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Recovery and Characterization of a Minute Virus of Canines[1]

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Four antigenically related transmissible agents were recovered from canine fecal specimens. The agents produced cytopathic effects in a continuous dog cell line developed in this laboratory. Increased antibody titers were demonstrated in three of the four dogs which provided the isolates. The virus did not produce cytopathic effects in primary canine kidney or thymus cell cultures, or in cell cultures of human, simian, porcine, bovine, feline, and murine origin. The agent is resistant to ether, chloroform, and heat treatment, and the growth of the virus is inhibited by 5-iodo-2-deoxyuridine. After acridine orange staining, green fluorescent intranuclear inclusions are seen in infected cell cultures. By electron microscopy, the virions measure approximately 20 to 21 nm in overall diameter and are present in the nuclei of infected cells. These properties are consistent with membership in the parvovirus or picodnavirus group. The agent hemagglutinates rhesus red blood cells at 5 C and by hemagglutination-inhibition tests could be readily distinguished from H-1, rat virus, and the minute virus of mice. Canine gamma globulin contains high titers of neutralizing antibody and neutralizing antibody was found in a high percentage of military dogs and in beagles of a breeding colony.

In contrast to many other domestic mammalian species, comparatively few new canine viruses have been described. This may be attributed to either the limited number of studies conducted, or perhaps more importantly, the lack of suitable canine virus indicator systems.

During an investigation of respiratory disease in military dogs (2), a canine cell line developed in this laboratory was utilized in the search for transmissible agents. From rectal specimens of four apparently normal dogs, similar transmissible agents were restrictively recovered in this cell line. This report describes the isolation and characterization of these viruses, designated the minute virus of canines (MVC).

MATERIALS AND METHODS

Specimens. Specimens from the rectum were collected with cotton swabs and placed in veal infusion broth containing 0.5% bovine serum albumin V. The specimens were stored at −65 C until processed for virus isolation tests (1). The first sera were obtained from blood collected on the day the rectal specimens were obtained. Second serum specimens were obtained a month later.

Cell cultures. A canine cell culture line was initiated in November 1965 from a subdermoid cyst of an irradiated male dog. The tissue was trypsinized and grown in medium 199 with 10% fetal bovine serum and 0.075% sodium bicarbonate. After seven to eight subcultures, the composition of the culture changed from fibroblast to epithelial cells. The canine cell line, designated Walter Reed canine cell (WRCC) line, was used for culturing virus from the 43rd passage on. At the present time, the WRCC line has been subcultured more than 170 times. Primary dog kidney (PDK) and thymus cells were prepared and maintained as previously described (11). Other primary and continuous cell cultures were obtained from commercial sources (Microbiological Associates, Inc., Bethesda, Md. or Flow Laboratories, Inc., Rockville, Md.). All cell culture media contained 100 units of penicillin, 100 μg of streptomycin, and 2.5 μg of amphotericin B per ml.

Virus. After recovery and establishment of the virus, the virus was purified by three successive terminal dilutions, and seed virus pools were prepared at the 8th-to-10th WRCC passage. Virus titrations were carried out in the WRCC line by using 0.1-ml volumes per tube. The cell cultures were examined for specific cytopathic effects (CPE) usually for 10 days, and the 50% end points were calculated (9).

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Determination of chemical and physical properties. The procedures to determine the effect of ether, chloroform, acid (*p*H 3.0), and 5-iodo-2-deoxyuridine (IUDR) on the virus are described elsewhere (11). The effect of heat on the virus was determined by diluting the seed virus preparation 1:10 in Leibovitz medium L-15 and heating in a water bath for the desired time. The virus was filtered through membranes (Millipore Corp., Bedford, Mass.) of graded porosity to estimate its size. Infectious canine hepatitis (ICH) virus was mixed with MVC prior to filtration for control purposes. After filtration, titrations for ICH were done in PDK, whereas the MVC virus was titered in the WRCC line after neutralization of the ICH virus with specific antiserum. Infected and control cell cultures were examined after acridine orange staining by the procedure of Star et al. (12).

