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Behavior of Human Cytomegaloviruses in Cell Cultures of Bovine and Simian Origin[1] (37814)

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The cytomegaloviruses (CMV) generally are considered to be species specific in terms of host preference when propagated *in vivo* and *in vitro*. Kim and Carp (1), however, have shown that murine CMV will replicate *in vitro* in nonmurine cells and will abortively infect human diploid cells (2). We have referred briefly to observations (3) indicating that bovine embryonic fibroblasts and Vero cells, of simian origin, when inoculated with human CMV exhibit a cytopathic effect (CPE) and that virus may be isolated for 5 weeks thereafter from the Vero cells. Recently, abortive infection of guinea pig cells by human CMV has also been described (4).

We here present the first evidence that human strains of CMV replicate in nonhuman cells (Vero) with production of infectious cell-associated virus. Human CMV also produces cytopathic changes in bovine cells and induces synthesis of viral antigens in the absence of evidence of viral replication.

Materials and Methods. General experimental design. Bovine or Vero (simian) cell cultures in tubes or on coverslips in Leighton tubes were inoculated with $10^{4.5}$–$10^{5.5}$ infectious units of CMV. After 1 hr absorption at 36° with agitation at 10-min intervals, the inoculum was aspirated, the cultures washed 2× with Hank's balanced salt solution (HBSS) and supplied with Basal Medium Eagles (BME). At 1, 3, 5, 7, 14, 21, and 28 days after inoculation, paired inoculated and control cultures were examined for cell-associated and cell-free infectious virus, for CMV antigenic constituents by fluorescent antibody (FA) and complement fixation (CF) tests, and studied morphologically by light and electron microscopy.

Cells. Cultures of Vero cells (passages 181–212), primary bovine embryonic fibroblasts, and primary human fibroblasts derived from fetal skin muscle were obtained from Microbiological Associates, Bethesda, MD. Bovine cell cultures were also prepared from skin-muscle tissues of fetuses obtained locally. All cell cultures were maintained on BME supplemented with 5% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cell cultures were grown at 36° in 160-ml milk dilution bottles, 16 × 150 mm stoppered culture tubes, or on 10.5 × 35 mm coverslips in Leighton tubes. Tests for mycoplasma were performed according to the method of Morton (5).

Virus. The Davis strain of CMV (6), passages 57–70, was used routinely. Additionally, the AD169 and Esp strains of CMV (6), passages 41–60 and 36–40, respectively, were studied in beef and Vero cell cultures to examine viral replication. The viral inocula were prepared when infected human fibroblast cell cultures in milk dilution bottles showed 90% CPE. Infected cells were mechanically harvested in 7 ml of culture fluid, disrupted with approximately

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20 strokes of a Ten-Broeck tissue grinder, and the suspension clarified by low-speed centrifugation. The supernatant was passed through a 0.4-μm polycarbonate filter and the filtrate used as the inoculum. Such preparations regularly contained $10^{5.0}$–$10^{6.0}$ infectious units/ml of CMV as assayed by the fluorescing cell assay technique (FCA) (7). Control inocula were prepared in the same manner from uninfected human fibroblast cultures maintained in parallel.

Virus isolation and cytopathic effect. Attempts to isolate virus from bovine or Vero cells were performed by a cocultivation procedure. Trypsinized bovine or Vero cells from inoculated tube cultures were mixed in suspension with twice their number of human fibroblasts. The resulting cell suspensions were used to prepare 6 culture tubes. Culture fluids removed from bovine or Vero cultures prior to trypsinization were also filtered through 0.4 μm filters and inoculated into 4 human fibroblast cultures to detect cell-free virus; these cultures were kept 4 weeks and observed every 2nd day for the appearance of CPE. Isolates were subcultured to human fibroblast cultures and their identity confirmed as CMV by typical CPE and neutralization with specific anti-Davis strain CMV antiserum produced in rabbits (3).

