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Persistent Shedding of Cytomegalovirus in the Urine of Healthy Rhesus Monkeys (37897)

D. M. ASHER, C. J. GIBBS, JR., D. J. LANG, AND D. C. GAJDUSEK
(Introduced by R. M. Chanock)

National Institute of Neurological Diseases and Stroke, Bethesda, Maryland 20014 and Department of Pediatrics, Duke University School of Medicine, Durham, North Carolina 27706

We have demonstrated that a cytomegalovirus (CMV) is still shed in the urine of healthy adult rhesus monkeys 4 years after the virus was first isolated from the same monkeys as juveniles (1). Rhesus CMV differs from known CMVs of the African green monkey and of man.

Materials and Methods. Rhesus monkeys. Eleven healthy young Indian-born *Macaca mulatta* were obtained in two lots from the Animal Quarantine Unit of the National Institutes of Health, Bethesda, MD, and kept in separate buildings. Pairs of rhesus monkeys were caged in rooms with other macaques, African green monkeys, new-world monkeys, and chimpanzees.

Urine specimens. Monkeys were sedated with phencyclidine (Sernylan) 1 mg per kg im and given mannitol 2 g and 0.9% saline 100–200 ml iv to fill the bladder. Urine was taken by suprapubic needle puncture.

Virus isolations. Each urine was diluted with an equal volume of tissue-culture maintenance medium and, if necessary, brought to neutral pH with sodium bicarbonate. Isolation attempts and titrations were performed in stationary WI38 tube cultures by standard techniques (2). Medium was changed every 5 days. Cultures were held up to 100 days in 1968, 30 days in 1972. All isolates were passaged at least once in WI38 cultures.

Cell cultures. WI38 fetal human lung diploid fibroblast cultures were purchased from HEM Laboratories, Inc., and from Microbiological Associates, Inc., which also provided tube cultures of MA112 fetal human mixed diploid fibroblasts, MA184 and MA407 human foreskin fibroblasts, MA101 fetal rhesus monkey lung diploid fibroblasts, MA105 fetal rhesus monkey skin muscle diploid fibroblasts, MA117 fetal African green monkey lung diploid fibroblasts, MA121 fetal African green monkey liver diploid fibroblasts, and MA188 horse testis diploid fibroblasts, and primary cultures of human embryo kidney, rhesus monkey kidney, African green monkey kidney, and rabbit kidney cells. All cell cultures were maintained on Eagle's Basal or Minimal Essential Medium in Earle's salt solution with 5% inactivated fetal calf serum and antibiotics. Samples of each type of cell culture showing cytomegalic cytopathic effect (CPE) as well as representative negative cultures were disrupted and passaged into WI38 cultures for confirmation.

Virus strains. All studies of biological and physical properties of rhesus monkey CMV were performed with strain 68-1, the first isolate from the urine of female rhesus No. 1. Several other isolates were also included in studies of range of cell cultures susceptible to the virus. The Ad.169 strain of human CMV (3) and the CSG strain of African green monkey cytomegalovirus (AGM CMV) (4) were provided by Drs. Wallace Rowe and Janet Hartley, National Institute of Allergy and Infectious Diseases. Vaccinia and Herpesvirus hominis (Kaplan strain) obtained from the Bureau of Bio-

logics, and Pan 5, a simian adenovirus isolated in our laboratories (5), served as controls in heat- and chloroform-stability tests.

Morphologic studies. Cells were fixed and mounted in collodion and stained with hematoxylin and eosin (6). Acridine orange-stained cell cultures were prepared in the laboratory of Dr. Kendall O. Smith, Bureau of Biologics (7). Infected cell cultures showing 75% CPE and control cultures were trypsinized, the cells gently centrifuged, the pellet resuspended in 1% phosphate-buffered osmium tetroxide, and later embedded in Epon for electron microscopic study by Dr. Peter Lampert, University of California at San Diego.

