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Received May 15, 1967. P.S.E.B.M., 1967, v126.

Viruses Recovered from Laboratory Dogs with Respiratory Disease. (32386)

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Respiratory disease with high morbidity and mortality has occurred repeatedly in newly acquired mongrel dogs at this institute. The disease occurred notwithstanding canine infectious hepatitis (ICH) and canine distemper virus (CDV) prophylaxis with attenuated vaccines. Previous attempts to relate bacteria to the respiratory disease episodes were unrewarding. Moreover, the outbreaks could not be controlled by intensive antibiotic treatments. Therefore, attention was directed to the possible role of viruses in respiratory disease in dogs.

Prior to 1962, CDV was the only known viral agent that was unequivocally associated with respiratory disease of the dog. In 1962, a new canine adenovirus was isolated from dogs with laryngotracheitis(1) and in 1963(2) and 1966(3) reovirus type 1 was isolated from dogs with respiratory disease signs. In addition there is also serological evidence of canine herpes virus infection in dogs with similar signs(4). These findings suggest that in addition to CDV other viral agents may be involved in respiratory disease in dogs.

Initial studies were conducted to determine the presence of viruses in throat specimens and post-mortem tissues of dogs with respiratory disease. The isolation of canine adenoviruses, canine herpes virus and a virus serologically related to SV-5 from dogs with respiratory disease are described here. The serological reactions of test dogs and pathogenicity studies on isolated viruses will be presented separately.

Materials and methods. Dogs. Seventy-five mature dogs of mixed breeds were purchased from a commercial vendor. At time of arrival, each dog was examined by a veterinarian and only those which appeared to be free of overt disease signs were accepted. All dogs were given inactivated phenolized rabies vaccine and half the dogs received inactivated ICH vaccine.‡ The CDV vaccination was omitted to avoid interference with serological testing to determine the occurrence of CDV infection. The 75 dogs were kept in the same room in 19 runs. Groups of 4 dogs were kept in 4′ × 12′ runs with 1-1/2′ × 6-1/2′ pallets raised 1-1/4′ above the concrete floor. The dogs were fed a commercial ration which was always available. With the exception of cats, no other species was kept in this building. The dogs were observed daily for clinical signs of respiratory disease and their rectal temperatures were recorded. Sick dogs were isolated and treated symptomatically and with antibiotics.

Specimens. Specimens for virus isolation were taken from each dog the first day signs of respiratory disease were detected. A cotton

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swab sample was collected from the throat and eluted into 5 ml of veal infusion broth containing 0.5% bovine albumin fraction V. These specimens were stored at —65°C. Blood specimens for serologic studies were collected from each dog on the day of arrival, the first day of clinical illness, 14-21 days post onset, and on days 21 and 41 after arrival.

A post-mortem examination was performed on each dog with a fatal infection. Specimens of tonsils, bronchi, trachea, lungs, liver, spleen, kidney and urinary bladder were taken for virus isolation tests. Additional portions of these tissues were fixed in 10% neutral formalin for histopathologic examination.

Virus isolation procedures and identification. The post-mortem tissues were prepared as 10% suspensions in Hanks' balanced salt solution containing 1.5% phenol red broth base and 0.5% lactalbumin hydrolysate, pH 7.4. The tissue suspensions and throat specimens were treated with penicillin, 1,000 units/ml; streptomycin, 1 mg/ml; amphotericin B, 25 units/ml; and centrifuged at 1,500 × *g* for 30 minutes at room temperature. The throat swab specimens were inoculated into primary dog kidney (DK), HEp-2 cell cultures,§ and 8-day-old embryonating eggs by the Gorham technique(5). In addition, the throat specimens were inoculated into either primary African green monkey kidney (GMK)§ or the Madin-Darby canine kidney cell line‖ (MDCK). The postmortem tissue suspensions were inoculated into primary DK, GMK and HEp-2 cell cultures. The preparation of primary DK cell cultures is described elsewhere (4). The dog and GMK cell cultures were maintained in either medium 199 or Eagle's basal medium (BME) with 2% fetal bovine serum. The HEp-2 cells were maintained in BME with 2% chicken serum. Penicillin, streptomycin and amphotericin B at concentrations of 100 units, 100 μg and 2.5 units per ml respectively, were included in all culture media. For control purposes, uninoculated cell cultures of each type were maintained, subcultured and examined as the inoculated cultures. All cell cultures were incubated at 36°C.

Cultures were observed 10 to 14 days for cytopathic effects (CPE). A subpassage was made of inoculated and control cell cultures which failed to develop CPE. A 0.4% suspension of guinea pig red blood cells was added to each cell culture which did not develop CPE on the 14-21 day of incubation. The cultures were then incubated at 4°C for 20 minutes and examined for hemadsorption. The embryonating eggs were candled daily for 6 days. Embryos and chorio-allantoic membranes (CAM) were harvested from dead eggs and CAM and allantoic fluids were harvested from viable embryos. The CAM were examined for pock formation, and the allantoic fluids were tested for the presence of agglutinins for guinea pig red blood cells at 4°C. A single subpassage of harvested egg materials was made in embryonating eggs which was tested similarly.

