# Recovery and Characterization of a Herpes-like Virus from Dog Kidney Cell Cultures. (30615)

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The spontaneous degeneration of primary dog kidney tissue cultures (DKTC) by infectious canine hepatitis virus (ICH) has been described by Simonyi(1). In April 1963, a spontaneous cytopathic effect (CPE), markedly different from that produced by ICH virus, appeared in primary DKTC derived from puppies approximately 2 weeks of age. From these cultures, a transmissible filterable agent was recovered which had many characteristics similar to those recently described for agents isolated from fetal and neonatal puppies(2-4). This report presents information on the isolation and characterization of this agent as a member of the herpes group of viruses. In addition, serological evidence of its occurrence in mature dogs is presented.

*Materials and methods. Tissue cultures.* Primary kidney cell cultures of dog, mouse, pig, rat, cow, hamster, horse, and cat and primary embryo cell cultures of mouse, rat, and chicken were prepared from trypsinized cell suspensions. These cells were propagated as monolayers in nutrient fluid consisting of Hanks' balanced salt solution supplemented with 10% heat-inactivated calf serum and 0.5% lactalbumin hydrolysate. Eagle's minimum essential medium, containing 2% fetal bovine serum, was used as maintenance medium for the primary cell cultures as well as the following cell strains and lines: L929, human embryonic diploid brain, human embryonic diploid kidney, HeLa and AV-3.‡ All media contained 100 U of penicillin and 100 μg streptomycin per ml.

Plaque formation was tested by inoculating serial 10-fold dilutions of the agent onto monolayers of DKTC in 4-oz prescription bottles. Inoculated cultures were incubated one hour at 37°C and then overlaid with medium consisting of 1.2% Noble agar, 3% bovine serum, 0.5% lactalbumin hydrolysate, 1.5% of a 1:1000 solution of neutral red, and 100 U of penicillin and 100 μg of streptomycin per ml in Hanks' balanced salt solution. The infected cell cultures were incubated at 37°C.

*Viruses and antisera.* The A-9 strain of herpes simplex virus (HSV) was obtained from Dr. M. Artenstein of this Institute, and commercially prepared anti-HSV guinea pig serum‡ was used. The HSV was prepared and tested in primary monkey kidney cell cultures. The E-2492 strain of herpes simiae (B-virus) was obtained from Dr. R. N. Hull of Lilly Research Laboratories, Indianapolis, Ind., and the anti-B virus horse serum was obtained from Upjohn Co., Kalamazoo, Mich. (5). B-virus was grown and tested in primary rabbit kidney cell cultures. The pseudorabies virus (PRV) was obtained from Dr. A. S. Kaplan of Albert Einstein Medical Center in Philadelphia and the anti-PRV swine serum was received from Dr. D. P. Gustafson of Purdue University. The PRV was grown and tested in primary DKTC. Infectious bovine rhinotracheitis virus (IBR) was obtained from Dr. F. R. Abinanti of National Institutes of Health and anti-IBR calf serum was received from Mrs. F. S. Yancey of University of Maryland. The IBR virus was grown and tested in primary bovine embryonic kidney cell cultures. Antiserum was prepared against the isolated strain designated S4-63 in 3-4 kg rabbits by intravenous injection of 1.0 ml volumes of infected tissue culture fluids on days 1, 3, 5, 7, 9, 13, and 15. In addition, on the 15th day a 5 ml suspension of the agent was mixed with an equal volume of complete Freund's adjuvant and

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‡ Obtained from either Microbiological Associates Inc., Bethesda, Md., or Flow Laboratories, Rockville, Md.