To quantitate infectious virus present in the cells in inoculated cultures, bovine and Vero cells from duplicate culture tubes after two washes with HBSS were harvested by scraping into 1 ml of BME and disrupted in a Ten-Broeck tissue grinder. The cell debris was removed by centrifugation at 250*g* and the supernatant assayed for virus by the FCA.

For morphologic studies by light microscopy, cell cultures on coverslips were fixed with Bouin's and stained with hematoxylin and eosin (H & E).

Electron microscopy. Cell cultures in tubes were fixed *in situ* with 2% glutaraldehyde in phosphate buffer (PB) (0.1 *M*, pH 7.2), and rinsed thoroughly with PB containing 0.2 *M* sucrose. Fixed cells were scraped free with a glass rod and sedimented by low-speed centrifugation. The cell mass was treated with 1% phosphate-buffered osmium tetroxide, dehydrated in graded ethanols, and embedded in Spurr resin (8). Sections were cut with a Sorvall MT 2B ultra-microtome, stained with uranyl acetate and lead citrate, and examined in a Phillips 300 electron microscope.

Fluorescent antibody (FA). Cell monolayers on coverslips were prepared for indirect immunofluorescence tests as described previously (7). Controls consisted of preparations from the parallel control cultures treated in the same manner.

Complement fixation (CF). Complement antigens were prepared as described previously (3) from inoculated Vero or beef cells by the alkaline-buffer extraction technique of Krech and Jung (9). Uninfected cell cultures maintained in parallel and treated in the same manner provided control antigens. The test was performed according to the microtiter method described by Lennette (10), using a human reference serum of known CF reactivity.

Ultraviolet inactivation of CMV. Virus was obtained as described above except that the culture fluid was replaced with HBSS. Between $10^{5.5}$ and $10^{6.5}$ infectious units of CMV in 5 ml of HBSS were transferred to a 60 × 15 mm Petri dish and, using a Mineral-light Model SL 3660 uv source at a distance of 8 cm, were irradiated for 15 min at room temperature with intermittent agitation.

Results. Cytopathic effect (CPE). Cytopathic changes in bovine cell cultures appeared between 18 and 48 hours after inoculation with Davis strain CMV. Approximately 40% of the cells became rounded and more refractile. In the H & E preparations, short cords of multinucleate cells and darkly stained rounded cells that occasionally had paler paranuclear cytoplasmic material were seen (Fig. 1a). The CPE was not progressive and many affected cells appeared to detach from the monolayer. By the 5th–7th days after inoculation the altered cells had disappeared and the cell sheet appeared "normal." In a single experiment the bovine cells were then susceptible to a new inoculum of CMV.

