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Isolation and Growth of Rat Cytomegalovirus *in Vitro* (34010)

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Cells characteristic of cytomegalovirus (CMV) infection have been observed histologically in salivary glands of guinea pigs, rats, mice, hamsters, moles, dogs, monkeys, and man (1). The cytomegaloviruses of mice, guinea pigs, horses, monkeys, and man have been grown *in vitro* in cell cultures and the viruses have shown marked species specificity with the exception of monkey CMV which grows in human as well as monkey cells, and the equine CMV which grows in rabbit cells (1-3).

In 1934, Kuttner and Wang described cytomegalic inclusion disease in the salivary glands of 50% of wild rats trapped in Peking, China and they were able to transmit the disease to other rats with cell-free filtrates (4). We have been unable to find any reports of isolation of the rat CMV in culture.

During an investigation of the prevalence of trypanosomes in roof rats (*Rattus rattus*) in the province of Panama, Republic of

Panama, 116 adult rats were trapped during the first 7 months of 1968 and brought to Gorgas Memorial Laboratory, where they were autopsied after one or more bleedings. Blocks of submaxillary, sublingual, and parotid glands were obtained from 84 rats (46 females and 38 males) and histopathologic evidence of CMV infection was found in 41 (23 females and 18 males). Characteristic intranuclear and intracytoplasmic inclusions were found in the submaxillary glands of all 41, the sublingual glands of 11, and the parotid glands of 2. (Fig. 1). The kidneys, lungs, and brains of the rats were examined and no CMV lesions were noted. The present report describes the isolation and growth of rat CMV *in vitro* from salivary glands of these wild Panamanian rats.

Materials and Methods. Collection of specimens. The right salivary glands of 15 rats were dissected with sterile instruments, transferred to sterile containers, and frozen at -70° . The left salivary glands were examined histopathologically and 6 of the 15 had evidence of CMV infection. The frozen

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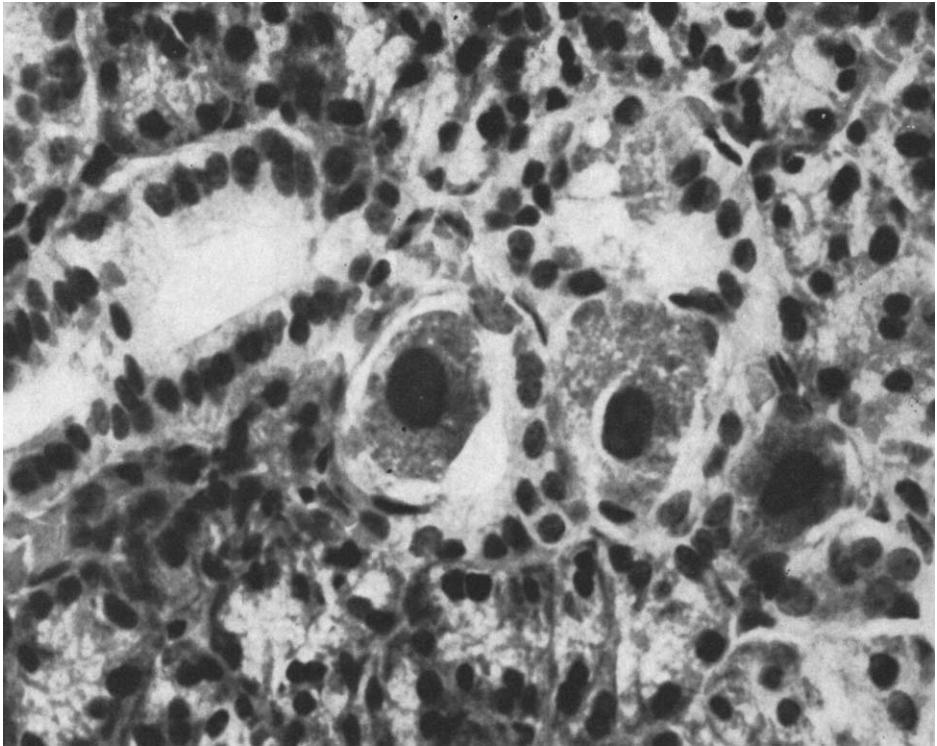


FIG. 1. Section of submaxillary gland of Panamanian wild rat with cytomegalic inclusion disease. Large cells with intranuclear and intracytoplasmic inclusions are seen in the ducts; hematoxylin and eosin; $\times 610$.

glands from these animals were sent to NIH in Bethesda, Md. for virus isolation.

