Herpes-Like Virus Isolated from Neonatal and Fetal Dogs

Abstract. *A herpes-like virus has been isolated from fetal and neonatal dogs that died with an acute hemorrhagic disease. The virus differs from the other herpes-like viruses in its immunologic and growth characteristics. It is capable of passing the placental barrier and of causing disease and death of some of the fetuses, but in other apparently healthy animals the virus may remain latent, becoming activated under certain conditions.*

In this report we describe the isolation of a herpes-like virus from fetal and 12-day-old dogs that died with an acute hemorrhagic disease. This virus appears to differ in its immunologic and growth characteristics from other herpes-like viruses.

While searching for a susceptible host for viruses or agents present in human leukemia, two newborn Guernsey calves were inoculated with cell cultures made from the white blood cells of children with acute leukemia (*1*). The cultured cells were centrifuged and suspended in a small volume of culture medium; 0.25 to 0.5 ml of the suspension was injected subcutaneously in each of four different areas. Six weeks later one calf developed a lymphadenopathy of the peripheral nodes. At this time samples of blood were obtained and cultures were made from the white blood cells; also, a node near the iliac crest was taken for biopsy and culture. The cells were superimposed on primary cultures of fetal bovine spleen.

Four newborn mongrel pups were inoculated intraperitoneally with 5 ml of the culture fluid containing cells scraped from the 2-week-old spleen cultures. The four pups died or became moribund within 12 days; all had a lymphadenopathy. One moribund pup was killed at 12 days and portions of the enlarged hemorrhagic nodes were removed aseptically, homogenized in buffered saline, and made up to a 10 percent cell-free extract. Eight newborn mongrel pups were each inoculated intraperitoneally with 5 ml of the cell-free extract. Again within 12 days all were dead or moribund. A moribund pup with severe rectal hemorrhage and subcutaneous petechiae was killed; the kidneys, which had subcapsular hemorrhages, were removed aseptically for culture and histologic examination. Monolayer cultures of trypsinized cells (*2*) were prepared in T30 flasks with 109 medium (*3*) and 10 percent fetal bovine serum.

The cells from the diseased dog kidneys grew out into monolayers of normal appearance. Four days after culture, however, the cells began to round up and within 48 hours cell degeneration became prominent. Fluids from the cultures were frozen and stored at −60°C for passage into cultures of kidney cells from normal fetal dogs. The cells were prepared as in (*2*) except that they were trypsinized overnight at 4°C. After one passage on such cultures a characteristic cytopathogenic effect became evident (Fig. 1).

Examination with the electron microscope of thin sections of cells from these cultures revealed the presence of virus particles in the nuclei and cytoplasm. In the nuclei (Fig. 2*A*) the particles, round or oval in shape and 800 to 1000 Å in diameter, were disposed in groups forming small inclusions. All the particles were surrounded by a membrane 100 Å thick; the inner structure was variable. Frequently, three or four dense formations were disposed symmetrically against the inner suface of the limiting membrane. The clear space between these formations delineated a characteristic cross or a three-branched star. The particles observed

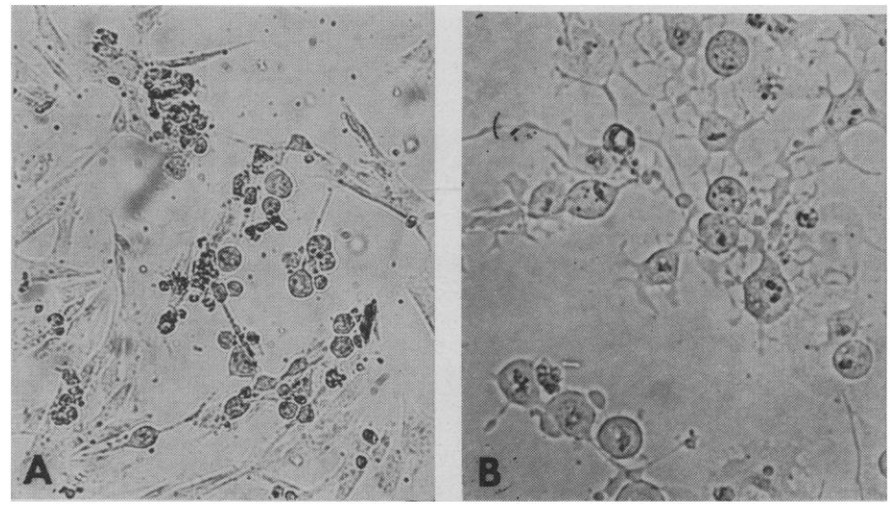

Fig. 1. Dog kidney tissue culture showing (*A*) rounded cells characteristic of the early cytopathogenic changes and (*B*) swollen cells with many cytoplasmic projections characteristic of the late cytopathogenic changes. (*A*, × 125; *B*, × 210)