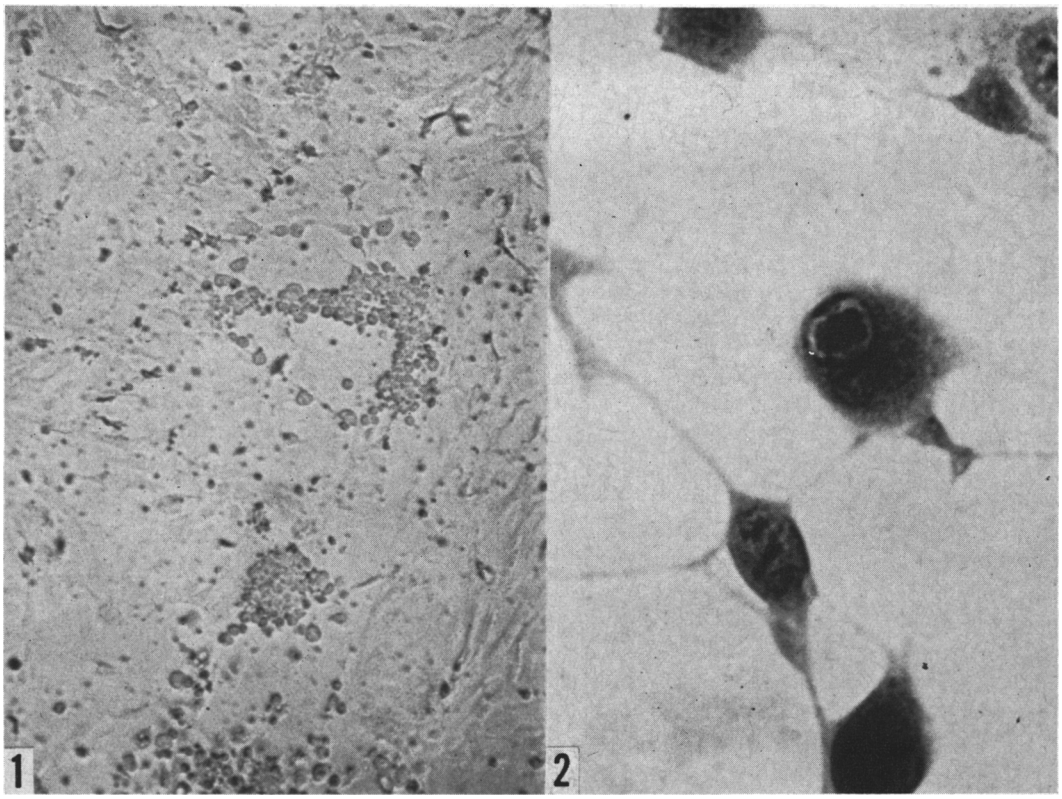

FIG. 1. The CPE of S4-63 in primary DKTC 24 hr post infection. 84 ×.
FIG. 2. Primary DKTC infected with S4-63 24 hr post infection showing a type A intranuclear inclusion. (H & E stained, 1260 ×.)

injected intramuscularly. The animals were exsanguinated on day 22.

*Virus titration.* Virus titrations were carried out by inoculating tube cultures of dog kidney cells with 10-fold dilutions of the virus. End points were determined by the appearance of the typical CPE and were calculated according to the method of Reed and Muench(6).

*Neutralization tests.* S4-63 plaque reduction or tube neutralization tests were carried out by adding approximately 100 plaque-forming units (PFU) or 100 tissue culture infectious doses 50 ($TCID_{50}$) to serial 2-fold dilutions of serum. After the virus-serum mixture had incubated at room temperature for one hour, 0.2 ml of each dilution was inoculated onto monolayers of DKTC. Following one hour adsorption at 37°C, the infected DKTC were overlayed with agar medium or maintenance medium. Virus controls were prepared in a similar manner, using a 1:4 dilution of normal rabbit serum. The plaques were counted 6 or 7 days following inoculation. Serum titers were recorded as the highest dilution of serum which resulted in 50% plaque reduction or in the case of tube neutralization tests, the highest serum dilution which inhibited the CPE.

*Fluorescence microscopy.* Infected cells on coverslips were stained with acridine orange and examined by fluorescence microscopy according to the method of Starr *et al*(7). With this method, DNA-containing compounds fluoresce green and RNA-containing compounds fluoresce red.

*Determination of physical and chemical properties.* Size was estimated by filtration through cellulose§ and gradacol membranes of graded porosity.

Sensitivity to lipid solvents was determined by treatment of the agent with chloroform(8).

5-Iodo-2-deoxyuridine (IUDR) which has

§ Millipore Filter Corp., Bedford, Mass.

been shown to inhibit the multiplication of DNA viruses(9) was used to determine the type of viral nucleic acid. Monolayers of DKTC were pretreated for 2 hours with maintenance medium containing 30 $\mu$g IUDR per ml prior to inoculation with the virus. After adsorption for one hour, fresh maintenance medium containing 30 $\mu$g IUDR per ml was added to the cultures which were then incubated at 37°C. Virus controls were infected with an equivalent amount of virus, but were not exposed to the drug. All cultures were assayed for infectious virus 3 days after inoculation.

*Results. Isolation of the virus.* In April 1963, a transmissible agent (S4-63) was obtained from primary cultures of dog kidney cells which had undergone spontaneous degeneration. Cytopathic effects were noted on the 8th day following preparation of the tissue cultures (Fig. 1). The agent produced a focal type of CPE with rounding of the cells and swelling of the nuclei. The agent could be readily transmitted in primary DKTC, but did not produce CPE or multiply in other cell cultures cited in the *Materials and methods.* Attempts to grow the cytopathic agent in a variety of bacterial and PPLO media were unsuccessful.