Cytopathic effect in Vero cultures developed between the 7th and 14th days after

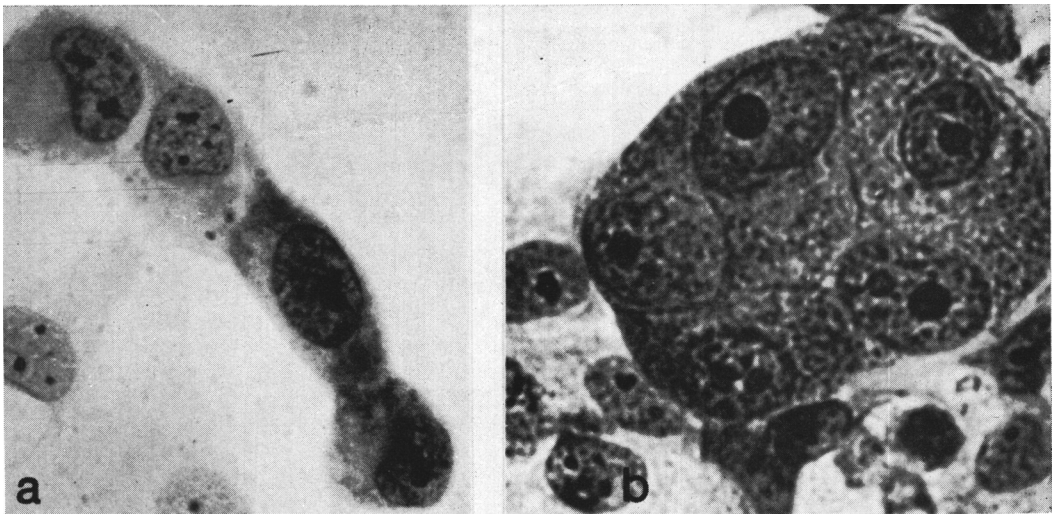

FIG. 1. Cytopathic changes in bovine and Vero cell cultures infected with CMV. Magnification 960×. (a) A multinucleate cord in a beef culture 24 hr after inoculation. (b) A Vero giant cell 2 weeks after inoculation.

inoculation; these changes could be seen in H & E preparations as early as the 5th day. Affected cells showed increased cytoplasmic staining and eccentric displacement of their nuclei. Prominent nucleoli and a cytoplasmic paranuclear granular body became apparent at about 7 days. At this time, between 20% and 30% of the cells were involved. Multinucleate giant cells appeared between 7 and 14 days and persisted for an additional 2 weeks before degenerating (Fig. 1b). Giant cells were readily visible in unstained cultures.

Detection of CMV antigens by FA. A specific FA reaction was observed in approximately 40% of beef cells between 24 and 48 hours after inoculation with virus. Fluorescence was primarily cytoplasmic although nuclear fluorescence was occasionally seen (Fig. 2a). By the 5th day, however, only

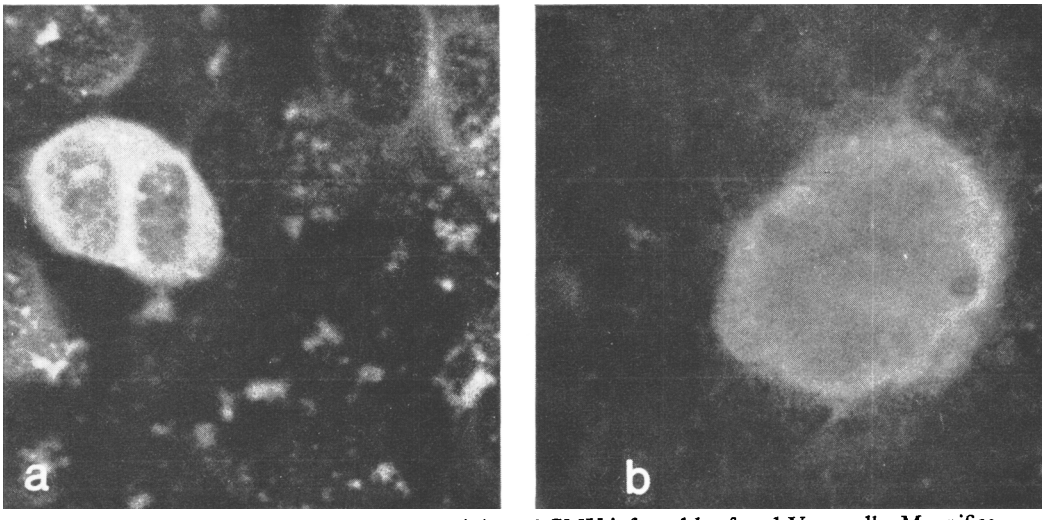

FIG. 2. Indirect immunofluorescent staining of CMV-infected beef and Vero cells. Magnification 960×. (a) A binucleate beef cell 48 hr after inoculation. (b) A Vero giant cell 2 weeks after inoculation.

TABLE I. Summary of Findings on Inoculation of Bovine and Vero Cell Cultures with Human CMV.