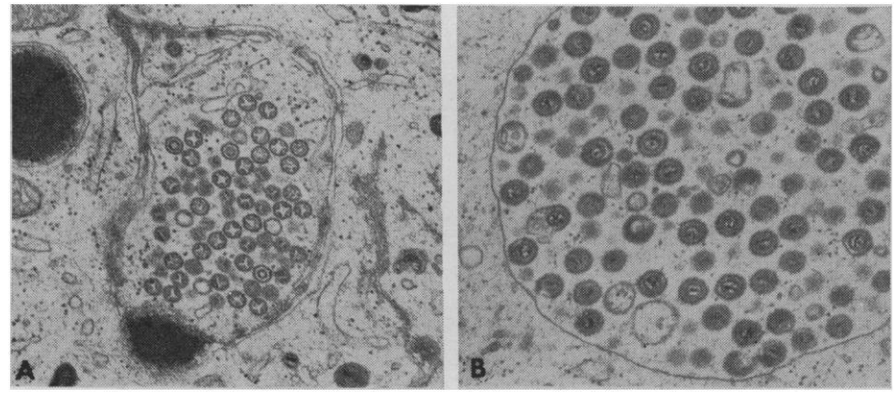

Fig. 2. Dog kidney tissue culture. (*A*) Section of a nuclear evagination containing an inclusion of the characteristic intranuclear particles. (*B*) Intracytoplasmic vacuole filled with particles usually seen in the cytoplasm and outside the cell. (*A* and *B*, about × 24,000)

Table 1. Results of neutralization tests of virus SL18HLV with specific viral antiserums (*11*). All antiserums were tested against 1000 $TCID_{50}$ of the virus.

Antiserum	Dilution	Cytopathogenic effect*
Equine rhinopneumonitis (Ky B strain)	1:2 and 1:4	+
IBR (C484R)†	1:2 and 1:4	+
Pseudorabies chick antiserum	1:2 and 1:4	+
Canine hepatitis	1:2 and 1:4	+
Canine distemper	1:2 and 1:4	+
SL18HLV virus‡	1:2 – 1:64	Negative

* Effect on primary cultures of embryonic dog kidney after 48 hours of growth. † Infectious bovine rhinotracheitis. ‡ Antiserum obtained from adult dog inoculated with SL18HLV virus.

in the cytoplasm (Fig. 2*B*) were generally inside vacuoles. They were bigger than those in the nucleus, approximately 1400 to 1700 Å in diameter, and were covered by an outer membrane. The nucleoid was a vesicle 800 to 1000 Å in diameter and contained an irregular (probably filamentous) dense structure. The external and internal surfaces of the outer membrane of the cytoplasmic particles were each covered with a layer of amorphous material.

Among two litters of fetal pups (close to term) obtained by cesarean section from apparently healthy mothers (*4*), several were diseased and runted. In one litter two of eight and in the other three of eight were diseased. Tissue cultures were prepared from the kidneys of the diseased fetuses, as well as from the apparently healthy littermates, precautions being taken to prevent cross contamination. The minced tissues were trypsinized at 4°C overnight. On culture the cells from both the diseased and the normal animals formed monolayers and then degenerated producing the same cytopathogenic effect noted with the original isolate (Fig. 1). Virus was recovered from the fetuses of both litters after one passage in kidney cultures from normal fetal dogs. The virus in both instances, as shown by culture characteristics, appeared to be the same as the original isolate.

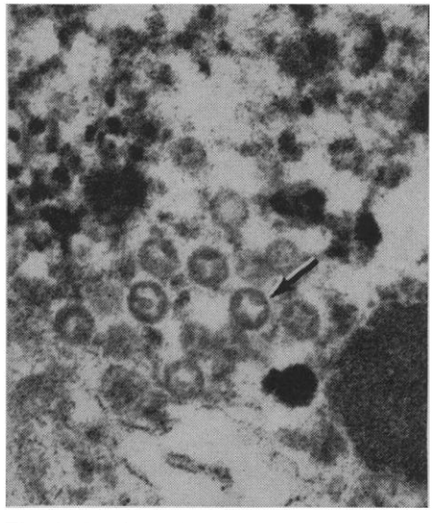

Fig. 3. Portion of a cell from the kidney of a diseased fetal pup. The nuclei of such cells were disrupted but it was still possible to recognize the characteristic particles (arrow). (× 49,000)

Examination of cells of these cultures with the electron microscope revealed nuclear and cytoplasmic particles identical to those observed in the original isolate. Sections from portions of the kidneys from the diseased fetal pups were also examined by electron microscopy and in one instance particles with the typical cross-shaped inner structure were observed in the cell nucleus (Fig. 3).

