice with PBS and treated with antihuman gamma globulins conjugated with fluorescein isothiocyanate, prepared in rabbits, for 30 min at 37 C. Cultures were then washed twice with PBS and mounted with Elvanol.

Complement-fixing and precipitin antigen preparation. Infected cells were removed by treatment with 0.25% trypsin and 0.1% EDTA at different time intervals postinfection. The cells were sedimented at 1,000 rpm for 10 min and resuspended in PBS, pH 7.2, at a 5% concentration. This suspension was sonically treated for 2 min. The resultant material was used for complement-fixing (CF) and precipitin antigens. CF antigen was assayed by the microtiter technique (10).

Gel-precipitin test. Molten agarose (0.4%) dissolved in Ca- and Mg-free PBS, pH 7.2, containing 0.01% Merthiolate was added to glass petri dishes (9-cm). Wells (8 mm in diameter) were made with centers 12-mm apart. The center well was filled with human antiserum, and the adjacent wells were filled with antigen. Petri dishes were incubated in moist boxes at 4 C for 1 week. Precipitin lines were observed with the positive antigen used as a control after 2 days of incubation.

Drugs. Actinomycin D (Sigma Chemical Co.), 0.01 μg/ml; cycloheximide (Nutritional Biochemicals Corp.), 0.5 μg/ml; bromodeoxyuridine (BUdR) (Nutritional Biochemicals Corp.), 5 μg/ml; and 5-fluorodeoxyuridine (FUdR) (Nutritional Biochemicals Corp.), 10 μg/ml, were dissolved in maintenance medium. Drug doses were chosen after previous cytotoxicity assays showed them to be nontoxic.

Electron microscopy. Confluent monolayers of GP cells in 35-mm petri dishes were cooled at 4 C for 15 min, and then were infected with 0.5 ml of virus suspension (5×10^6 tissue culture infective dose [$TCID_{50}$]/ml). For the experiment in which the cells had to be fixed within the 1st h at 37 C, the CMV inoculum was concentrated 20 times by ultracentrifugation. After infection, the cultures were incubated for 1 h at 4 C, and then the temperature was quickly raised to 37 C (zero time) to synchronize the initiation of virus entry. After 1 h of incubation, cells were washed three times with PBS, refed with medium, and incubated at 37 C. Infected cells were fixed at 5, 15, 30, and 60 min and at 6, 24, 48, and 72 h. After prefixation in situ with 2% of glutaraldehyde in PBS for 30 min at 4 C, cells were rinsed two to three times with PBS, fixed with 1% OsO_4 in Millonig buffer for 1 h at 4 C, dehydrated, and embedded in Epoxy resin. Thin sections were doubly stained with uranyl acetate (saturated solution in 50% ethanol) and 0.2% lead citrate. Uninfected cells were fixed at zero time and after 72 h.

RESULTS

CPE. Confluent monolayers of GP cells were prepared in tubes with or without cover slips. The virus inoculum (10^6 $TCID_{50}$/0.2 ml) was allowed to adsorb for 1 h at 37 C. The cultures were then washed three times and incubated with fresh medium. At the indicated time intervals (Table 1), two cover slips were stained with May-Grünwald Giemsa, and cells and medium from three tubes were harvested and assayed for virus in WI-38 cells.

We observed a cytopathic change which was characterized by the presence of round, swollen cells (Fig. 1). No intranuclear inclusions were seen. Cell rounding began 5 to 6 h postinfection, and at the 24th h 50% of the cells were round. On days 2 and 3 many cells disintegrated but, at the same time, a high mitotic activity was observed which resulted in a reconstitution of the monolayer. During the following days, no significant changes were seen.