In February and March of 1964, transmissible agents were recovered from primary DKTC following the development of spontaneous CPE. Each of these agents produced CPE similar to that produced by the S4-63 agent.

On examination of infected dog kidney cells by acridine orange fluorescence microscopy 24 hours following infection, large, DNA-containing intranuclear inclusions were revealed. Eosinophilic, intranuclear inclusions could be seen in infected cells stained with hematoxylin and eosin (Fig. 2). In some cells, the inclusions appeared to almost completely fill the nucleus.

*Plaque formation and virus purification.* Monolayers of DKTC infected with S4-63 and overlaid with agar developed small, 1-1.5 mm plaques with irregular borders in 3 days (Fig. 3). From a well isolated plaque, infected material was obtained with a capillary pipette and passed in tube cultures of dog kidney cells. This procedure was repeated twice and from the 3rd plaque isolate, stock virus was prepared in DKTC for further study.

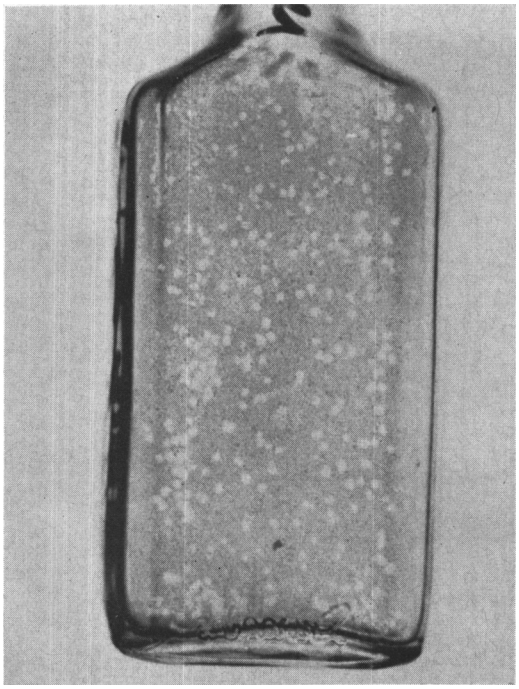

FIG. 3. Plaque formation of S4-63 in primary DKTC.

*Results of plaque reduction tests.* Hyperimmune rabbit serum at a dilution of 1:4 completely suppressed plaque formation by 100 PFU of S4-63; the 50% plaque-reduction titers of each of 2 rabbits were 1:32 and 1:64, respectively.

*Size.* By the use of cellulose membrane filters it was shown that virus titer was unaffected after passage of the virus suspension through membranes with a pore diameter of 450 m$\mu$. However, passage of the suspension through membranes with a pore size of 220 m$\mu$ resulted in an approximate 100-fold loss in infectivity titer: infectious virus could not be detected in suspensions which had been passed through membranes with a pore diameter of 100 m$\mu$.

Similar results were obtained using gradacol membranes. Infectivity could be detected after passage of virus suspensions through membranes with a pore size of 160 m$\mu$; however, gradacol membranes with a pore diame-

TABLE I. Effect of Chloroform on S4-63.

| Virus | Virus titer* Treated | Untreated |
|---|---|---|
| S4-63 | <1.0 | 5.0 |
| ICH | 2.5 | 2.8 |
| SV5 | <1.0 | 2.8 |

* Expressed as $TCID_{50}$ per 0.1 ml.

TABLE II. Effect of IUDR on Development of Infectious Virus.

| Virus | Virus titer* Treated | Untreated |
|---|---|---|
| S4-63 | 1.0 | 3.8 |
| ICH | .7 | 3.5 |
| SV5 | 4.0 | 4.5 |

* Expressed as $TCID_{50}$ per 0.1 ml.

ter of 110 mμ prevented passage of the agent. From these data, the size of this agent was estimated to be 150-170 mμ.

*Sensitivity to lipid solvents.* S4-63 was completely inactivated by treatment with chloroform. The results are given in Table I. Myxovirus SV5 and ICH viruses were used as chloroform sensitive and resistant controls.

*Treatment with IUDR.* IUDR at a concentration of 30 μg per ml of nutrient fluid interfered with replication of S4-63. The results are summarized in Table II. As controls, SV5, an RNA virus, and ICH, a DNA virus were used. These findings provided evidence that the viral nucleic acid type was DNA and were consistent with the acridine orange staining properties of the virus.

*Hemagglutination.* The agent did not agglutinate guinea pig, human O, adult chicken, or dog red blood cells when tested at a pH range of 5.0 to 8.0 and when incubated at either 4 or 37°C. Spontaneous agglutination of dog red blood cells occurred below pH 6.0.

