INFECTION AND IMMUNITY, Feb. 1977, p. 568-575
Vol. 15, No. 2

Printed in U.S.A.

Detection of Latent Cytomegalovirus in Murine Salivary and Prostate Explant Cultures and Cells

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Received for publication 8 July 1976

After infection of adult mice, cytomegalovirus was detectable in salivary gland suspensions by tissue culture inoculation for up to 3 months. After these cultures had become negative, virus apparently latent in these tissues could be detected in explants of salivary and prostate glands and in cell lines derived from these explants. In some cases cycles of virus-induced cell injury and regrowth were observed. Murine cytomegalovirus plaque efficiency and morphology were evaluated in prostate and salivary gland cell cultures derived from previously infected and uninfected mice. No evidence of interference was detected, although plaque size was altered (larger) in prostate cells from previously infected mice. These studies indicate the presence of a range of suppression, latency, or effects of murine cytomegalovirus detectable after the resolution of active infection and provide methods for additional study of the establishment and activation of virus latency.

Cytomegalovirus (CMV) is a species-specific member of the herpesvirus group which may cause latent or chronic infections in appropriate hosts. Chronic CMV infection with shedding of virus in saliva is common in wild mice (*Mus musculus*) trapped in both urban and rural areas (5, 13). Laboratory-induced CMV infection in Swiss Webster mice produces prolonged chronic infection, with recovery of virus from the salivary gland and throat secretions (3, 10).

In our studies of the initiation of latency, activation, and transmission of CMV, we have used adult mice of both inbred and outbred strains. We have shown that after the inoculation of adult mice with 10^4 plaque-forming units, murine cytomegalovirus (MCMV) is demonstrable by tissue culture assay of salivary gland homogenates for a maximum of 10 to 12 weeks. Three months after the initiation of infection, virus can no longer be detected by these methods. Nevertheless, we have reported that virus can be reactivated in the salivary gland after heterologous blood transfusion (K. S. Cheung, H. M. Smith, and D. J. Lang, Pediatr. Res. 9:339, 1975), indicating the presence of latent or persistent MCMV infections in organs and tissues of previously infected mice. We report herein the demonstration of latent MCMV in explants and/or alterations in cell lines derived from mouse salivary and prostate glands after virus was no longer detectable by tissue culture assay of organ suspensions.

MATERIALS AND METHODS

Mice. Inbred C_3H and BALB/c strains and outbred specific-pathogen-free CD-I Swiss mice were obtained from Charles River Laboratories and maintained in the animal facilities at Duke University Medical Center. Comparable results were obtained when experiments were performed with each of these strains or with F-1 hybrids. Therefore, no further distinction will be made pertaining to the variety of mouse used in each specific experimental trial.

Virus. MCMV Smith strain was originally obtained from June Osborne of the University of Wisconsin and has been maintained in our laboratory by repeated passages in CD-I mice. MCMV virus pools were prepared by inoculating weanling mice (3 to 4 weeks old) intraperitoneally with 2×10^4 plaque-forming units of virus and harvesting the salivary glands 10 to 12 days later. The salivary glands were homogenized in medium 199 (10%, wt/vol) with a mortar and pestle, centrifuged to remove cellular debris, and stored in liquid nitrogen in 1-ml aliquots.

Cell culture medium. Medium 199 and BME medium with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer were both obtained from Grand Island Biological Co., New York. Medium was supplemented with either 10% inactivated fetal calf serum (growth medium) or with 2% fetal calf serum (maintenance medium).

Mouse embryonic fibroblast (MEF) cultures. CD-I embryos (14 days old) were used for the preparation of tissue cultures. Cell suspensions prepared by trypsinizing the minced embryos were filtered through sterile gauze to remove tissue fragments and cultured in 32-ounce (ca. 1-liter) glass prescription bottles with 40 ml of M-199 growth medium. Secondary embryonic cultures, made from these primary cultures by trypsinization, were routinely used for virus assay.

