Characterization of a Cytomegalo-Like Virus Isolated from Spontaneously Degenerated Equine Kidney Cell Culture* (33492)

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Cytomegaloviruses have been isolated from several animal species including man, monkey, dog, mouse, and guinea pig (1-4). During our studies on latent virus infections in primate and nonprimate kidney tissues, several horse kidney tissues were included for examination. The present report describes the isolation of a virus from a horse kidney cell culture which underwent spontaneous degeneration after prolonged cultivation; the isolate appears to be an equine representative of the cytomegalovirus group.

Materials and Methods. Tissue cultures. Primary equine kidney cell cultures: Portions of the cortex of fresh equine kidney tissue were cut into small pieces and trypsinized according to the method previously described for monkey kidney tissue (5). The growth medium, at the beginning of the study, consisted of Hanks' balanced salt solution, 0.5% lactalbumin hydrolysate and 20% calf serum. Later, Eagle's basal medium with Hanks' salt solution and 10% calf serum was used for growth. The cell cultures were propagated in large bottles until confluent monolayers had developed. The cells were then removed from the glass with 0.2% trypsin and were subcultured in Leighton tubes containing 11 × 22 mm coverslips. These subcultures were incubated at 37° until complete cell sheets had formed and were maintained in Earle's salt solution, containing 0.5% lactalbumin hydrolysate and 2% calf serum, as long as the cells were in good condition.

Equine dermis or ED cell line (CCL57): This cell line was initiated on September 16, 1965 by A. J. Kniazeff, W. A. Nelson-Rees, and R. O. Owens from a skin biopsy of a horse. Through the courtesy of A. J. Kniazeff we obtained the ED cell line and propagated it readily in Eagle's basal medium with Earle's salt solution and 10% calf serum.

Cytopathology. Whenever CPE was observed or when the cultured cells degenerated, fluids from the culture tubes were removed and the coverslips containing the cells were either fixed with Zenker's acidic fluid and stained with hematoxylin-eosin or fixed in Carnoy's fluid for Feulgen reaction (5).

Size determination. Ultrafiltration of the new isolate was performed by Millipore membrane filtration using membranes of 50, 100, and 300 mμ limiting pore diameters, a method previously described (6).

The electron microscopic techniques used were essentially the same as those described in an earlier report (7). In brief, the infected cells were removed from the glass, fixed in 2% glutaraldehyde, postfixed in osmium tetroxide, embedded in maraglas and examined with a Siemens electron microscope.

Sensitivity to lipid solvents. Equal volumes of diethyl ether and virus suspension were mixed and incubated for 1 hr at room temperature with intermittent shaking.

Serological tests. Complement fixation tests. The modified CF procedure (5) was employed, using overnight refrigeration fixation with 2 full units of complement. Antigens consisted of infected cells plus culture fluid which were concentrated by osmotic dialysis against Carbowax 20.

Neutralization tests were carried out by adding approximately 100 $TCID_{50}$ of virus suspension to serial 2-fold dilutions of serum. After the virus–serum mixture had incubated at room temperature for 1 hr, 0.2 ml of each dilution was inoculated into monolayers of horse kidney cells or ED cell cultures.

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TABLE I. History of the Horses[a] and the Growth of Their Kidney Tissue Cells.

Horse no.	Age	Immunization or other usage	Date sacrificed	Growth of cell culture
53	12	Supply normal serum	12- 6-66	Good
165	30	Supply normal serum	12-20-66	Good
82[b]	23	Immunized with simian adenovirus SV_{17}	1-10-67	Good
185	22	Immunized with human globulin	1-10-67	Good
186	26	Immunized with human serum	1-17-67	No growth
210	23	Immunized with human adenovirus type 19	3- 1-67	Poor
72	10	Supply normal blood	3-16-67	Contaminated with molds
55	19	Immunized with human adenovirus type 27	3-30-67	Good
1184	30	Immunized with diphtheria toxin	8-17-67	Excellent

[a] All horses were geldings.

[b] The 82-A virus was isolated from this animal; stock virus of SV_{17} was prepared in LLC-MK_2 cells using plaque purified virus.