Day after inoculation	Cytopathic effect		Antigen detectable by FA		Antigen detectable by CF		Herpes-like particles demonstrated by electron microscopy	
	Bovine	Vero	Bovine	Vero	Bovine	Vero	Bovine	Vero
1	+	−	+	−	2[b]	2	−	−
3	+/−[a]	+/−	+/−[a]	+/−	−	2	−	−
5	−	+	+/−	+	−	4	−	−
7	−	+	+/−	+	−	8	−	+
14	−	+	−	+	−	2	−	+
21	−	+	−	+	−	2	NT	NT
28	−	+	−	+	NT[c]	NT	−	+

[a] Indicates variable results.
[b] Reciprocal of the highest dilution of antigen exhibiting complete fixation.
[c] NT, not tested.

an occasional reactive cell was visible and no reacting cells were detected 7 days after inoculation (Table I).

In Vero monolayers, a positive immunofluorescent reaction, which was restricted to the cytoplasm, occurred in less than 5% of the cells within the first 3 days after inoculation. However, between the 5th and 7th days, specific staining was evident in both cytoplasm and nucleus of 10–20% of the cells. Immunofluorescent staining of the multinucleate giant cells (Fig. 2b) was demonstrable thereafter for the duration of the experiment (Table I).

Detection of CMV–CF antigens. Slight CF antigen reactivity was detectable at 24 hr on extraction of inoculated Vero and beef cells. Titers of antigen increased in the extracts of Vero cultures reaching maximum levels in harvests obtained at about 7 days. Thereafter decreasing but detectable amounts persisted for another 2 weeks. However, in extracts of beef cells harvested more than 3 days postinoculation (Table I), no CF antigen was detected.

Virus isolation. Virus was isolated from inoculated cultures of bovine and Vero cells by cocultivation with human fibroblasts at 3, 5, and 7 days after inoculation. Thereafter, inoculated Vero cells continued to

TABLE II. Recovery and Quantitative Assay of Virus from CMV-Inoculated Bovine and Vero Cell Cultures

Time (days)	Virus isolation by cocultivation of cells		Virus isolation from cell-free culture fluid		Infectious units/ml recovered from disrupted cells[a]	
	Bovine	Vero	Bovine	Vero	Bovine	Vero
1	NT[e]	NT	NT	NT	$10^{1.08}$	$10^{1.94}$
3	3/6[b] (14–16)[c]	6/6[b] (9–14)[c]	2/4[d] (10–15)[c]	2/4 (10–15)	$10^{0.60}$	$10^{1.34}$
5	4/6 (14–18)	6/6 (9–13)	1/4 (9)	2/4 (8–10)	$10^{0.60}$	$10^{1.10}$
7	4/6 (18–22)	6/6 (8–10)	1/4 (11)	1/4 (10)	$10^{0.40}$	$10^{0.90}$
14	0	6/6 (8–11)	0/4 (0)	0/4 (0)	0	0
21	0	3/6 (9–16)	0/4 (0)	0/4 (0)	0	0
28	0	2/6 (9–20)	0/4 (0)	0/4 (0)	0	0

[a] Infectious units as assayed in human fibroblast cell cultures by fluorescing cell assay method.
[b] Number of cultures showing CPE out of 6 prepared.
[c] Time (days) in which CMV–CPE first appeared.
[d] Number of cultures showing CPE out of 4 inoculated.
[e] NT, not tested.

yield virus on repetitive samplings throughout the 28 days of observation, but virus was not recovered from the bovine cultures after the 7th day. The yield of virus from Vero cell cultures decreased after 14 days (Table II), as indicated by a decreasing number of positive cultures per isolation attempt. Cell-free virus was isolated from the culture fluids of bovine and Vero cells at 3, 5, and 7 days after inoculation. Comparable results also were obtained when bovine and Vero cultures were inoculated with the AD169 or Esp strains of CMV.

In contrast to the continued recovery of virus from Vero cells by cocultivation, repetitive quantitation of disrupted cells by FCA gave negative results after the 7th day with both types of cells (Table II).