Serologic studies. Sera were stored frozen and were inactivated immediately prior to study at 56°C for 30 min. Neutralization tests were performed in tube cultures of WI38 cells as outlined by Rowe *et al.,* (3) with 10–100 tissue-culture 50% infectious doses ($TCID_{50}$) of virus prepared by freezing and thawing infected monolayers in culture fluids, followed by low-speed centrifugation to remove cellular material. Results were recorded between 12 and 20 days later as indicated by control titrations. No attempt was made to study complement-dependent neutralizing antibody. Complement-fixation (CF) tests were performed by microtechnique (8) using two full units of complement and 4–8 units of antigen. Antigens were prepared by freezing and thawing CMV-infected cultures (when CPE was almost complete) and uninfected control cultures of WI38 cells in culture fluids. Antibody titers were recorded as reciprocals of the highest dilutions of serum completely neutralizing CPE or fixing complement. Dr. David Carver, Johns Hopkins University School of Medicine, provided pooled human serum which neutralized and fixed complement with the Ad.169 strain of human CMV.

Chloroform inactivation. The procedure of Feldman and Wang (9) was followed.

Results. In Table I are summarized isolations of cytomegalovirus from urines of immature monkeys in 1968 and from the same monkeys and their progeny 4 years later. Ten of the 11 original monkeys remained well throughout the 4-year period; one (No. 8) died of bacterial pneumonia a year after the study began. Two babies (Nos. 12 and 13) were born during the last year of observation; one died at age 7 months with bacterial pneumonia.

TABLE I. Persistence of CMV in Urine of Rhesus Monkeys as Juveniles and Adults.

		CMV isolations from urine (Number positive/number tested)	
Monkey no.	Sex	April–July 1968	February–December 1972
1	F	4/4	2/2
2	F	1/2	2/2
3	M	2/2	1/2
4	M	2/2	0/1
5	M	2/2	1/1
6	M	1/1	1/1
7	F	1/1	0/1
8	F	1/1	— (Died Dec. 1969)
9	F	2/2	1/1
10	F	0/1	1/1
11	M	1/1	1/1
12 [1 × 4]	F	—	1/1[a]
13 [2 × 5]	M	—	0/1[b] (Died Dec. 1972)
Total		17/19	11/15

[a] Eight months old.
[b] Six months old.
[ ] = parental Nos.

Ten of 11 monkeys had demonstrated viruria in 1968 and virus was isolated again from eight of ten that were still alive in 1972. Urine of one of the two offspring also yielded virus. Of 19 urine specimens from 11 monkeys examined in 1968, 17 were positive; in 1972 virus was isolated from 11 of 15 urines collected from 12 animals. Hematoxylin–eosin-stained tissues of monkey No. 8 showed no evidence of cytomegalic inclusion disease except for a single swollen cell with an intranuclear in-