Virus assay. MCMV was assayed by a plaque procedure using secondary fibroblasts incubated in

plastic culture plates (Linbro Scientific Co., Inc., New Haven, Conn.) maintained at 37°C in an atmosphere of 5% CO_2. Dilutions of virus were introduced into the cell cultures and incubated for 60 min at 37°C. Thereafter, the inoculum was removed, and the cell sheet was overlaid with 1% Methocel in BME medium with 2% fetal calf serum (11). Five days later, the plates were fixed and stained with methylene blue, and the plaques were enumerated.

Explant and cell cultures. Explant cultures were initiated by placing organ fragments on stainless wire grids in 60-mm organ culture dishes (Falcon Plastics, Oxnard, Calif.). The tissue fragments were bathed in BME medium supplemented with 10% fetal calf serum and 10% horse serum. Cell cultures, initiated from explants by Pronase treatment, were attempted at various times after the tissue explant was established. The methods used in establishing the explants and the cell cultures derived from them were essentially the same as described by Chen and Heidelberger (4).

RESULTS

Salivary gland explants and cell lines. Explants of salivary glands obtained from both uninfected and previously infected animals usually survived in vitro cultivation and initially yielded comparable thin sheets of tissue covering the supporting wire grids. In most cases round refractile cells appeared in the cell sheets derived from previously infected animals within a week after the explant was established, and, concurrently, virus was recovered from the culture medium. The likelihood of virus recovery was inversely proportional to the time between the infection of the animal and the preparation of the explant. Virus was not initially recovered from salivary gland explants obtained from mice infected 2 to 3 months or more previously. However, after long periods of cultivation, virus was isolated from cell lines initiated from salivary gland explants derived from two mice infected 10 and 13 weeks previously (Table 1).

A salivary gland explant obtained from a mouse infected 10 weeks previously was maintained in culture for 5 weeks without apparent abnormalities. Thereafter, a suspension of cells was prepared from this explant and seeded in 12 25-cm^2 plastic flasks. The monolayer of cells derived from these cultures appeared healthy until 3 weeks later, when cytopathic effects (CPE) typical of MCMV appeared and destroyed virtually the entire monolayer. A few cells that survived this virus-induced injury replicated and formed a confluent culture after 7 more days. At this time the viral CPE reappeared and destroyed most of these cells. This cycle of regeneration and cell destruction was then repeated. Although the initial cycle of CPE had involved virtually the entire cell

TABLE 1. *Recovery of MCMV from prostate and salivary tissues*

Age at infection (weeks)	Interval between infection and sacrifice (weeks)	Recovery of virus				Remarks
		Salivary gland			Prostate explant	
		Suspension	Explant	Cell line		
4	2	Positive	Positive	ND[a]	Negative	Culture fluid from salivary gland explant was positive from the beginning
4	2	Positive	Positive	ND	Negative	
11	2	Positive	ND	ND	ND	Prostate suspension found positive for MCMV in very low titer
10	4	Positive	ND	ND	ND	
3	10	Negative	Positive	ND	Negative	Culture fluid positive for MCMV after salivary gland explant established for 2 weeks
6	10	ND	Negative	Positive	ND	Cell line initiated 5 weeks after explant; CPE appeared 20 days after cell line established
6	13	ND	ND	ND	Negative	
7	13	Negative	Negative	ND	Positive	Culture medium positive for MCMV 2 weeks after explant established
10	13	Negative	Negative	Positive	ND	Cell culture initiated 1 week after explant; CPE appeared 6 months later
12	34	Negative	Negative	ND	Positive	Culture medium became positive for MCMV 2 weeks after explant established

[a] ND, not determined.

sheet, the cellular destruction observed in the subsequent cycles was always incomplete and cycles of virus-induced injury occurred concurrently with cell regrowth, preventing the monolayer from reforming into more than small patches. Figure 1 records this sequence of events. The culture was observed for 3.5 months after its initiation from the salivary gland explant, until it was lost through fungus contamination. Virus recovered from this culture induced CPE typical of MCMV when grown in secondary MEF. The virus also produced characteristic salivary gland cytopathology when inoculated into mice.