Results. Isolation of virus 82-A from spontaneously degenerated horse kidney cell cultures. As shown in Table I, attempts were made to propagate, *in vitro*, kidney tissues obtained from a total of nine horses; good growth was obtained from six. From January to May 1966, horse no. 82 had received seven injections of suspension of live simian adenovirus, SV_{17}, for the production of hyperimmune serum. This horse was not utilized again until sacrifice on January 10, 1967. Immediately after the horse was sacrificed, kidney tissue was obtained, and cell cultures were prepared. Complete cell sheets developed by January 30. Subcultures were made on February 1, 10, and 20. Spontaneous degenerations were first noted in several subcultures on March 8 but extensive CPE was not obtained until April 2. Spontaneous CPE appeared first in foci of small rounded cells and progressed very slowly. Extensive cellular destruction occurred only after 3–4 weeks of the initial appearance of CPE. Such cellular destruction occurred in other primary horse kidney cell cultures even after six serial passages, thus establishing a transmissable agent. Currently, the 82-A virus is being propagated in the ED cell culture. Typical foci of rounded cells in ED cultures similar to those produced by 82-A virus in primary horse kidney cell cultures as shown in Figs. 1 and 2.

Nucleic acid type. In the H&E stained preparations, basophilic intranuclear inclusions were observed in the infected cells within the degenerated areas (Figs. 3 and 4); these inclusions were Feulgen-positive indicating that they were DNA in nature.

Size determination. Electron microscopic examination of thin sections of infected primary horse kidney cell revealed particles that were similar to herpesvirus (8). Virus particles with or without electron dense central cores were usually seen in the infected nuclei (Fig. 5), and occasionally within the cytoplasmic vacuoles which are not shown in Fig. 5. All viewed particles had an inner membrane as shown in Fig. 5, and appeared to be oval in shape; the size of the virus without outer membrane was approximately 106 mμ in diameter.

Ultrafiltration. As shown in Table II, the virus particles passed through a 300 mμ membrane with some reduction in virus titers, while infectivity was removed when virus suspensions were filtered through membranes of 100 and 50 mμ. According to previ-

TABLE II. Filtration and Ether Treatment of 82-A Virus.

Methods of treatment	Virus titers ($TCID_{50}$/0.1 ml)
Filtration through Millipore membrane (mμ)	
Unfiltered control	3.5
300	2.0
100	0
50	0
Treatment with diethyl ether	0