Electron microscopy. Prior to negative staining (3), partially purified virus preparations were obtained by differential centrifugation of supernatant fluid at 10,000 × *g* and 100,000 × *g*. Infected and control cell samples were fixed with glutaraldehyde and osmium tetroxide (10) and embedded in a mixture of Epon and araldite (8). Sections were cut on a Porter-Blum microtome and stained with uranyl acetate and lead citrate. The specimens were examined with a Hitachi 7S electron microscope.

Serological tests. Neutralization tests were conducted in the WRCC line as described previously (1). Hemagglutination tests were performed at 5 C with 1% rhesus erythrocytes at *p*H 5.8. The highest dilution of hemagglutinin (HA) having complete agglutination was considered one unit. Initially, the HA was prepared from infected cell cultures maintained in medium 199 without serum. Subsequently, high titer HA preparations were made from 20% infected cell suspensions in medium 199. The cell suspensions were frozen and thawed three times, and heated to 56 C for 0.5 hr. The HA was clarified by centrifugation at 800 × *g* for 20 min to remove cellular debris. Prior to hemagglutination-inhibition (HI) tests, the sera specimens were treated with receptor-destroying enzyme (RDE) obtained from Microbiological Associates, Inc., Bethesda, Md., and adsorbed with rhesus erythrocytes. Four units of antigen were employed in the HI tests, and the serum-antigen mixtures were incubated at 25 C for 1 hr. After addition of the erythrocytes, the test was placed at 5 C. Antisera and HA antigens to the rodent picodnaviruses were obtained from the National Cancer Institute, Viral Oncogenesis Branch.

MVC antiserum. Antiserum against the purified reference isolate was prepared in rabbits. The immunizing antigen was made from infected cell cultures maintained in medium 199 without serum. Each rabbit was injected by both the intravenous and intramuscular routes. Intravenous injections of 1.0 ml were given on days 1, 7, 14, 21, 30, and 37, and the intramuscular injections of 10 ml of a 1:1 mixture of virus and Freund's incomplete adjuvant mixture (Difco) were given on days 1 and 21. The rabbits were bled before inoculation and on days 37 and 44.

Animal pathogenicity tests. Newborn and weanling mice, hamsters, and guinea pigs were obtained from the laboratory animal production facilities of this institute. Rabbits and ferrets were obtained from commercial sources. Rabbits were obtained from Rowmar Rabbitry, Mt. Airy, Md., and ferrets from George Mazur, Washington, D.C.

RESULTS

Isolation. Ten rectal specimens were inoculated into the WRCC line, PDK, African green monkey kidney, and human embryonic kidney cell cultures. On the sixth day after inoculation of the WRCC line, similar CPE were evident in cell cultures from two specimens (Table 1). After a subpassage in the WRCC line, two other specimens provided agents with the same type of CPE. The agents were transmissible in the WRCC line, and the development of CPE was markedly dose dependent. Undiluted inocula could produce CPE in 1 day, however, 10- to 14-days were required to reach end points of titrations. Infected cells became rounded and developed distinct cell membranes and cytoplasmic strands were seen, and finally, the cells detached from the glass container. Transmissible agents were not recovered in the other types of cell cultures.

Each of the four agents were reisolated from the original fecal specimen. The 2X66 isolate was selected as a reference strain, and, after purification, virus pools were prepared for characterization studies and immunization of rabbits. The rabbit antiserum neutralized all the original isolates. A rise in serum neutralization and HI antibody was demonstrated in three of the four dogs (Table 1). Sera from the fourth dog were not available.

Host range. Except for the WRCC line, attempts to produce transmissible CPE with the reference 2X66 isolate in primary and continuous cell cultures were unsuccessful (Table 2). The 2X66 isolate was not lethal for newborn or weanling mice, hamsters, guinea pigs, or rabbits. Embryonating hens' eggs survived 10 days after 2X66 virus was inoculated into the allantoic sac, yolk sac, amniotic sac, or chorioallantoic membrane.