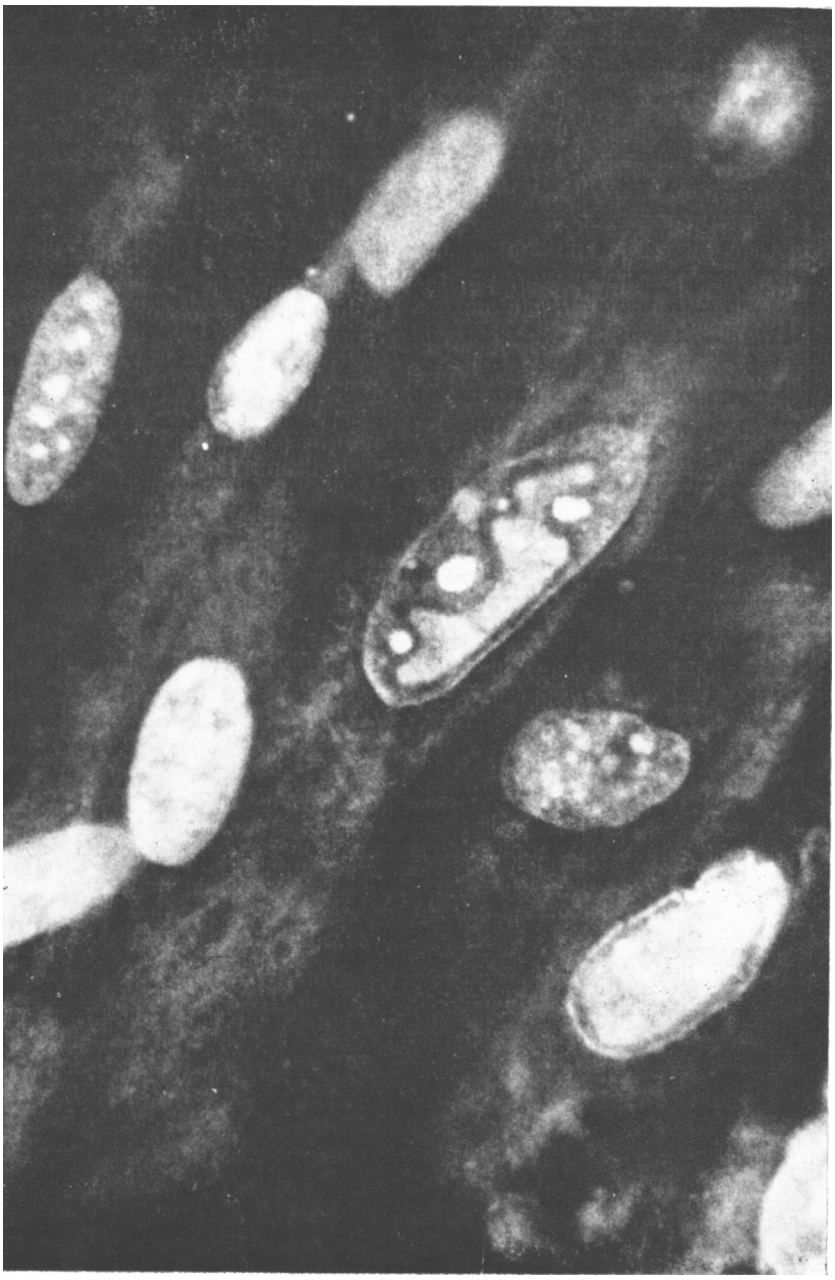

FIG. 1. Rhesus CMV-infected WI38 cells. Fluorescence micrograph showing large bizarre intranuclear inclusion and swollen nucleoli. (Acridine orange, approx. ×1000.)

clusion seen in kidney. Tissues of monkey No. 13 showed no cytomegalic cells.

The 28 isolates of virus all behaved similarly in WI38 tissue cultures, producing focal areas of cellular enlargement typical of CMV. CPE appeared from 5 to as late as 81 days after inoculation of urine into cultures. On serial passage in cell cultures CPE often appeared as early as 3 days after inoculation. CPE usually progressed to complete destruction of the monolayer within 10–30 days after it first appeared. Infectivity titers ranged from less than 10 to 1000 $TCID_{50}$ per ml of urine. Virus passaged in WI38 cell cultures sometimes reached titers of 10^6 $TCID_{50}$ per ml.

Rhesus CMV produced CPE similar to that in WI38 in other human diploid fibroblast lines (MA112, MA184, and MA407), in rhesus monkey diploid fibroblasts (MA101 and MA105) and in African green monkey dipolid fibroblasts (MA117 and MA121), but not in equine dipolid fibroblasts (MA188) or in primary cultures of human embryo kidney, rhesus monkey kidney, African green monkey kidney, and rabbit kidney cells. No disease was observed in a rabbit inoculated im with undiluted infected cells and supernatant fluid or in suckling mice inoculated ic with undiluted material or with a 10^{-1} dilution.

Cytomegalic CPE was confirmed by hematoxylin–eosin-stained cultures which showed characteristic enlarged cells with eosinophilic intranuclear inclusions, first as isolated cells, later as oval nests of swollen cells oriented parallel to the grain of the normal fibroblasts, finally entirely replacing normal cells.