Cell cultures. Monolayer cultures of a cell line derived from kidneys of (Lew-BN) F_1 rats were used for initial virus isolation and subsequent passage and plaque assays. Newborn Syrian hamster kidney cell cultures were prepared from explants and by trypsinization. All cultures were grown and maintained in a medium composed of 20% fetal bovine serum (FBS) and 80% RPMI no. 1640.

Plaque assay. Virus was titered by a plaque assay similar to that used by Plummer and Benyesh-Melnick for human CMV (5). Monolayers of Lew-BN rat cells in 25 cm^2 Falcon flasks were inoculated with 0.2 ml of virus preparations which had been diluted in growth medium (80% RPMI no. 1640 and 20% FBS). After 1-hr absorption at 37°, the inoculum was removed, the flasks were washed with medium and overlaid with

the methylcellulose medium described by Plummer and Benyesh-Melnick (5). After 7-days incubation at 36°, the overlay was removed and the cultures were stained with methylene blue. Distinct plaques were evident at 10–30 \times magnification with a dissecting microscope.

Plaque neutralization studies. All sera were inactivated at 56° for 30 min and diluted in growth medium; virus serum mixtures were incubated at 37° for 30 min and inoculated into 25 cm^2 Falcon flasks (0.2 ml/flask) containing monolayers of Lew-BN cells. After 1 hr absorption, the flasks were washed and overlaid with methylcellulose medium as described under "Plaque Assay."

Results and Discussion. The frozen salivary glands were thawed and minced into explants 1 mm in diameter. The explants were placed on monolayers of Lew-BN rat cells in 25 cm^2 Falcon flasks (25 explants/flask) and fed with 6 ml of medium. After 5-days incu-

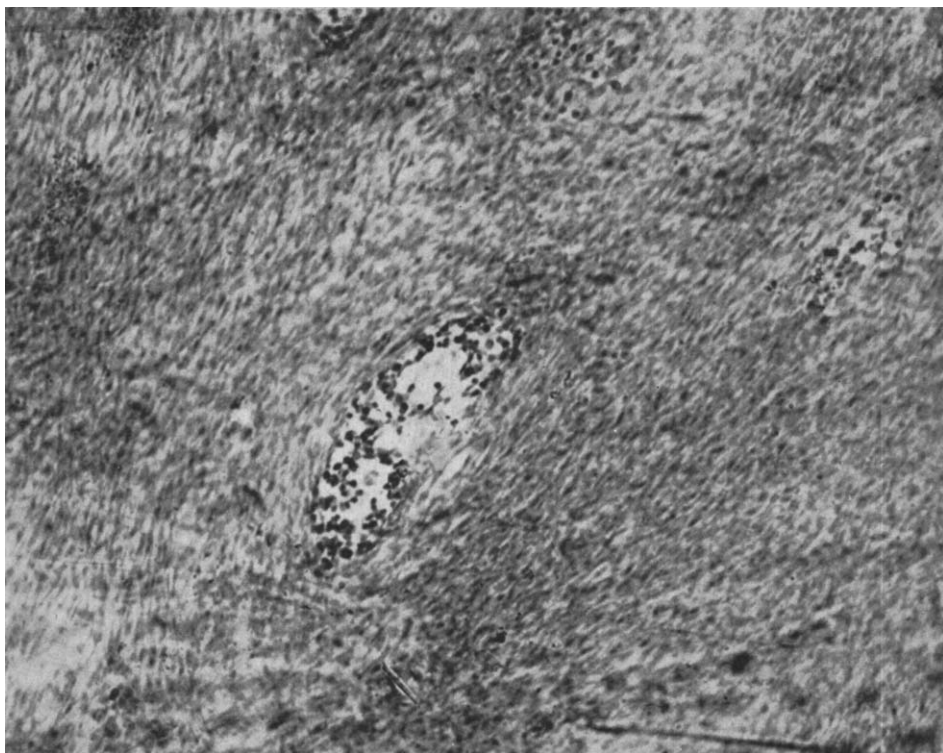


FIG. 2. Monolayer of rat kidney cells 7 days after infection with rat cytomegalovirus: culture was grown on coverslip in Leighton tube, fixed and stained with hematoxylin and eosin. Plaque with dark staining cells and some loss of cells in central portion is seen; $\times 85$.