Chemical and physical characteristics. The MVC was resistant to chloroform, ether, and acid treatment (Table 3). The virus was markedly resistant to heating (1 hr at 60 and 70 C). When heated at 80 C, the virus was recovered after 1 hr in one experiment, and after 30 min in a second experiment. The MVC isolate passed through the Millipore GS, VC, and VM filters. The control ICH virus passed through the Millipore GS and VC filters but not through the VM filter. These findings indicate that the MVC is smaller than 50 nm. Treatment of WRCC line with IUDR

TABLE 1. *Recovery of minute virus of canines (MVC)*

Dog no.	Day CPE noted (passage)	Rabbit anti-2X66 serum neutralization titer[c]	Dog antibody titer	
			Neutralization	Hemagglutination inhibition
2X66[a]	6 (Original)[b]	16,304 (1.5)	64/256[d]	40/160
7X01	6 (Original)[b]	4,096 (2.5)	<16/256	20/80
5A37	4 (Subpassage)[b]	4,096 (1.5)	<16/64	<10/40
6X54	9 (Subpassage)[b]	1,024 (3.0)	Not available	Not available

[a] Reference strain.
[b] Agent reisolated.
[c] Log 10 challenge virus dose indicated in parentheses.
[d] Serum specimen on day rectal specimen obtained/serum specimen at approximately 30 days later.

TABLE 2. *Primary and continuous cell cultures[a] resistant to cytopathic effects of 2X66 (minute virus of canines)*

Primary cell cultures	Serial cell cultures
Dog kidney	WI-38 human embryonic lung
Dog thymus	KB human carcinoma of nasopharynx
African green monkey kidney	HEp-2 human epidermoid carcinoma of larynx
Human embryonic kidney	
Hamster kidney	FL amnion
Bovine embryonic kidney	Monkey heart (Salk)
Porcine kidney	LLC MK2 rhesus monkey kidney
Mouse kidney	MA104 embryonic rhesus monkey kidney
Rat embryo	BHK-21 baby hamster kidney
Feline kidney	LLC-RK1 rabbit kidney
Guinea pig kidney	MA bovine embryonic kidney cells
Rabbit kidney	

[a] Except for primary dog kidney and thymus cells, all cell cultures were obtained from either Microbiological Associates, Inc., Bethesda, Md., or Flow Laboratories, Inc., Rockville, Md.

inhibited the growth of MVC and the reference deoxyribonucleic acid (DNA) ICH virus but not the reference ribonucleic acid (RNA) SV5 virus. These tests provided evidence that virus contains DNA.

Cytological characteristics. At 48 hr after WRCC cells were infected, intranuclear inclusions were evident in hematoxylin-and-eosin-stained preparations. Large intranuclear inclusions nearly filled the nuclei, and a clear zone was observed between the inclusion and nuclear membrane. Acridine orange staining imparted a green color to inclusions. In control uninfected cultures, no similar inclusions were seen.

Electron microscopic appearance. Partly purified, negatively stained virus particles measured approximately 20 to 21 nm in overall diameter and many particles had hexagonal profiles (Fig. 1). In ultrathin sections of infected cells, small electron-dense particles were evident in the nuclei at 48 hr postinfection. Similar particles could not be observed in uninfected cells. These particles were 13 to 14 nm in diameter, and the minimum distance between two dense particles was always about 7 nm. This suggests the presence of an electron translucent shell of about 3.5 nm in thickness. Therefore, the total diameter of the virus can be estimated to be 20 to 21 nm. This size is consistent with that determined from micrographs of negatively stained virus particles.

Hemagglutination characteristics. Initially HA antigens of supernatant fluids from disrupted cell cultures maintained in medium 199 elicited low titer (1:4 to 1:8) reactions with rhesus or African green monkey erythrocytes at 5 C but not at 25 or 37 C. Subsequently, high titer (≧ 1:4096) HA antigens were prepared from 20% infected cell suspensions. Guinea pig, human O, dog, goose, rat, sheep, bovine, and pig erythrocytes were not agglutinated at 5, 25, or 37 C by the HA antigen.