Failure of ultraviolet inactivated CMV to induce CPE and FA antigens in bovine and Vero cells. Cytomegaloviruses inactivated by uv irradiation did not induce CPE in bovine or Vero cells nor were CMV antigens produced that were detectable by indirect immunofluorescence. In parallel control experiments, cultures inoculated with infectious CMV responded as previously described.

Virus isolation from subcultures of CMV infected Vero cells. Infected Vero cultures were trypsinized and subcultured at weekly intervals for 4 passages. Half of the trypsinized cells from each passage were used alone to prepare 2 cultures and the rest cocultivated with human fibroblasts as described above. Culture fluids were tested for cell-free virus at the time of passage and 5 days afterward. Virus was recovered from cells through the 3rd passage and from culture fluids through the 2nd passage (Table III).

TABLE III. Virus Recovery during 4 Passages of CMV-Infected Vero Cultures.

Passage number	Virus recovery from: Cocultivated cells	Virus recovery from: Cell-free fluid
1	6/6[a] (10–12)[b]	2/4[c] (9–12)[b]
2	6/6 (8–15)	1/4 (10)
3	2/6 (12–19)	0/4 (0)
4	0/6 (0)	0/4 (0)

[a] Number of cultures showing CPE out of 6 prepared.

[b] Time (days) when CMV-CPE first appeared.

[c] Number of cultures showing CPE out of 4 inoculated.

Electron microscopy. In one experiment, inoculated bovine cells that had floated free during the first 48 hr were concentrated and examined by electron microscopy. Virus particles were not seen in this material nor on repeated examination of bovine cells fixed *in situ* during the course of the experiments.

In contrast, on examination of Vero cultures, herpes-like particles at various stages of maturation were seen in the nuclei of 4–10% of the cells at 7 and 14 days and in less than 1% of the cells at 28 days. Virus particles were rarely demonstrable in the cytoplasm of infected cells (Fig. 3).

Discussion. Bovine and Vero cells inoculated with human CMV exhibited cytopathic changes and specific immunofluorescence. These reactions could not be induced with uv inactivated virus. Virus was recovered by cocultivation from infected bovine cells at 3, 5, and 7 days after inoculation and from infected Vero cells throughout the 28-day course of the experiment. The amount of virus recovered from bovine cells was less than that from Vero cells as evidenced by the percentage of positive cultures and by the rapidity of appearance of CPE. The bovine cell isolations probably represented residual inoculum which remained viable in the cultures. The continued recovery of virus from infected Vero cells, however, by cocultivation at 14, 21, and 28 days and the demonstration by EM of virus particles in cells 7 days after inoculation indicated that CMV replicated in Vero cells. These isolations may reflect the ability of herpes viruses to induce cell fusion between infected cells and adjacent uninfected cells with the subsequent transfer and expression of the virus (11). The CPE of CMV infected Vero cells appeared, in fact, to be a syncytial and phagocytic phenomenon as reported for CMV infected human fibroblasts by Diosi (12).

Human CMV, therefore, will infect bovine and Vero cells. The bovine infection is abortive. Cytomegalovirus antigens are produced but viral particles are not synthesized. Infected Vero cells, in contrast, support synthesis of infectious particles that can