In another instance, explants were derived from salivary gland fragments obtained from a female mouse infected 3 months previously. Cell cultures were initiated from the explanted tissues 1 week later. Simultaneous explants and subsequent cell cultures were initiated from an uninfected female mouse. The cell cultures derived from the infected and uninfected

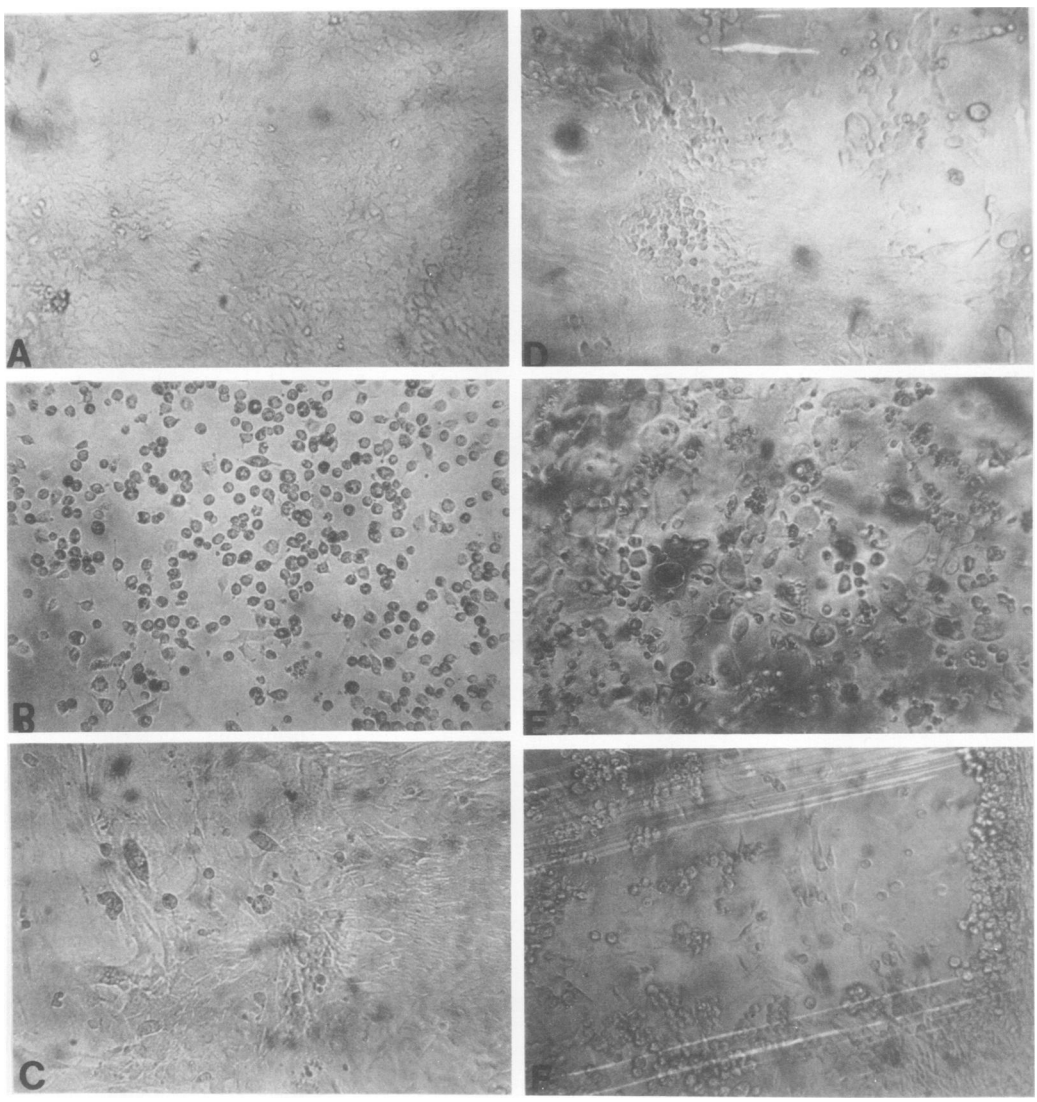

FIG. 1. *Cyclic appearance of CPE of MCMV in a cell line derived 20 days earlier from a 5-week-old salivary gland explant (ISG) of a mouse infected at 10 weeks of age and sacrificed 7 weeks later. (A) Cell monolayer derived from explant; (B) appearance of extensive MCMV CPE; (C) subsequent regrowth of monolayer; (D) reappearance of MCMV CPE; (E) further cell destruction secondary to MCMV replication; (F) simultaneous appearance of CPE and regenerating cells.*