A linear relationship between the number of round cells and the titer of the inoculum was demonstrated when cultures were infected with 10-fold dilutions of the virus suspension (Table 2). Additional evidence supporting the idea that this cytopathic change depended upon the presence of infectious viral particles in the inoculum was obtained by high-speed centrifugation, by neutralization, and by UV inactivation (Table 2). Virus suspension was centrifuged for 1 h at 25,000 rpm, and GP cultures were inoculated with the supernatant fraction, with the pellet resuspended in fresh medium to the original

TABLE 1. *Virus titer, cytopathic effect, and mitotic index after infection of guinea pig cells by cytomegalovirus*

Days postinfection	Virus titer ($TCID_{50}$/0.2 ml)		CPE[a]		Mitotic index[b]	
	Cells[c]	Medium	Control	Inf.	Control	Inf.
0[d]	10^4					
1	10^1	10^0	0	47.0	27	31
3	10^0	$<10^0$	0	5.0	1	41
5	$<10^0$	$<10^0$	0	0	1	2
7	$<10^0$	$<10^0$	0	0	ND[e]	ND[e]

[a] Cytopathic effect (CPE) expressed as the percentage of round cells.
[b] Calculated by counting 1,000 cells.
[c] Cell associated virus titrated after sonication.
[d] After 1 h of adsorption.
[e] ND, Not done.

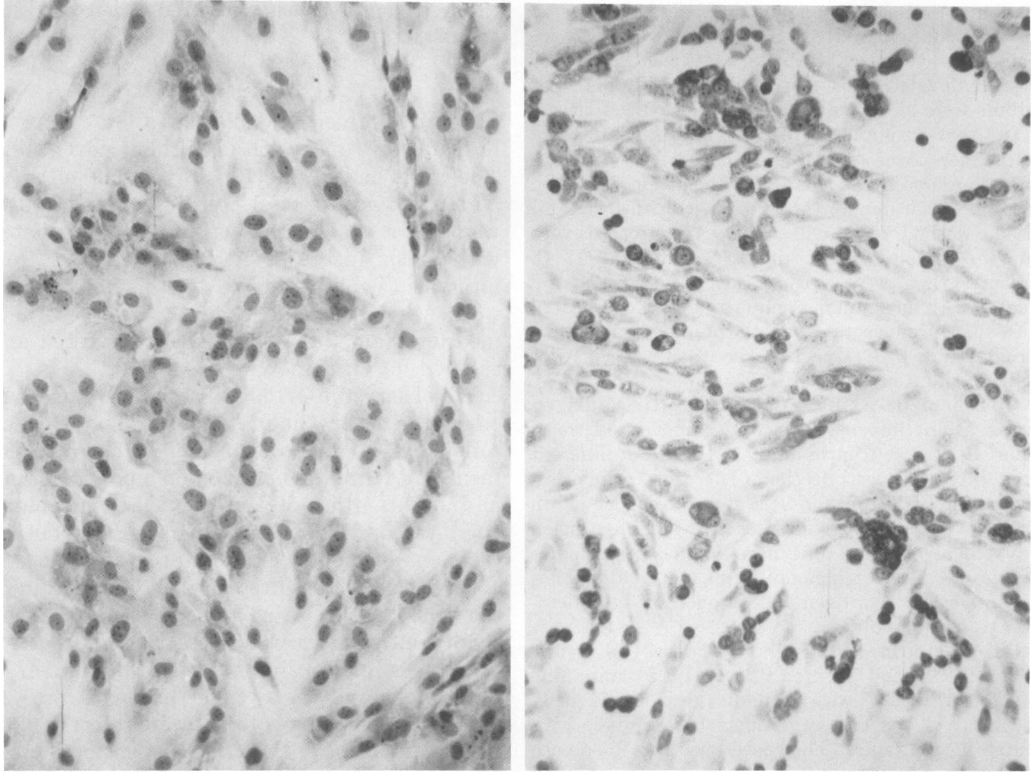

FIG. 1. *Morphological changes in guinea pig cells after infection. A, Uninfected cells. B, Cells 24 h postinfection. Most of the infected cells are rounded.*