bation, typical CMV plaques were seen on the monolayers and these progressed to involve the entire culture in 14–21 days. Fluid from infected cultures with cytopathic effects could be passed to new monolayers with the appearance of typical plaques in 3–5 days. Monolayers of rat cells grown on coverslips in Leighton tubes were infected and when plaques were evident they were fixed in formal-sublimat^e and stained with hematoxylin and eosin. Intranuclear and intracytoplasmic inclusions typical of CMV infection were seen (Fig. 2 and 3).

Virus pools were prepared in Lew-BN cells in 75 cm² flasks. When more than 75% of the cells showed cytopathic effects, the cultures were frozen and thawed 2 times, centrifuged at 200g for 5 min and the supernatant fluids were frozen in 1-ml amounts in glass vials at -70° . Pools prepared in this manner contained $10^{4.5}$ to $10^{5.0}$ pfu/ml.

Sera from 35 wild Panamanian rats were tested at a 1:10 dilution for neutralizing antibody against 100 pfu of virus. Twenty of the 35 sera neutralized more than 80% of the pfu. No neutralizing activity has been found at 1:10 dilution in 4 sera from NIH Laboratory rats (Osborne-Mendel, Buffalo, ACI/N), 2 black spider monkeys, and 5 humans living in the area in which the rats with cytomegalic inclusion disease were trapped.

Primary explant cultures of newborn Syrian hamster kidneys were infected with $10^{5.15}$ pfu of the rat CMV from a pool prepared as described above in an attempt to produce some type of "transformation" in cells of a heterologous species. Instead, typical CMV plaques appeared in 7 days and spread to involve most of the cells in the cultures within 14 days. The virus was passed serially in monolayer cultures of primary hamster kid-

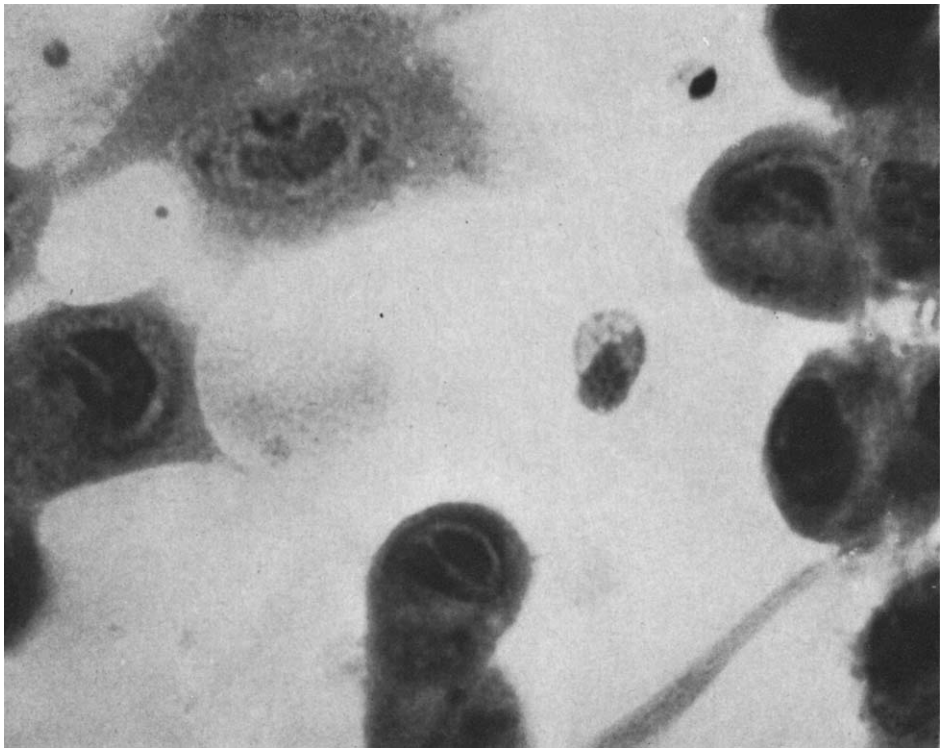


Fig. 3. Same as Fig. 2 at higher magnification: cells in plaque contain intranuclear and intracytoplasmic inclusions typical of cytomegalovirus infection; hematoxylin and eosin; $\times 1340$.

ney (3 passages) with production of intranuclear and intracytoplasmic inclusions similar to those seen in infected rat cells.