Serological examinations. Antisera against the following known or reported canine viruses, canine distemper, canine herpes, rabies, infectious hepatitis, Toronto A26/61, reovirus type 1, and echovirus 6, failed to neutralize MVC. Nor was the MVC neutralized by antisera (obtained from the Department of Virus Diseases, Division of Communicable Diseases and Immunology, Walter Reed Army Institute of Research) against polioviruses types I to III, coxsackie B1 to B6 and A9, and human echoviruses types 1 to 32. From the characterization and electron microscope studies, MVC had properties con-

TABLE 3. *Chemical and physical properties of minute virus of canines (MVC)*

Treatment	Virus (strain)	1/TCD_{50}(log 10)		
		Not treated (A)	Treated (B)	Change (A-B)
Chloroform	MVC (2X66)	6.9	6.3	0.6
	ICH (Utrecht)	4.8	4.1	0.7
	SV5 (C958)	5.8	<1.0	≧4.8
Ether	MVC (2X66)	6.9	6.7	0.2
	ICH (Utrecht)	4.8	4.1	0.7
	SV5 (C958)	5.8	<1.0	≧4.8
*p*H 3.0	MVC (2X66)	7.0	6.5	0.5
	ICH (Cornell)	6.0	5.5	0.5
	Canine herpes	4.7	≦2.5	≧2.2
60 C, 1 hr	MVC (2X66)	7.6	5.9	1.7
70 C, 1 hr	MVC (2X66)	7.1	3.9	3.2
80 C, 1 hr	MVC (2X66)	7.8	2.1[a]	5.7
IUDR ($10^{-3.5}$ M)	MVC (2X66)	4.7	2.0	2.7
	ICH (Utrecht)	3.5	<1.0	≧2.5
	SV5 (C958)	3.0	4.0	(+)1.0
Filtration-VC[b]	MVC (2X66)	7.0	6.5	0.5
	ICH (Cornell)	5.5	5.5	0.0
Filtration-VM[b]	MVC (2X66)	7.0	6.5	0.5
	ICH (Cornell)	5.5	<2.0	≧3.5

[a] Virus present in one of 2 experiments; virus survived 30 min in other experiment.
[b] Millipore Filter Corp., Bedford, Mass.

sistent with the parvovirus or picodnavirus group. Serological studies were carried out to compare the isolate with the established murine parvoviruses (Table 4). The homologous titers of the rodent picodnavirus antisera were at least 32-fold higher than the titers to the MVC. Antiserum against the MVC did not react with any of the rodent parvoviruses tested.

High neutralizing antibody titers (1:4096) against the MVC were found in commercial canine gamma globulin (Globulon, Pitman-Moore Co., Indianapolis, Ind). Twenty of 27 mature German shepherd and 7 of 7 beagles had neutralizing antibody.

DISCUSSION

An apparently new canine virus was obtained from rectal specimens of four mature German shepherd dogs. The viruses were recovered in a continuous canine cell line, WRCC, initiated in this laboratory. Each isolate produced a similar type of CPE and each was neutralized by antiserum prepared against a purified reference isolate. Antisera against other viruses isolated from dogs failed to neutralize the virus.

The canine origin of the agents was verified by reisolation and the demonstration of a rise in neutralization and HI antibody titers in three of the four dogs that provided the isolates. The demonstration of MVC neutralizing antibodies in beagles and German shepherd dogs provided further evidence of the canine origin of the virus.

The virus had a remarkably limited host range, producing CPE only in this cell line. CPE was not evident in primary canine cells or primary and continuous cell cultures from many different species. Attempts to induce overt signs of disease or death in laboratory rodents were unsuccessful.