*Stability.* S4-63 in maintenance medium was completely inactivated at 50°C for one hour; when kept at 37°C for 24 hours, infectivity was reduced approximately 100-fold. The addition of 40% fetal bovine serum increased the stability of the agent in storage. Freeze-drying of S4-63 with 40% fetal bovine serum resulted in a 100-fold loss in titer; however, the residual infectivity remained constant throughout 9 months of storage at —65°C. The pH stability of the agent was measured according to the method of Kettler (10). At pH 5.5, 6.0, 6.5 and 7.0 there was only slight loss in infectivity. The infective titer decreased 20-fold at pH 5.0 and was completely inactivated at pH 4.0.

*Animal pathogenicity.* Suckling and weanling mice were resistant to infection following oral, nasal, intraperitoneal, intracranial and subcutaneous inoculation of approximately $10^{4.5}$ $TCID_{50}$. Chick embryos could not be infected by the allantoic, amniotic, or chorioallantoic routes. Lesions did not develop in rabbits following inoculation of the skin and cornea. Two ferrets which had been inoculated subcutaneously with the agent developed no signs of disease within a 4-week observation period. However, each of these animals developed classical distemper when challenged with a known virulent canine distemper virus (CDV).

*Antibody studies of dogs.* Paired sera were obtained from 50 newly acquired mongrel dogs. Neutralizing antibodies to S4-63 developed in 7 of these dogs 22-38 days after arrival. Five of the sero-positive dogs developed an upper respiratory illness 7-14 days after their arrival. The 2 animals which were not clinically ill had the lowest antibody titers. Other dogs in this group developed a clinically-indistinguishable illness without antibody development to S4-63. Therefore, the association of the antibody to S4-63 to disease could neither be affirmed nor denied. Further evidence associating S4-63 with dogs was the presence of neutralizing antibody in the sera of 9 dogs which had been under observation for approximately one year. Low levels of neutralizing antibody were also found in 3 lots of commercial canine globulin.

*Serological comparisons.* Rabbit immune serum against S4-63 did not neutralize CDV, ICH, or rabies and antisera prepared against these agents did not inactivate S4-63. Cross neutralization tests were used also to compare S4-63 with other herpes viruses. On the basis of these tests, which are summarized in Table III, S4-63 was unrelated to PRV, B-virus, IBR, and HSV. It was of interest to note that a commercial lot of human globulin did not contain any activity against the canine herpes virus.

TABLE III. Serological Comparisons of S4-63 with Other Herpes Viruses by Cross Neutralization.

| | *Serum titer *vs* the indicated virus | | | | |
|---|---|---|---|---|---|
| Antisera | S4-63 | IBR | Pseudo-rabies | B-virus | H simplex |
| S4-63 | *64* | 0 | 0* | 0 | 0 |
| IBR | 0 | *16* | <4 | ND | ND |
| Pseudorabies | 0 | ND | >*64* | " | " |
| B-virus | <4 | " | <4 | *64* | <25 |
| H simplex | <4 | " | <4 | ND | *16* |
| Human globulin | <4 | " | <4 | <16 | *256* |

* Undilute serum did not neutralize the virus.
ND = not done.

*Discussion.* The herpes viruses are characterized by a DNA core, sensitivity to lipid solvents, a size ranging from 100-150 mμ, heat sensitivity and the formation of type A intranuclear inclusions(11). The physical, chemical, and biological properties of S4-63 are consistent with each of these criteria, therefore it was classified as a member of the herpes virus group. The lack of a serological relationship to other herpes viruses and the inability of the virus to grow on cells other than those of canine origin provide evidence that the virus is distinct from other members of the herpes virus group. While these studies were in progress, we learned of a similar isolate at Cornell University by Dr. Carmichael. Antisera against S4-63 inactivated Dr. Carmichael's F-205 cytopathic agent, indicating the viruses are closely related(3).

Since the virus was first recognized in tissue cultures which had undergone spontaneous degeneration 8 days following preparation of the cultures, the problem of viral contamination of cell cultures used in the production of vaccines is further emphasized.

Herpes viruses are important causes of neonatal death and respiratory disease in several species(11). The reports(2-4) of fatal disease in neonatal puppies and the appearance of neutralizing antibody in dogs with respiratory disease are consistent with the pathogenicity observed for other herpes viruses. However, further pathogenicity studies in dogs are required to define the pathogenicity of this agent. The presence of neutralizing antibody in commercial canine globulin suggests that the virus may be widespread in dog colonies.

*Summary.* A transmissible agent was recovered from primary DKTC which developed a spontaneous CPE. Characterization and serological studies of the virus indicated that the virus was a new member of the herpes virus group.

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