The three virus isolations were also shown to be the same by serum neutralization tests and by their cytopathogenic effect in tissue culture. The virus, designated as SL18HLV, appears to be specific for dogs. Fetal human, bovine, and porcine kidney cultures and the chorioallantoic membrane of the chick embryo were resistant to the cytopathogenic effect of the virus; nor did these cells support its growth since fluids taken from inoculated cultures were negative when put on dog kidney cells.

Morphologically the particles observed in the nucleus were identical to those seen by Tajima *et al.* (*5*) and by Reczko and Mayr (*6*) in tissue culture cells infected with the equine abortion virus. Since this virus resembles, morphologically, a herpes-group virus, serum neutralization tests were carried out on dog kidney cultures with antiserums against herpes-like viruses from dog (pseudorabies), cow (rhinotracheitis-vulvo vaginitis) (*7*) and horse (equine abortion virus) (*8*). The virus was also tested against antiserums for canine hepatitis and distemper viruses (Table 1). Lack of neutralization by these serums and the fact that the other herpes-like viruses all grow on porcine kidney cells while SL18HLV does not, rules out its identity with any of these.

Antibodies against SL18HLV were demonstrated in three of eight adult dogs tested. The titers ranged from 1 : 4 to 1 : 64 against 1000 $TCID_{50}$ (tissue culture infective dose, 50-percent effective). Normal bovine serums and serum from a researcher who had worked with this virus for over 1 year showed no viral neutralizing antibodies.

The virus is destroyed by heating at 56°C for 15 minutes and is inactivated by chloroform. It does not produce a hemagglutinin when grown on dog kidney cultures.

Both the fetal and neonatal dogs from which the virus was recovered had an acute nephrosis of the proximal convoluted tubules of the kidneys and massive interstitial hemorrhage in many of the organs. A similar hemorrhagic disease in pups thought to be caused by a mycoplasma was described recently (*9*). Mycoplasma were not detected in our cultures when the media recommended by Barile *et al.* (*10*) and anaerobic conditions were used. No mycoplasma-like particles were observed by electron microscopy.

Latency among the group of herpes-like viruses is frequently encountered. The activation of latent herpes-simplex by numerous agents under various conditions is well known. Our results indicate that the virus recovered from the 12-day-old pups shows a similar type of latency. Since the same virus was isolated from both diseased fetal pups and from pups appearing healthy, the virus must pass the placental barrier and remain latent in those animals which survive infection in the uterus. Thus it seems reasonable to conclude that our original isolate was a virus that was latent in the newborn pups which received the inoculum.

SARAH E. STEWART, J. DAVID-FERREIRA
ELIZABETH LOVELACE, JOHN LANDON
National Cancer Institute,
Bethedsa, Maryland 20014

NEAL STOCK
South Jersey Medical Research
Foundation, Camden, New Jersey

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11. The antiserums were provided by E. R. Doll, Univ. of Kentucky (equine rhinopneumonitis); J. H. Gillespie, Cornell Univ. (IBR); T. Tokumaro, Children's Hospital, Philadelphia (pseudorabies); Fromm Laboratories, Grafton, Wis. (canine hepatitis and canine distemper).

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Separation and Partial Purification of Plasma-Membrane Fragments from Ehrlich Ascites Carcinoma Microsomes

Abstract. *Membrane vesicles arising from the surface of Ehrlich ascites carcinoma cells were separated from the bulk of other microsomal components in Ficoll density gradients. Initial separation depends on the differential action of magnesium ion on various microsomal components. Immunologic and enzymic markers were used to monitor separation and purification steps.*

The membrane components of Ehrlich ascites carcinoma microsomes are made up primarily of broken endoplasmic reticulum, and also of the fragmented plasma membrane of these cells (*1–4*). We have previously examined the distribution of mixtures of microsomal membranes in density gradients of Ficoll, a sucrose polymer of low osmotic activity, and demonstrated that the component membranes exist as fluid-filled vesicles whose volumes are determined by electrostatic mechanisms and by osmotic effects (*4*). We followed the plasma-membrane fragments by means of a specific immunologic tag and by use of an enzyme marker, and showed vesicles derived from the surface membrane of these cells to be relatively insensitive to changes in ionic environment, in contrast with vesicles arising from the cell interior, which shrink with decreasing *p*H and with increasing ionic strength. We now report a purification of plasma-membrane vesicles which relies on their stability in the presence of divalent cations, which shrink and aggregate most other membrane structures. In that it relies on specific markers and depends on differences between the electrostatic properties of various membrane types, this approach differs from other membrane separation methods (*5*).