TABLE 2. *Evidence for dependence of cytopathic effect on infectious viral particles*

Treatment	Virus titer after treatment ($TCID_{50}$/0.2 ml)	CPE[a]
None	10^6	47.0
Dilution, 1:10	10^5	4.4
Dilution, 1:100	10^4	0.2
UV	10^4	4.4
Anti-CMV serum	<10	0
High-speed centrifugation	10^2 [b]	0
	ND[c]	50

[a] Cytopathic effect (CPE) expressed as the percentage of round cells.
[b] Supernatant fraction.
[c] Pellet; ND, not done.

volume, or with virus suspension before centrifugation. CPE did not appear in those cultures which were infected with the virus-free supernatant fraction.

Virus suspensions containing 10^6 $TCID_{50}$/0.2 ml were incubated at 37 C for 1 h with an equal amount of human anti-CMV serum or seronegative serum, and then they were used to infect GP cultures. After 1 h of adsorption, the cultures were washed three times and incubated for 24 h. No CPE appeared in cultures infected with virus neutralized by anti-CMV serum, but it did occur in cultures infected with the control mixture.

Virus inoculum was exposed to UV light for 15 min. Monolayers were infected with 10-fold dilutions of unexposed virus or with undiluted irradiated virus. UV irradiation reduced the virus titer by 99% and the CPE by 90%.

Despite the profound CPE, we found no evidence of virus replication. The amount of infectious virus associated with the cells or present in the medium decreased very quickly during the first 24 h, and the decrease continued during subsequent days.

Adsorption. Virus adsorption to GP cells and to human cells (WI-38) was compared. Monolayers were exposed to 1.5×10^5 $TCID_{50}$/0.2 ml. At various intervals, inocula were removed from three cultures, the cells were washed three times, trypsinized, and disrupted by sonic treatment. The unadsorbed virus and the cell-associated virus were then titrated by plaque assay in WI-38 cells.

After 30 min of exposure, 93% of the virus had

adsorbed to GP cells and 95% to WI-38 cells. After 1 and 2 h of exposure, the amount of unadsorbed virus and the amount of the virus associated with the cells did not change significantly.

Penetration. The capacity of the virus to penetrate the cells was tested by titration after adsorption and treatment with anti-CMV serum. GP and WI-38 cells were infected with 1.5 $\times 10^5$ $TCID_{50}$/0.2 ml, and, after 1 h of adsorption, cultures were washed and treated with rabbit anti-CMV serum or with preimmune serum. The cultures were washed 1 h later, and some were removed for virus assay, and others were incubated for 24 h and stained for CPE. Residual infectious virus was titrated by plaque assay in WI-38 cells.

By separate in vitro tests, the rabbit antiserum was shown to neutralize 90% of the inoculum. The virus recovered from antiserum-treated GP and WI-38 cells was reduced by 60% in comparison with the virus recovered from cells treated with preimmune serum. These results imply that during the adsorption time, 30% of the virus entered both GP and WI-38 cells. This percentage of virus was apparently sufficient to cause CPE, because both GP and WI-38 cultures treated with antiserum showed a CPE 24 h after treatment which was quantitatively similar to controls treated with preimmune serum.

Activity of actinomycin D, cycloheximide, FUdR, and BUdR on CPE. Cultures exposed to virus inoculum for 1 h were washed and then were incubated either in the presence or absence of inhibitors added at different times postinfection. Cultures were stained and observed for CPE at 24 h postinfection.

Early CPE was strongly reduced if actinomycin D or cycloheximide were added during the first 6 h postinfection (Table 3). Equal CPE was produced in the presence or absence of FUdR or BUdR.