mice were morphologically indistinguishable. After an initial period of slow growth these two cell lines grew so rapidly that it was necessary to perform transfers every 3 to 4 days. For over 6 months both lines appeared normal in vitro and did not show evidence of a productive MCMV infection, even when inoculated into susceptible mice. After 6.5 months of cultivation, CPE characteristic of MCMV appeared in the cells originally derived from the infected mouse. Cycles of CPE and cell regeneration were observed thereafter. The changes noted in this cell line are pictured in Fig. 2. The virus recovered was transmissible to mice and murine embryonic tissue cultures, yielding a characteristic salivary gland infection and CPE.

Prostate gland homogenates. MCMV was recovered from tissue homogenates of prostate in only one of seven attempts. In this instance the prostatic tissues were obtained 2 weeks after infection of MCMV. The virus titer in the prostatic suspension was 1 to 3 plaque-forming

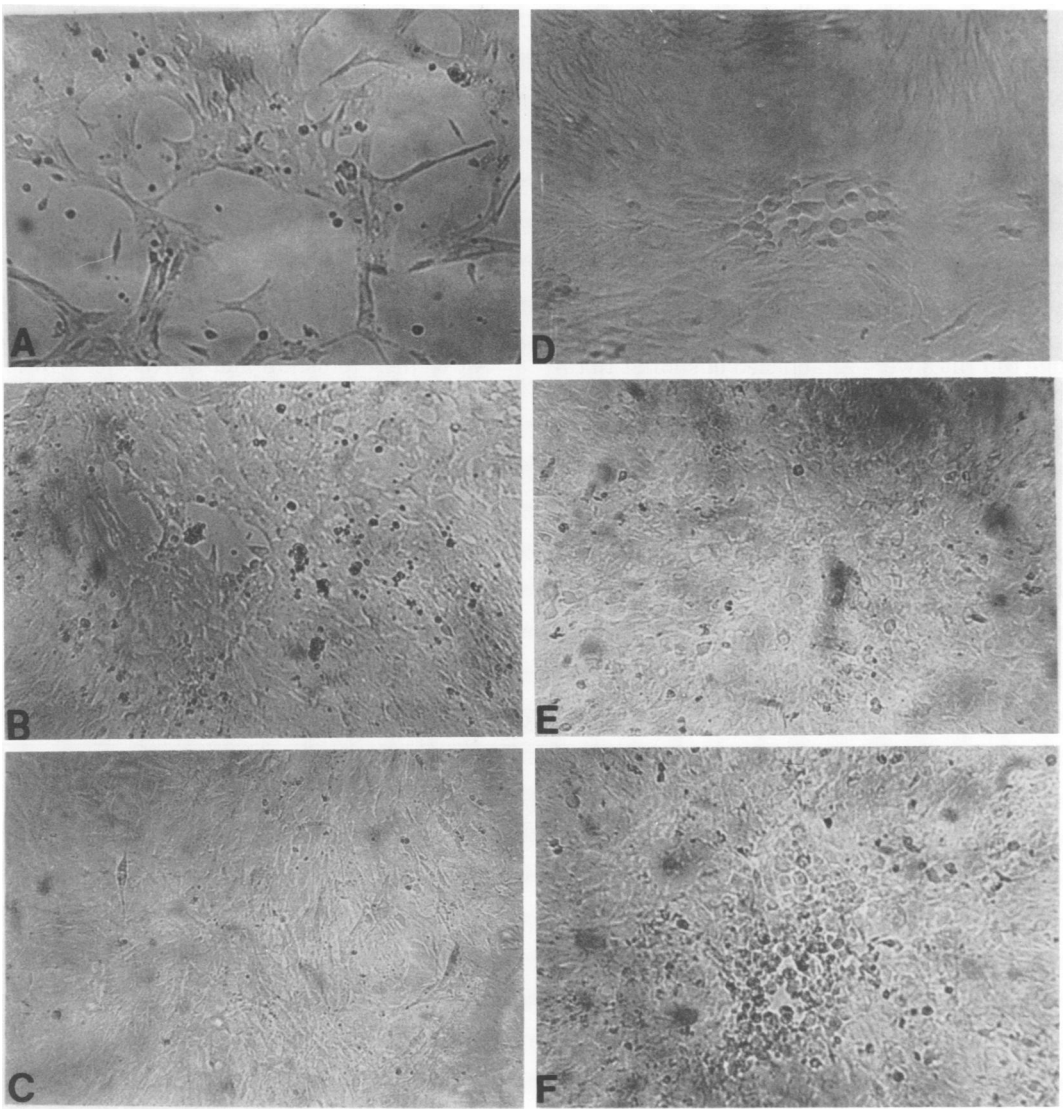

FIG. 2. *Late appearance of CPE of MCMV in a cell line derived 6 months earlier from a 1-week-old salivary gland explant of a female mouse infected at 8 weeks of age and sacrificed 3 months later. (A) Cell line derived from explant (passage 1); (B) cell line at passage 7; (C) subsequent normal appearance of cell line; (D) initial appearance of CPE 6 months after establishment of cell line; (E) re-established monolayer after cycle of CPE; (F) reappearance of CPE.*

units/0.1 ml of a 10% (wt/vol) preparation. Infectious virus was not recovered from prostatic homogenates of two animals infected more than 3 months previously.

Prostate gland explants and cell lines. Explants of prostate were established from uninfected mice as well as from animals previously infected with MCMV. Although infectious virus was not recovered from the prostatic homogenates of animals infected more than 3 months previously, MCMV was recovered from two of three explants of these glands (Table 1).