Purification of Ficoll, preparation and sampling of density gradients, density measurements, centrifugations, and determinations of protein and of rates of adenosine triphosphate hydrolysis have been described (*3, 4*). Oxidation of reduced diphosphopyridine nucleotide (DPNH-diaphorase activity) was measured in a medium containing $10^{-4}M$ DPNH, $6.6 \times 10^{-4}M$ potassium ferricyanide, $0.01M$ tris-hydroxymethyl aminomethane (Tris) (*p*H 7.4), and 4 to 20 μg of membrane protein in a volume of 1.1 ml. Oxidation of DPNH was followed by the change of absorbance at 340 mμ. The extinction coefficient used was 6.22×10^3 liter mole^{-1} cm^{-1}.

Distribution of cell-surface antigens (*2, 4*) was estimated by the ability of various fractions to absorb antibody capable of agglutinating intact tumor cells. Antiserum was prepared by immunization of a horse with whole microsomal membranes. The γ-globulin (antibody fraction) was prepared from the antiserum as described (*4*).

Absorptions were performed as follows: about 100 μg of protein from each fraction was incubated with 480 μg of the γ-globulin for 15 minutes at 37°C in a mixture of 0.5 ml of $0.15M$ NaCl, $0.01M$ Tris (*p*H 7.4), and 0.2 percent gelatin. The volume was increased to 2 ml with the same medium, and the mixture was centrifuged at 40,000 rev/min for 15 minutes. The supernatants were tested for agglutinating antibody, with serial twofold dilutions (*6*). A 2-plus reaction, defined as the formation of clumps about 0.2 mm in diameter, was chosen as end point. The unabsorbed γ-globulin was used as reference in each assay. With our γ-globulin preparation and 2×10^5 cells per milliliter of assay mixture, a 2-plus reaction was obtained with 3.8 μg of γ-globulin per milliliter. Results of antibody absorptions are expressed as changes in the concentration of γ-globulin required to produce agglutination. When absorption reduced the agglutinating titer by more than four doubling dilutions, the absorption was repeated with less protein. In those fractions giving no detectable absorption in the initial test, absorptions were repeated at higher concentrations of protein.

To isolate plasma membranes, tumor cells were separated as before (*7*) and then ruptured in a mixture of $0.25M$ sucrose, $0.005M$ Tris (*p*H 7.4), and $0.0002M$ $MgSO_4$ by intracellular cavitation of nitrogen (*8*). The homogenate was then made to $0.001M$ with respect to EDTA, and nuclei, mitochondria, and lysosomes were sedimented by centrifugation for 15 minutes at 12,500 rev/min and 4°C (rotor 9RA, Lourdes Betafuge). Microsomes were sedimented by centrifugation for 45 minutes at 40,000 rev/min and 4°C (rotor No. 40, Spinco L-2 preparative ultracentrifuge). The microsomal pellet was resuspended to a concentration of 1.0 to 1.5 mg of protein per milliliter in $0.01M$ Tris (pH 8.6) and resedimented by centrifuging at 40,000 rev/min for 45 minutes. The washing was repeated with $0.001M$ Tris, *p*H 8.6. The initial washing removes trapped soluble proteins; intravesicular soluble proteins are released in the second washing step because of the transient leakiness of the vesicle membranes caused by osmotic stress at very low ionic strength (*9*). The microsomes were then homogenized in $0.001M$ Tris

Table 1. Distribution of microsomal protein and of various membrane markers after centrifugation of Ficoll-Mg barrier at 25,000 rev/min for 15 hours at 4°C in SW-25 rotor; 51.5 mg of protein per tube; percentages are of total recovery.

Location	Protein (%)	DPNH-diaphorase		Na^+-, K^+-ATPase*		Surface antigen	
		mmole mg^{-1}min^{-1}	%	Pi (μmole-mg^{-1} hr^{-1})	%	Δ(γ-globulin) mg^{-1}	%
At barrier	20.2	0.068	3.7	7.95	67.1	1.14	88.3
In barrier	11.5	.213	6.3	2.80	13.4	0.12	4.5
Pellet	62.9	.553	90.0	0.74	19.4	.03	7.3

* Adenosine triphosphatase; P_i, inorganic phosphorus.