When monolayer cultures of primary hamster kidney cells were substituted for the Lew-BN rat cells in the plaque assay, typical CMV plaques were produced in 7 days; however, a sample of virus which produced 400 plaques in a flask of rat cells produced only 125 plaques in hamster cells.

The CMV plaques produced by hamster cell passaged virus could be neutralized by rat sera that had neutralizing antibody in the rat cell system.

We have been unable to produce cytomegalic inclusion disease in rats with the virus grown in rat cell cultures suggesting that the agent has become attenuated *in vitro*. Newborn and adult Sprague-Dawley rats were inoculated with 10^4 to 10^5 pfu intraperitoneally and intracerebrally but no evidence of illness occurred during a 2-month observa-

tion period. No histologic evidence of cytomegalic inclusion disease could be found in animals killed 1-2 weeks after infection.

Summary. Histopathologic evidence of cytomegalovirus infection was found in the salivary glands of 49% of wild rats trapped in the Republic of Panama. Rat cytomegalovirus was isolated from these glands in monolayer cultures derived from (Lew-BN) F_1 rat kidneys and serially passed in these cells with production of typical cytomegalovirus plaques and intranuclear and intracytoplasmic inclusions. The virus could also be serially passed in primary hamster kidney cells with production of plaques and inclusion bodies. Sera of 20 of 35 wild Panamanian rats contained neutralizing antibody, but no antibody was found in sera of 5 humans living in the area in which the infected rats were trapped.

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Nucleic Acid Content of Visna Virus* (34011)

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Visna virus produces a progressive and fatal neurological disease of sheep (1, 2). Many months or years may pass between introduction of the virus into the animal and the first appearance of disease, which establishes the "slow" nature of the infection.

Visna virus was first propagated in primary cell structures prepared from sheep choroid plexus (SCP) (3), and most studies of the virus have been performed with such cultures. In SCP cells virus growth occurs after a latent period of 16–20 hr; infective virus increases exponentially from 20–36 hr, followed by a gradual increment until 72–96 hr after infection (4, 5). Virus replication is accompanied by cytopathic changes and cell destruction.

Visna virus has also been shown to multiply in a continuous cell line derived from pig kidney [PK(15)] (6). Peak titers of infective virus in these cells are reached 96–168 hr after infection, and neither cytopathic changes nor cell depletion are observed.

Electron microscopic observations indicate that virus particles are formed by budding from the cytoplasmic membrane of host cells

in a manner similar to the RNA-containing avian leukosis and murine leukemia viruses (7), and histochemical observations demonstrate that virus-infected cells harbor increased amounts of cytoplasmic RNA (8) and contain aggregates of viral antigen which have the staining characteristics of RNA (9). Although these data strongly suggest that visna virus is an RNA-containing virus, direct evidence of the nature of the nucleic acid in the infective virus particle is still lacking.

This report describes the purification of visna virus by density-gradient centrifugation in cesium chloride, attempts to incorporate radioactive nucleic acid precursors into virus particles, and studies on the effect of actinomycin D on RNA synthesis in virus-infected cells. The results indicate that visna virus contains RNA and suggest that actinomycin D suppresses virus-specific RNA synthesis.

Materials and Methods. Cell cultures. Sheep choroid plexus (SCP) cells were prepared by trypsin dispersion of choroid plexuses removed from the brains of exsanguinated domestic Hampshire or Suffolk sheep as previously described (5). Cells were grown in reinforced Eagle's medium (10) containing 10% fetal bovine serum in 250-ml plastic flasks and incubated at 37°. SCP cells in their fourth serial passage were suspended in medium containing glycerin, frozen and stored in a liquid nitrogen refrigerator; cells were reconstituted when needed and used in their sixth to ninth passage.

Porcine kidney [PK(15)] cells were obtained from the American Type Culture Col-

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