Prostate cell lines were derived from explants established from control and infected animals (Table 2). Three prostate cell lines (NP_J, NP_K, and NP_O) were initiated from 3-week-old explants derived from uninfected young adults. Two prostate cell lines designated IP_1 and IP_2 were initiated from mice infected with MCMV more than 3 months previously.

All five prostatic cell lines initially appeared to contain a mixture of cell types. Each of the prostate lines underwent a series of crises during which only a small population of cells survived to repopulate the culture. Eventually, five cell lines were established of similar fibroblastic morphology. There was no recognizable morphological difference between the cell lines derived from control or previously infected animals (Fig. 3).

Superinfection of prostate and salivary cell lines with MCMV. Morphologically normal prostate and salivary gland cell lines derived from infected and control animals were challenged with MCMV to search for the presence of interference as a manifestation of residual or latent virus. The plaque procedure was used for this purpose to evaluate both the plaque morphology and number.

Two prostate cell lines were derived from mice infected at the age of 3 to 4 weeks with a low dose of MCMV. At the time of sacrifice for explant the mice were 10 and 17 weeks old, respectively. The cell lines were initiated after the prostate explants had been maintained in culture for 5 weeks. Prostate cell lines were similarly established from uninfected mice for comparison.

To obtain a salivary gland cell line from an infected animal, it was necessary to wait at least 3 months after the original infection before initiating an explant. Tissues explanted prior to that time usually resulted in the rapid appearance of CPE. A cell culture was established from the salivary gland explants of a mouse infected more than 3 months previously (ISG_2). Although CPE appeared spontaneously in this cell line after 6.5 months, the effect of MCMV superinfection was studied prior to that time. A salivary gland cell line-derived from an uninfected male was used for comparison in this study (NSG_2) (Table 2).