Stained with acridine orange (Fig. 1) the enlarged cells had pink cytoplasm and distorted nuclei containing one or two bright-green lobulated inclusions and swollen nucleoli, findings essentially the same as those produced by African green monkey CMV (7). Electron microscopic examination (Fig. 2) showed in both nucleus and cytoplasm of WI38 cells infected with rhesus CMV masses of particles indistinguishable from particles of Herpesvirus hominis and human CMV prepared in WI38 cultures as controls.

Incubation at 56°C for 10 min or exposure to chloroform for 10 min at 4°C completely inactivated 10^4 $TCID_{50}$ of rhesus CMV.

Sera obtained November 1968 from the 11 original monkeys in the study were screened at 1:10 dilution by neutralization test against strain 68-1 of rhesus CMV, as summarized in Table II. Nine sera neutralized 10 $TCID_{50}$ of virus; sera from monkeys Nos. 2 and 9, from both of which CMV was isolated, failed to neutralize this strain. Serum obtained from rhesus No. 1, prior to inoculation with human CMV, had a neutralizing antibody titer of 20. A 1:10 dilution of pooled human serum which neutralized the Ad.169 strain of human CMV failed to neutralize rhesus CMV.

Sera of the 11 original monkeys were also tested against rhesus CMV antigen (68-1 strain) by CF test: four sera had titers of 8, four were negative (at 1:8 dilution), and three were anticomplementary. In a small CF survey of other monkeys in our colony, four of six rhesus had no detectable antibody to 68-1 at a serum dilution of 1:8, one had a titer of 8, and one of 16. Four of five cynomolgus monkey sera tested were negative at a dilution of 1:8 and one had a titer of 8. Two African green monkey sera were negative at 1:8. Ten additional monkeys had anticomplementary sera. Serum of an investigator with prolonged exposure to infected monkeys was negative at a dilution of 1:2 in CF tests with both the Ad.169 strain of human CMV and rhesus CMV. However, pooled human serum of high titer in CF tests with Ad.169 had a CF titer of 8 with rhesus CMV.

Rhesus monkey No. 1 was inoculated with a total of 10^9 $TCID_{50}$ of human CMV/Ad.169, by ic, iv, ip, im, and sc routes. Table III shows subsequent antibody determinations: a marked increase was demonstrated in the monkey's neutralizing antibody to its own CMV (strain 68-1) without corresponding increases in neutralizing antibody to either human CMV/Ad.169 or to AGM CMV/CSG as measured by our conventional technique without addition of fresh complement. The