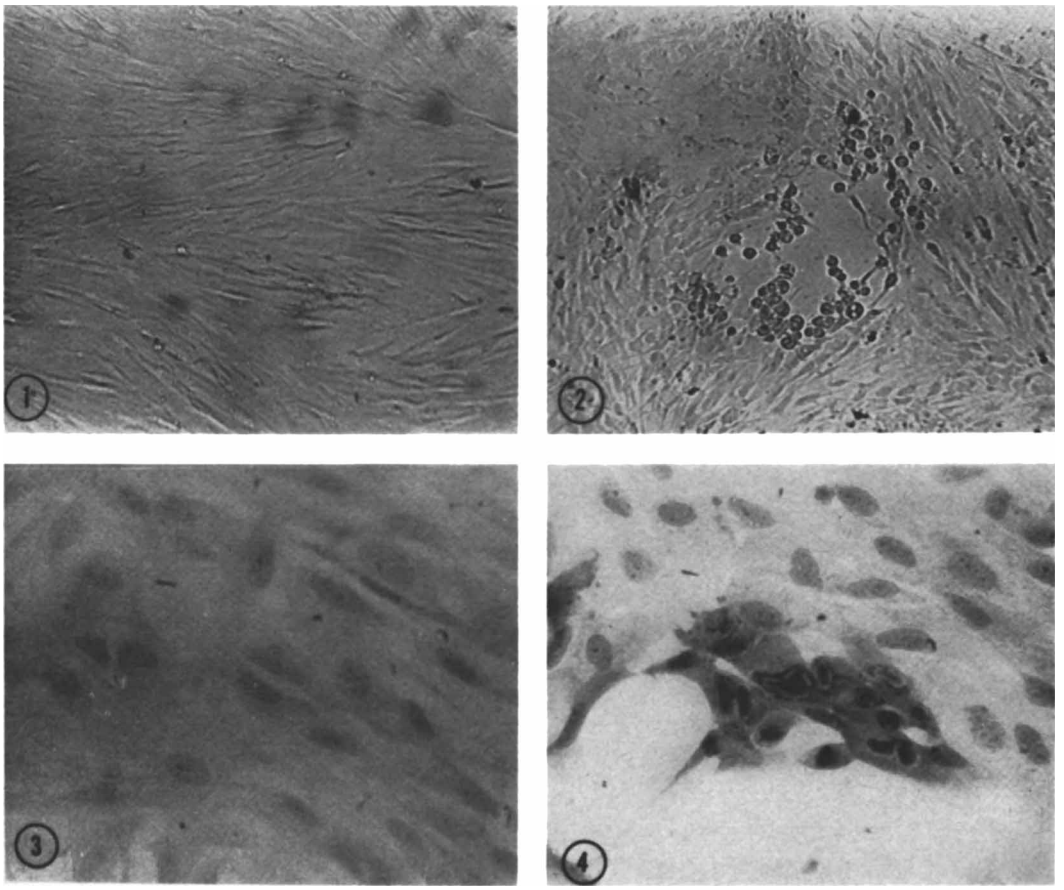

FIG. 1–4. Growth characteristics of 82-A virus in tissue cultures: 1. An uninoculated control culture of ED cell (100×). 2. A focus CPE induced by 82-A virus in ED cell culture 7 days after inoculation (100×). 3. An uninoculated control of primary horse kidney cell culture (hematoxylin–eosin, 400×). 4. Intranuclear inclusion induced by 82-A virus in primary horse cell culture 10 days after virus inoculation (400×).

ous data on the filtration of other animal viruses (6), the equine virus is in the large size range.

Lipid content. Virus infectivity was completely destroyed by treatment with diethyl ether for 1 hr at room temperature (Table II), demonstrating the presence of a lipid component in the virus particle.

Host range. Virus 82-A propagated equally well, if not better, and CPE appeared earlier in the ED cell cultures than in primary horse kidney cell cultures (Figs. 1 and 2). Primary rabbit kidney cell cultures infected with the 82-A virus also showed CPE but inconsistent results were obtained with primary human, monkey, and hamster kidney cells. No pocks were produced on the chorioallantoic membrane of embryonated eggs.

Serology. Concentrated 82-A virus-infected culture cells and fluids showed no fixation with human reference sera for adenovirus and cytomegalovirus, or with antiserum to herpes simplex virus. In the neutralization test the CPE produced by the 82-A virus was not inhibited by antiserum to equine herpesvirus type 1,[1] but was delayed in the presence of antiserum to LK virus strain of equine herpesvirus type 2[1] (9). Serum ob-

[1] Antisera to equine herpesvirus types 1 and 2 were obtained through the courtesy of Dr. G. Plummer, Loyola University, Chicago.