All of these prostate and salivary cell lines were infected with serial dilutions of MCMV to study the efficiency of infection. There was no significant difference in the efficiency of MCMV infection in infected and control cell lines as well as in MEF. The plaque size varied significantly, however (Fig. 4). MCMV superinfection of cell lines derived from salivary glands yielded microplaques with a similar efficiency and morphology whether from infected or uninfected animals. Medium size plaques were produced in MEF and in prostate cells derived

TABLE 2. *Cell lines derived from explants and subsequent MCMV recovery*

Expt. conditions	Age at sacrifice (explant; weeks)	Tissue explanted	Cell line designated	Age of explant when cell line established (weeks)	Age of cell line at time of MCMV recovery
Uninfected	7	Prostate	NP_J	3	No virus recovered
Uninfected	7	Prostate	NP_K	3	No virus recovered
Uninfected	7	Prostate	NP_O	2.5	No virus recovered
Infected, age 3 weeks	10	Prostate	IP_1	5	No virus recovered
Infected, age 4 weeks	17	Prostate	IP_2	5	No virus recovered
Uninfected	8	Salivary gland (male)	NSG_1	5	No virus recovered
Uninfected	8	Salivary gland (female)	NSG_2	1	No virus recovered
Infected, age 10 weeks	17	Salivary gland (male)	ISG_1	5	Virus recovered from cell line after 20 days
Infected, age 8 weeks	21	Salivary gland (female)	ISG_2	1	Virus recovered from cell line after 6.5 months