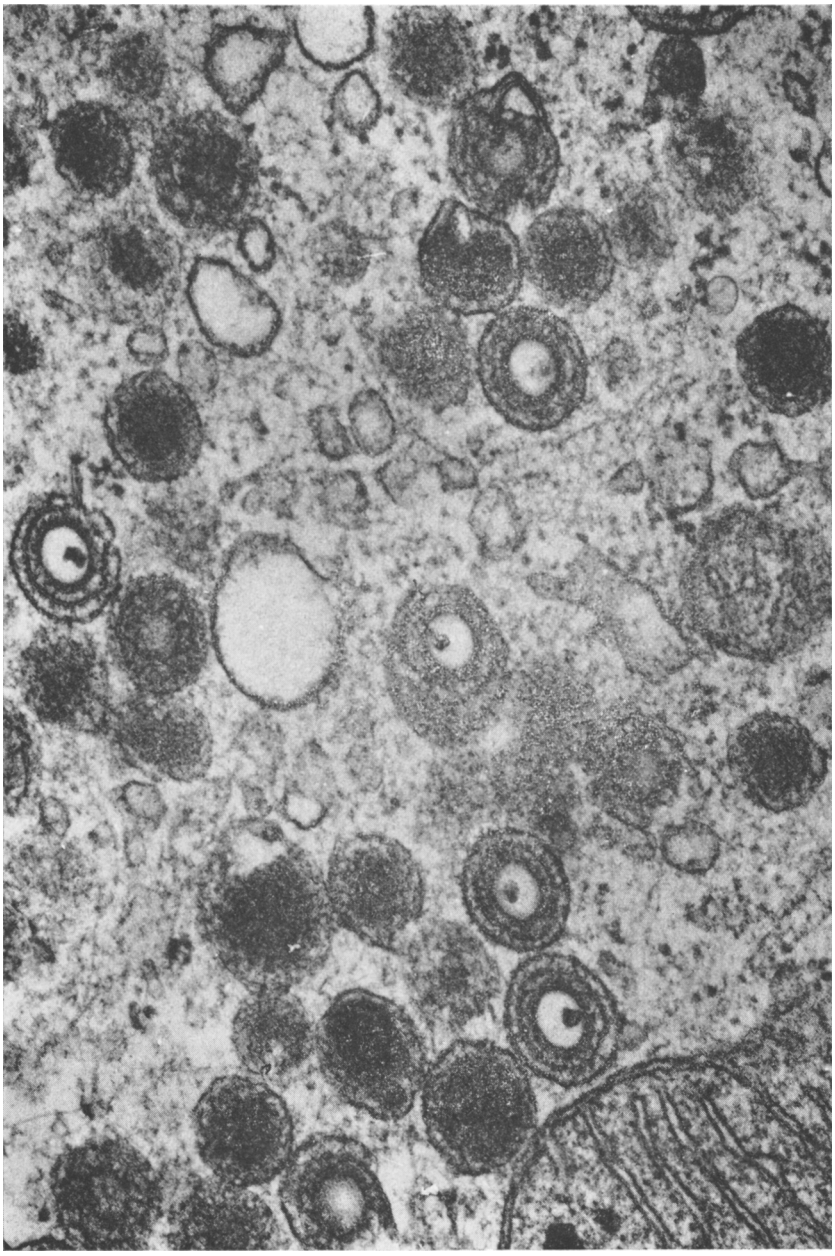

FIG. 2. Rhesus CMV-infected WI38 cells. Electron micrograph showing double-coated particles typical of the herpes virus group. (Osmium tetroxide, approx. ×100,000).

CF test showed an increase in antibody to Ad.169, but, surprisingly, no rise was detected in antibody to homologous rhesus CMV.

Discussion. These viruses satisfy criteria for CMV: they are sensitive to chloroform and heat, have the electron-microscopic morphology of the herpes group and produce in human and monkey fibroblast cultures pathognomonic changes including enlarged, distorted nuclei with bizarre inclusions (10). CMVs have been isolated from two other species of primates, the African green monkey (4, 7, 11–14) and the owl

TABLE II. Presence of Antibody to Rhesus CMV in Simian and Human Sera.

	CMV isolation from urine	Antibody to rhesus CMV	
		Neut[a]	CF[b]
Rhesus monkey No. 1	+	+	+
2	+	−	+
3	+	+	−
4	+	+	+
5	+	+	AC
6	+	+	−
7	+	+	AC
8	+	+	−
9	+	−	AC
10	−	+	AC
11	+	+	−
Other monkeys			
Six rhesus monkeys (4)	NT	NT	−
(2)	NT	NT	+
Five cynomolgus monkeys (4)	NT	NT	−
(1)	NT	NT	+
Two African green monkeys	NT	NT	−
Humans			
One serum negative with human CMV[c]	NT	−	−
One serum positive with human CMV[d]	NT	−	+

[a] Neutralization test: All sera tested at 1:10 dilution with 10$TCID_{50}$ of rhesus CMV, 68-1 strain in WI38 cells.

[b] Complement fixation test: All sera tested at 1:8 dilution with 68-1 strain of rhesus CMV in WI38 cells.

[c] Serum from an investigator with considerable exposure to rhesus monkeys, their urines, and the rhesus CMV, but negative in CF and neutralization tests with the Ad. 169 strain of human CMV.

[d] Pooled blood bank serum with antibody to the Ad. 169 strain of human CMV by CF and neutralization tests.

AC–anticomplementary; NT–not tested.

TABLE III. Neutralizing and Complement-Fixing Antibody Titers to Human and Simian CMVs in Sera from a Rhesus Monkey Inoculated with Human CMV (Ad. 169).