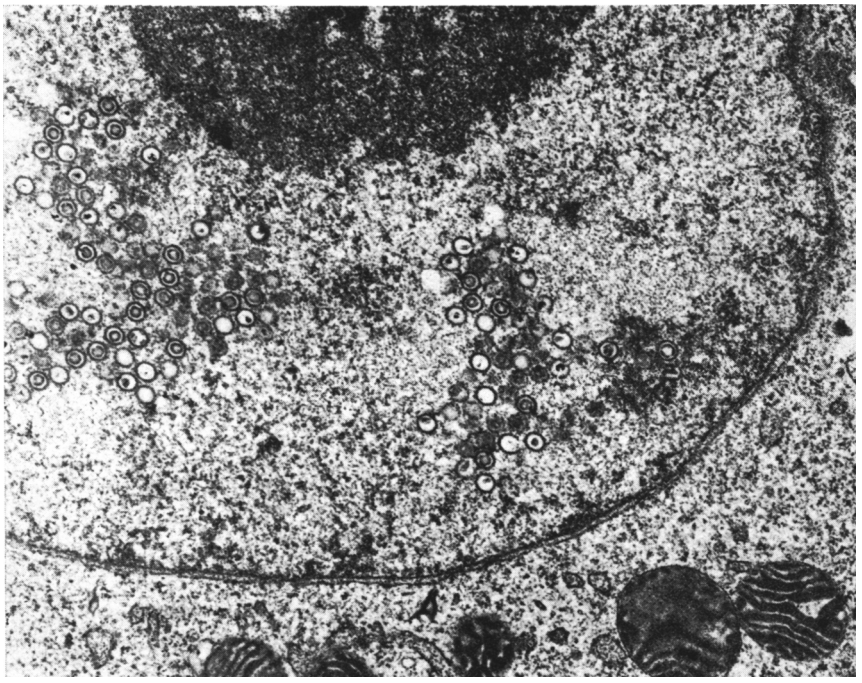

FIG. 3. Herpes-like particles in various stages of maturation in the nucleus of an infected Vero cell 14 days after inoculation. Magnification 25,000×.

be visualized in the nuclei but are rarely seen in the cytoplasm. Assays of infectious virus in successive passages of infected Vero cells indicate that virus is not freely transferred to new cells but is "diluted" out, probably by the death of individual infected cells. It appears, therefore, that a complete but inefficient cycle of replication occurs, resulting in individual cells that are persistently infected.

Summary. Bovine and Vero (simian) cells infected with human cytomegalovirus (CMV) exhibited cytopathic changes and produced specific antigens as demonstrated by immunofluorescence and complement fixation (CF). Infectious virus was recovered by cocultivation with human fibroblasts from the bovine cells only during the first week after inoculation, but from the Vero cells throughout the 4-week period of observation. Herpes-like virus particles were evident by electron microscopy in the nuclei of infected Vero cells at 1, 2, and 4 weeks after inoculation but were not demonstrable in the bovine cells.

1. Kim, K. S., and Carp, R. I., J. Virol. 7, 720 (1971).

2. Kim, K. S., and Carp, R. I., Infect. Immunity 6, 793 (1972).

3. Waner, J. L., and Weller, T. H., *in* "Perspectives in Virology" (M. Pollard, ed.), Vol. 8, p. 211. Academic Press, Inc., New York (1973).

4. Fioretti, A., Furukawa, T., Santoli, D., and Plotkin, S. A., J. Virol. 11, 998 (1973).

5. Morton, H. E., Smith, P. T., and Leberman, P. R., Amer. J. Syph. 35, 361 (1951).

6. Weller, T. H., Macauley, J. C., Craig, J. M., and Wirth, P., Proc. Soc. Exp. Biol. Med. 94, 4 (1957).

7. Waner, J. L., and Budnick, J. E., Appl. Micro. 25, 37 (1973).

8. Spurr, A. R., J. Ultrastruct. Res. 26, 31 (1969).

9. Krech, U., and Jung, M., Arch. Gesamte Virusforsch. 28, 248 (1969).

10. Lennette, E. H., *in* "Diagnostic Procedures for Viral and Rickettsial Infections" (E. H. Lennette and M. J. Schmidt, eds.), 4th ed., p. 52. American Public Health Association, New York (1969).

11. Roizman, B., Cold Spring Harbor Symp. Quant. Biol. 24, 327 (1962).

12. Diosi, P., Babusceac, L., and Gherman, D., J. Infect. Dis. 125, 669 (1972).

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