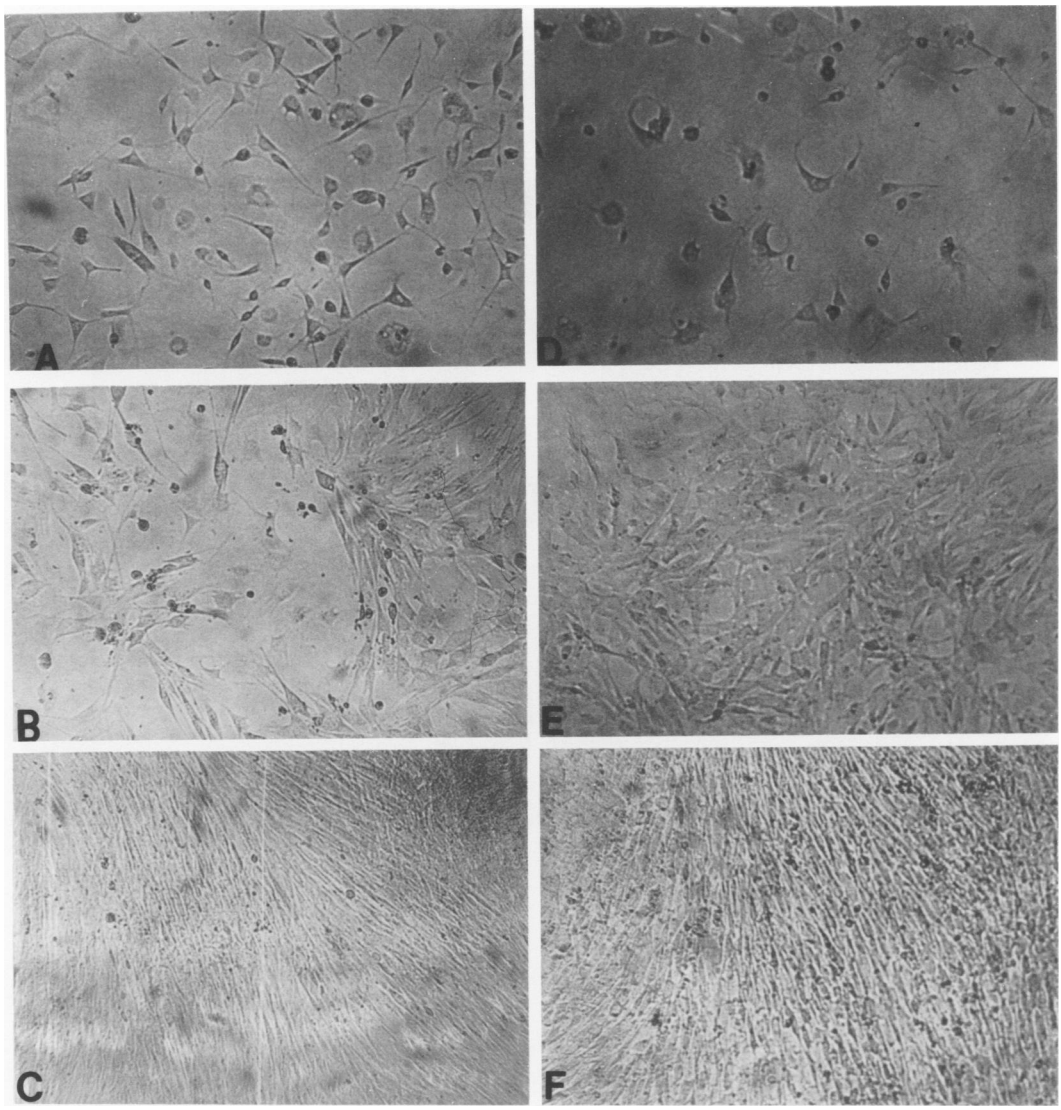

FIG. 3. *Prostate cell lines derived from explants. Appearance of cells from an uninfected mouse (NP_K): (A) initial appearance; (B) after 9 passages; (C) monolayer after passage 15. Appearance of cells from a mouse infected 7 weeks prior to sacrifice (IP_1): (D) initial appearance; (E) after 9 passages; (F) monolayer after passage 15.*

from uninfected animals, whereas, in contrast, much larger plaques were consistently produced in the prostate cells derived from infected mice.

DISCUSSION

In adult mice, infection with low doses of MCMV eventually resulted in the establishment of a quiescent or latent infection in the salivary gland. In many instances it was possible to reactivate and/or detect latent or suppressed salivary gland MCMV infection in explant tissues or in cell cultures derived from explants. The use of cell cultures and explants has facilitated the recovery of latent viruses in other systems (1, 2, 14, 15). It may be that activation of latent viruses is promoted by the enhanced cellular proliferation associated with in vitro survival and growth.

Cycles of CPE were observed in salivary gland explants of infected mice and in cell monolayers derived from them. Similar cycles of viral CPE and monolayer repair were described by Hampar in connection with herpes simplex