Electron microscopy. Sections made of cells taken within the 1st h after zero time showed several unenveloped particles outside the cell and in cytoplasmic vacuoles inside the cells (Fig. 2A). The number of virus particles inside the cell increased during the 1st h. At 1 h most virions appeared to be degraded, some showed a partial disruption of their envelope, and others were more completely digested (Fig. 2B). At the same time, some uncoated particles were observed free in the cytoplasm (Fig. 2C). At the

TABLE 3. *Percentage reduction of cytopathic effect[a] after various drug treatments of cytomegalovirus-infected guinea pig cells when inhibitors were added at different times after infection*

Drug (μg/ml)	Time of treatment (hour postinfection)						
	0[b]	2	4	6	8	10	12
Actinomycin D, 0.01	81	85	82	75	35	41	40
Cycloheximide, 0.5	75	79	60	61	39	33	30
BUdR, 5.0	0	0	0	0	0	0	0
FUdR, 10.0	0	0	0	0	0	0	0

[a] Cytopathic effect: quantitative data were obtained by counting the round cells per 1,000 cells.
[b] After 1 h of adsorption.

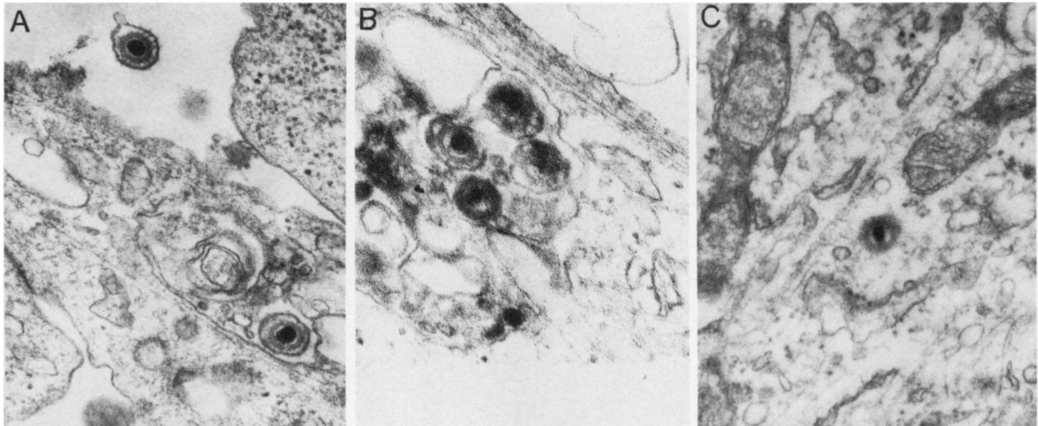

FIG. 2. *Electronmicrographs of thin sections of guinea pig cells infected with cytomegalovirus (CMV). A, Virus particles close to the cell surface and inside cytoplasmic vacuoles at 15 min postinfection; ×41,000. B, Degradation of virus particles inside cytoplasmic vacuole at 1 h postinfection; ×48,000. C, Unenveloped CMV particle free in the cytoplasm 1 h postinfection. The capsid of the particle is partially disrupted; ×41,000.*

6th h, only a few viruses could be seen, and all were located within the vacuoles. No virus particles could be detected thereafter. Ultrastructural alterations of cells during the 1st h consisted in a slight enlargement of the endoplasmic reticulum and Golgi complex and a great increase in pseudopod formations and pinocytotic vesicles next to the cell surface. The cells fixed 24, 48, and 72 h postinfection showed a severe, diffuse vacuolization, phagosomes, and myelinic figures. Another evident feature was the great enlargement of endoplasmic reticulum, whose vesicles contained a pale and amorphous substance.

Detection of immunofluorescent antigens. Fluorescent antigens were detected 4 h postinfection, when both the nuclei and cytoplasm were positive (Table 4). At the 6th h, round cells with strong fluorescence could readily be detected. The nuclei showed granular fluorescence, and in the cytoplasm the perinuclear area was strongly positive. Maximal fluorescence was seen 24 h postinfection, when 50% of the cells were positive. In subsequent days the number of the cells showing fluorescence decreased, and by the 7th day fluorescence had disappeared. No fluorescence was detected in controls using conjugated negative serum or in uninfected cells stained with positive serum.