The recovered canine virus has many properties in common with the proposed parvovirus or picodnavirus group of Mayor and Melnick (6). As originally described, the group consisted of the rodent viruses H-1 and rat virus and the defective adenovirus-associated viruses. In addition, three other viruses have been described with

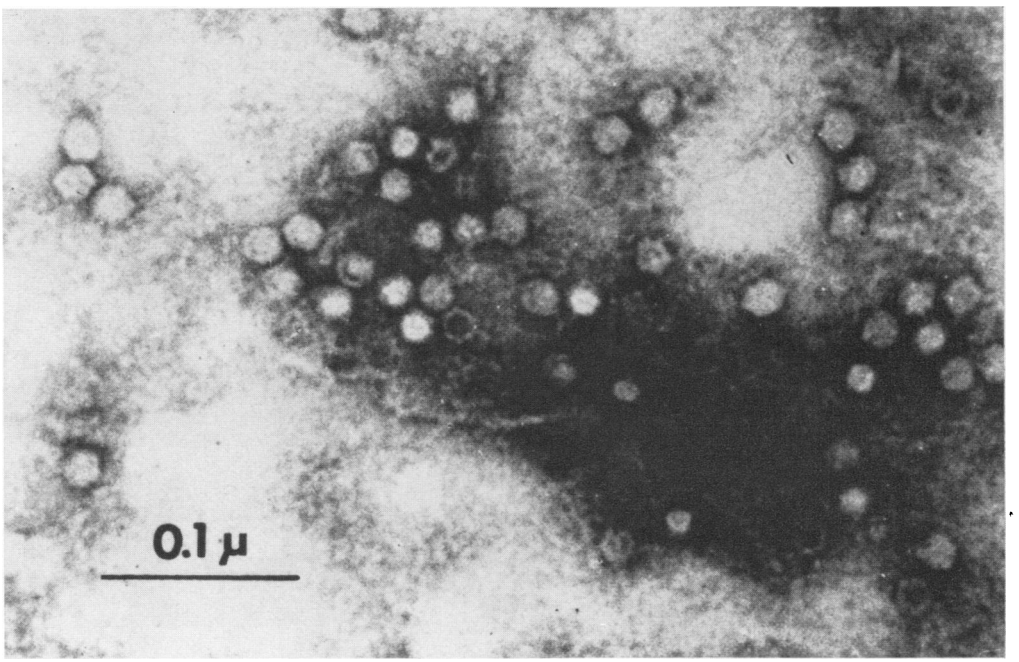

FIG. 1. *Negatively stained minute virus of canines which is approximately 20 to 21 nm in overall diameter.*

TABLE 4. *Antigenic relationship of minute virus of canines (MVC) and rodent picodnaviruses*

Antiserum	Test system	1/Hemagglutination-inhibition antibody titer	
		Homologous virus	MVC (2x66)
MVC (2X66)[a]	Rhesus RBC at 5 C	≧2,560	
H-1[b]	Rhesus RBC at 5 C	≧2,560	40
Rat virus[b]	Rhesus RBC at 5 C	320	10
MVM[b]	Guinea pig RBC at 5 C	1,280	20

[a] MVC rabbit antiserum did not inhibit H-1, rat virus, or MVM at a 1:10 or greater serum dilution.
[b] Antigens and mouse antisera received from the National Cancer Institute, Reference Reagents Program.

properties in common with this group. These include the minute virus of mice (4), a porcine picodnavirus (7), and the panleukopenia virus (5). The HI tests summarized in this report clearly establish the difference between the canine isolate and the rodent picodnaviruses. The canine virus has a different hemagglutinating spectrum for erythrocytes than the rodent, feline, and pig agents. The canine virus agglutinated simian erythrocytes but not guinea pig, human O, rat, or pig erythrocytes. Furthermore, the canine virus can only be grown in a continuous canine cell line. We propose that the canine virus described in this report be designated a member of the parvovirus group and designated the minute virus of canines.

The pathogenicity of the MVC for dogs is unknown and further work is required. From the limited serological tests conducted, it appears that this virus may occur frequently in canine populations. The comparatively few new canine viruses described in recent years may be due to the lack of suitable indicator systems. The present study indicates that the viral flora of the dog is not limited and, with suitable indicator systems, additional viruses may be found.

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