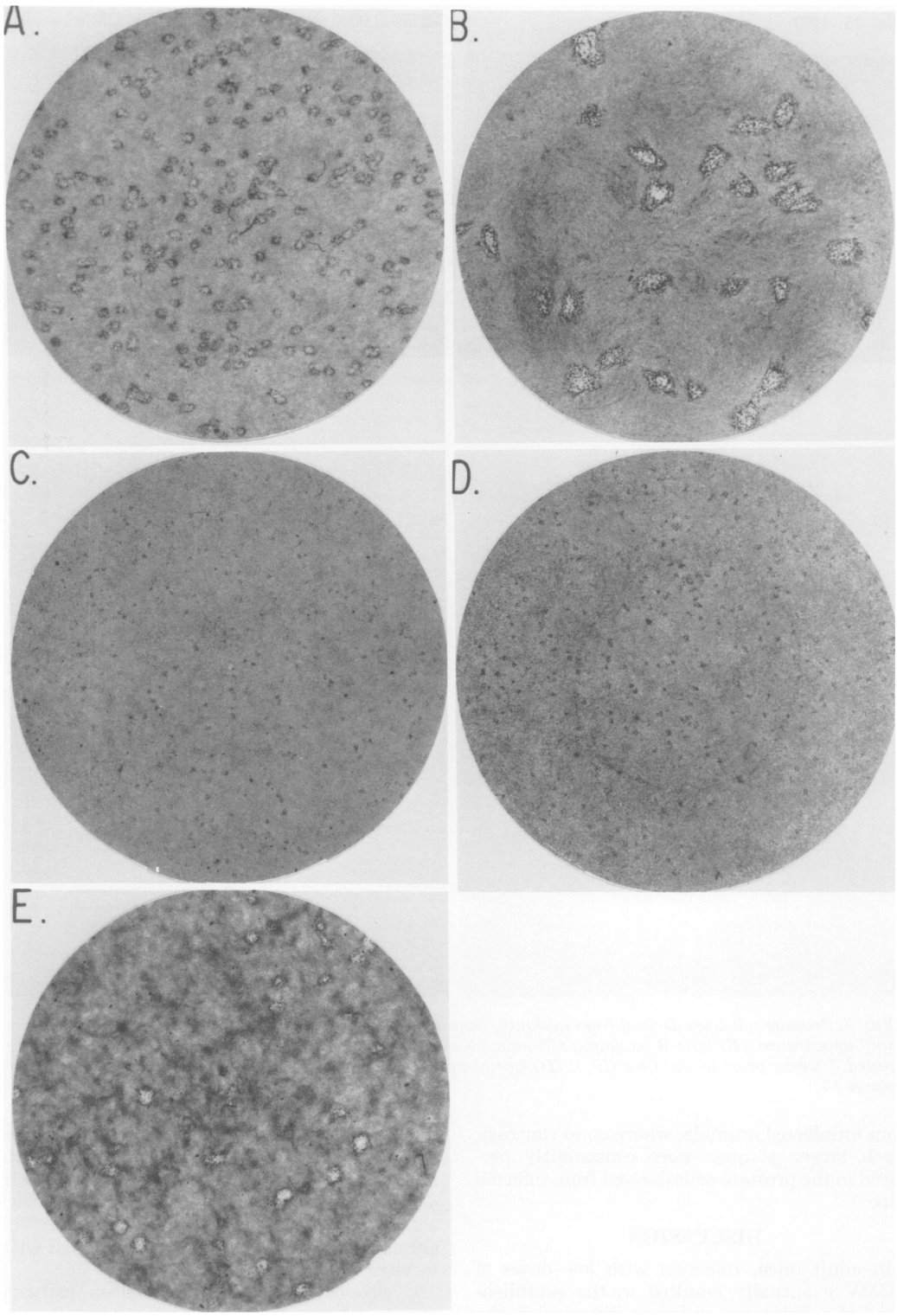

FIG. 4. *Appearance of plaques after superinfection with MCMV of the following cell cultures: (A) prostate cells from uninfected adult; (B) prostate cells from previously infected adult; (C) salivary cells from uninfected adult; (D) salivary cells from previously infected adult; (E) secondary culture of normal embryonic cells.*

virus infections of guinea pig cells (6–9). These observations may indicate the presence of cell types with variable susceptibility to virus replication and injury. The small plaques seen when salivary gland cell lines were superinfected with MCMV in vitro sugests a degree of intrinsic resistance to productive MCMV infection in these cells. It is possible that cycles of replication and healing occur in these relatively virus resistant cells in vivo and that this in part is responsible for viral persistence and intermittent shedding.

We have documented in this study that MCMV may be found persistent or latent in the mouse prostate gland, although the frequency and magnitude of the prostate infection was less than that noted in salivary gland cultures. Comparable efficiency of MCMV infection was observed in MEF and in the cells derived from infected and control salivary and prostate glands, indicating that residual or latent virus was not yielding significant interference. However, the size of the plaques induced in these cells differed significantly, perhaps reflecting differences in the ability of different cell types to support the growth and production of infectious virus.

Large plaques observed after superinfection with MCMV of prostate cells from previously infected mice may be due to the selective survival of a highly susceptible population of prostate cells. Alternatively, enhancement of plaque size with an unchanged efficiency of infection may reflect the rescue by complementation of incomplete MCMV residual in prostate cells. Superinfection with complete infectious virus may supply enzymes or structural proteins to facilitate full expression of the latent or residual MCMV genes. These findings are of particular interest in light of a recent publication by Rapp and associates reporting the demonstration of long-term persistence of CMV genome in cultured human cells of prostatic origin (12).

ACKNOWLEDGMENT

This work was supported by Public Health Service grant 5 RO1 HD04183-05 from the National Institute of Child Health Development.

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