Day of experiment	Rhesus CMV[a]		Human CMV[b]		African green CMV[c]	
	Neut	CF	Neut	CF	Neut	CF
0	20	8	<5	0	<5	NT
11	⩾320	<8	<5	>16	<5	NT
19	⩾320	8	<5	8	<5	NT
28	⩾320	8	<5	4	<5	NT
56	<160	8	NT	4	NT	NT

[a] Antigen prepared from WI38 cells infected with 68-1 strain of rhesus CMV.

[b] Antigen prepared fom WI38 cells infected with Ad. 169 strain of human CMV.

[c] Antigen prepared from WI38 cells infected with CSG strain of African green monkey CMV.

Neut = neutralizing antibody titer (reciprocal of highest dilution of serum completely inhibiting CPE). CF = complement-fixation antibody titer (reciprocal of highest dilution of serum-fixing complement). NT = not tested.

monkey (15) although inclusions suggestive of CMV infection have been observed in tissues of capuchin monkeys (16) and chimpanzees (17) as well as rhe us and African green monkeys (18). Since our initial isolations (1) CMV has been found in rhesus monkey urines by several other groups (19–21).

The relationship of rhesus CMV to AGM and human CMVs is not yet entirely clear. Both rhesus CMV and AGM CMV grow well in human and in heterologous monkey fibroblasts as well as in fibroblasts of the natural host, while human CMVs grow only in human cells. Black, Hartley, and Rowe (4) demonstrated that African green monkey sera with CF antibody to AGM CMV also fixed complement with the Ad.169 strain of human CMV, but that human sera with antibody to Ad.169 failed to react with AGM CMV. Dreesman and Benyesh-Melnick's observations (12) generally confirmed this one-way CF cross reaction, although they found one human serum which did react with AGM CMV to low titer; there was no cross neutralization. Our results suggest that some human sera may also fix complement with, but not neutralize, rhesus CMV. Most of our rhesus monkeys had demonstrable neutralizing antibodies to rhesus CMV; however, other investigators found that sera of rhesus monkeys usually failed to neutralize rhesus CMV even when neutralizing AGM CMV (7, 13). Sera from one rhesus with high titers of neutralizing antibody to rhesus CMV falied to neutralize AGM CMV. Thus, although AGM and rhesus CMVs are similar in biological behavior and are antigenically related to each other and to human CMV, they are not identical viruses.

One rhesus monkey inoculated with the Ad.169 strain of human CMV demonstrated a marked increase in neutralizing antibody to rhesus CMV, but none to Ad.169 or to the CSG strain of AGM CMV. This suggests that the antigenically related human CMV provoked an anamnestic antibody response by the rhesus monkey to its own CMV, a possible example of "original antigenic sin" as described for influenza A (22).

It remains to be learned if monkeys can be infected with heterologous simian CMVs. The demonstration of complement-dependent neutralizing antibodies to heterologous strains of CMV (23) and the development of sensitive kinetic neutralization tests for CMV (24) open further avenues for study of antigenic relationships among human and simian CMVs.

CMV is an important pathogen of man and it would be useful to have an experimental model for the epidemiology and pathogenesis of CMV infections. Experimental infections of the mouse with murine CMV have been well studied but have not given a model of the intrauterine congenital cytomegalic inclusion disease or of the other syndromes associated with human CMV infections (25–27). The chronic asymptomatic viruria found in rhesus macaques resembles that of man (28) and the monkey is an obvious choice for experimental studies of pathogenesis. However, since CMV infections are common among rhesus monkeys, it would be a formidable undertaking to establish colonies free of this agent, particularly since offspring of chronically infected mothers are themselves infected early in life, perhaps at birth or even in utero. Of course, studies of naturally infected animals may also give insights into the nature of human disease.

Summary. Cytomegalovirus persisted in the urine of healthy young rhesus monkeys for more than 4 years. The rhesus cytomegalovirus is similar to cytomegalovirus of the African green monkey in that it grows in cultures of human fibroblasts as well as in both rhesus and African green monkey fibroblasts. Rhesus cytomegalovirus differs antigenically from African green monkey cytomegalovirus although both viruses and human cytomegalovirus appear to share some antigenic determinants.

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