The appearance of fluorescent antigens was delayed and almost completely suppressed by treatment with actinomycin D and cycloheximide added immediately after adsorption; and FUdR did not reduce the production of fluorescence.

CF and precipitin antigens. Infected cultures were tested for CF antigen and precipitin antigen at 1, 3, 5, and 7 days postinfection. No antigens were detected.

DISCUSSION

The data presented in this paper indicate that human CMV infects guinea pig cells but does not replicate infectious virus. This conclusion is based upon the following evidence. (i) Virus adsorbed to and penetrated guinea pig cells to the same degree that it adsorbed to and penetrated permissive cells (WI-38). (ii) Guinea pig cells showed, 5 to 6 h postinfection, morphological changes which were similar to the "early CPE" that takes place in WI-38 cells 6 to 12 h postinfection (2). This "early CPE," caused only by high-titer CMV infection, is characterized by cell rounding involving nearly all the cells. At this stage there are no inclusion bodies, and no new virus is detected. (iii) New antigens detectable with indirect immunofluorescent staining appeared a few hours postinfection and reached the maximum at 24 h postinfection. (iv) The titer of the supernatant and cell-associated virus dropped during the day after infection, and no virus was detectable thereafter. (v) Investigation by electron microscopy showed the presence of viral particles inside the cells up to the 6th h postinfection. They were found inside cytoplasmic vacuoles and free in the cytoplasm. No particles at all were found there-

TABLE 4. *Intensity and location of immunofluorescent antigens detected in cytomegalovirus-infected guinea pig cells to which actinomycin D or cycloheximide was added*[a]

Time postinfection	Round cells (%)			Location and intensity of fluorescence					
				Nuclei			Cytoplasm		
	A	B	C	A	B	C	A	B	C
0 h	0	0	0	−	−	−	−	−	−
1 h	0	0	0	−	−	−	−	−	−
4 h	0	0	0	+	−	−	+	−	−
6 h	25	0	0	++	±	±	+++	−	−
8 h	25	0	0	++	±	±	+++	±	±
12 h	25	0	0	++	±	±	+++	±	±
24 h	50	10	10	+++	+	±	++++	+	±
3 days	25	0	0	+	−	−	+	−	−
5 days	0	0	0	±	−	−	±	−	−
7 days	0	0	0	−	−	−	−	−	−

[a] A, Untreated; B, actinomycin D; C, cycloheximide. All round cells showed some degree of fluorescence, and some nonrounded cells also were fluorescent.

after. (vi) CF and precipitin antigens were not detectable at any time postinfection.

Our evidence shows that the CPE only follows the inoculation of infectious virus, that it depends on the synthesis of new protein(s), and that it does not require the synthesis of DNA. Furthermore, the fluorescent antigens are apparently viral coded. These conclusions are based on the following evidence. (i) Viral suspensions treated with anti-CMV serum, centrifuged at high speed, or treated with UV light did not produce CPE. (ii) There was a correlation between the extent of the CPE and the titer of the virus inoculated. (iii) Actinomycin D and cycloheximide inhibited the appearance of the CPE and of fluorescent antigens, whereas FUdR and BUdR were ineffective. (iv) In staining for fluorescent antigens, we used human antiserum. Because viral replication occurs in man, the antiserum should have contained antibodies to virus structural proteins. If virus infection induced the synthesis of proteins coded for by guinea pig cell genome, it is unlikely that human antiserum would have had antibodies to it.

Analogous results were obtained with murine CMV (4), which was reported to produce CPE in human cells without any production of infectious virus, and with herpes simplex virus in LLC-MK_2 rhesus monkey cells (6) and in dog kidney cells (1). The mechanism for nonproductive infection remains to be explained.

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