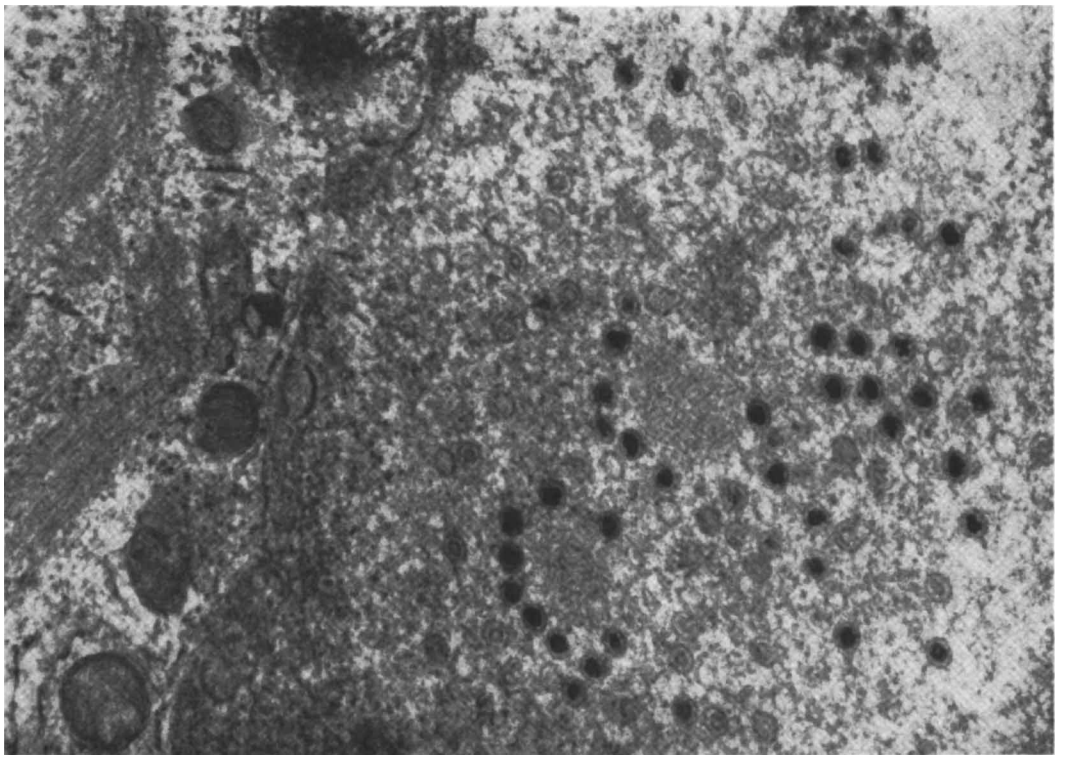

FIG. 5. Electron micrograph of virus particles as seen in thin section of primary horse kidney cell culture 21 days after infection with 82-A virus. 50,000×. Note dense and coreless centers enveloped by a membrane.

tained from horse no. 82 at the time the kidney was taken, showed 1:20 neutralizing titer to the virus isolate. In a separate experiment, 82-A virus was subjected to a neutralization test using antiserum obtained from rabbits hyperimmuned with SV_{17} stock virus. The 82-A virus was not inhibited by the rabbit antiserum. Since the stock virus of SV_{17} used for immunization of rabbit and horse 82 was derived from the same plaque purified virus grown in LLC-MK_2 cells, the lack of neutralization of the 82-A virus by this rabbit antiserum indicated there was no antigenic relationship between the 82-A virus and SV_{17} or other latent simian virus(es) that might be present in the SV_{17} stock.

Discussion. In accordance with size, morphology, intranuclear site of development, DNA composition, and sensitivity to ether, virus 82-A appears to represent an equine member of the herpesvirus group. Ultrafiltration indicated that the virus could be classified in the large size range and electron microscopy confirmed that enveloped virus particles observed in thin sections of infected nuclei had an average diameter of 106 mμ. Complement fixation tests showed that the virus was not related to the adenoviruses, human cytomegalovirus and the herpes simplex virus group. Although serological relationships to other herpesviruses of eqine origin have not yet been studied adequately, the morphological, physicochemical, as well as biological properties of the 82-A virus do justify grouping this isolate in the equine herpesvirus group. Although the 82-A virus multiplied readily in rabbit kidney cell cultures as well as in equine cells, its growth in human, rhesus monkey, or hamster cells was extremely limited. Recently we found that cytomegalovirus isolated from guinea pigs also grew well in primary rabbit kidney cell cultures (data to be published). Apparently growth in rabbit cells is an exception to the host specificity of cytomegaloviruses. Thus, the relative host specificity of the 82-A virus

for equine cells and its intranuclear growth characteristics suggest that it is an equine cytomegalovirus.

Viral latency in man and animals has been reported by various investigators. Cytomegalovirus is one of the common latent virus types isolated in cases of chronic disease in man (10). Had not the horse kidney cells been kept for a total of 50–80 days and through two subcultures, the presence of a cytomegalovirus in the horse kidney tissue would not have been recognized. This example indicates again that the recognition of latent viruses in cell cultures often requires long-term cultivation (11).

Summary. An equine virus designated as 82-A, was isolated from spontaneously degenerating equine kidney cell cultures after prolonged cultivation. Morphological, physicochemical, and biological properties of the virus suggest that it is a cytomegalovirus of the equine herpesvirus group.

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