Cell cultures with CPE or hemadsorption were harvested and the contents stored at —65°C. Isolates which produced an adenovirus-like CPE were identified by a hemagglutination (HI) test(6) with antisera against ICH or Toronto A26/61-like viruses¶(1). Isolates which produced a canine herpes type CPE were identified by a neutralization test using 16 units of anti-canine herpes rabbit sera and 100 tissue culture dose 50 (TCD50) of virus. The hemadsorbing agents were identified by use of a neutralization test with 16 units of rabbit antiserum prepared against a reference isolate. The reference isolates were purified by 3 terminal dilutions, and a virus pool was prepared for characterization and immunization of rabbits. Chloroform sensitivity, size, and effect of 5-Iodo-2-deoxyuridine (IUDR) were determined as previously described(4). The hemadsorbing virus was identified by use of neutralization tests in MDCK and human embryonic kidney (HEK) cell cultures employing myxoviruses and their respective antisera provided by the Reference Reagents Branch, National Institute of

§ Obtained from Microbiological Associates, Inc., Bethesda, Md.

‖ Obtained from Bacteriology & Immunology Branch, Armed Forces Inst. of Pathology, Washington, D. C.

¶ The Toronto A26/61 canine adenovirus was supplied through the courtesy of Dr. J. Ditchfield, Guelph, Ontario, Canada.

Allergy and Infectious Diseases of the National Institutes of Health, a commercial source,** and Dr. Mohanty, University of Maryland, College Park.

Serological tests. Seed stocks of isolated virus were prepared in primary DK cell and in MDCK cultures. The infected cultures were harvested when CPE was observed in 75% or more of the cells exhibiting CPE, except for hemadsorbing agents which were harvested on the 6th day. Infected cells were disrupted by alternate freezing and thawing and clarified by centrifugation at 500 × *g* for 20 minutes. The canine herpes virus pools were stabilized by addition of an equal volume of inactivated fetal bovine serum.

For neutralization tests, equal volumes of 4-fold dilutions of inactivated serum were mixed with the virus preparation containing 100 TCD50/0.1 ml. The mixture was incubated at room temperature for 1 hour. Two primary DK cell or MDCK tube cultures were inoculated with 0.2 ml of each virus serum mixture. After 1 hour, the maintenance medium was added and cultures were incubated for 6 days. The canine herpes virus neutralization tests were conducted in primary DK cell and MDCK were used for the canine adenovirus neutralization tests. Parainfluenza virus neutralization tests were done in either HEK or in MDCK cultures. Control virus titration and positive reference sera were included in each test.

The HI test for the parainfluenza viruses as done according to Chanock and Johnson procedure(7). The sera were treated with receptor destroying enzyme, and 4 units of antigen and guinea pig erythrocytes were used in the test.

Results. During a 6 week period of observation, 53 of 75 dogs developed significant signs of respiratory disease resembling CDV infection. The incidence and duration of respiratory disease was the same in the unvaccinated dogs and those receiving inactivated ICH vaccine. More than one-half of the sick dogs developed the first signs of disease between the 4th and 10th days after arrival. The illnesses were long lasting having a median duration of 25 days. Ten of the 53 sick dogs died with histopathologic evidence of respiratory disease. Giant cells were observed in the lungs of 2 dogs, D004 and D033, consistent with a diagnosis of canine distemper. One of these 10 dogs, D007, also had signs of encephalitis. Conjunctivitis, purulent nasal discharge, tracheitis, bronchopneumonia and an elevated body temperature were observed in 75% or more of the sick dogs. Most of the dogs had 4 or more signs of disease and 79% of the dogs had lower respiratory disease signs.

Isolations from throat secretion specimens. Twenty-four transmissible agents were recovered from 57 throat specimens of 53 dogs with signs of respiratory disease. Fourteen isolates were canine adenoviruses of which 11 were serologically similar to Toronto A26/61 virus and 3 to the "classical" ICH virus. The remainder comprised 9 hemadsorbing agents and one canine herpes virus. All except 3 of the isolates were recovered in primary DK cell cultures. The 3 exceptions were hemadsorbing viruses which were recovered on GMK cells only. These were obtained from samples that also contained adenoviruses. A canine adenovirus was also present in the throat swab specimen that provided the herpes virus. In 7 instances, the same isolates were recovered in either MDCK or GMK cell cultures, as well as primary DK cells. Transmissible agents were not recovered from either HEp-2 cell cultures or embryonating chicken eggs. With the exception of dog C963, a rise in antibody titer to the respective isolated agents was demonstrated in dogs which recovered from their respiratory illness. Canine herpes and a Toronto A26/61-like adenovirus were obtained from dog C963. In this dog a rise in titer to the herpes but not to the adenovirus was demonstrated. It did, however, have neutralizing antibodies to the adenovirus on the day of arrival 23 days prior to disease onset.

Isolations from post-mortem tissue specimens. Transmissible agents were recovered from 8 of 10 dogs dying with pathologic signs of bronchopneumonia or lobar pneumonia. The cell culture systems from which the various viruses were isolated were the same as those that provided the respective isolates

** Obtained from Microbiological Associates, Inc., Bethesda, Md.

TABLE I. Viral Isolation and Serologic Data from Dogs with Fatal Respiratory Disease.

Dog No.	Day onset* /Day death	Virus isolated	Source of isolate	1/Neut. antibody titers 1st serum (day)	2nd serum (day)
C955	6/30	ICH	Throat,† tonsil,† trachea†	<20 (6)	20 (21)
		A26/61	Lungs†	<20 (6)	20 (21)
C958	8/33	A26/61	Throat	<20 (8)	1280 (21)
		Hemad.	Throat, lungs†	< 4 (8)	<4 (21)
		Herpes	Tonsil, trachea-bronchi pool	< 1 (8)	<1 (21)
C960	13/33	A26/61	Throat, tonsil, lungs	<20 (13)	320 (21)
		Hemad.	Throat	< 4 (13)	<4 (21)
C982	9/69	ICH	Throat	<20 (13)	1280 (28)
C990	6/63	None		—	—
D001	14/16	None		—	—
D004‡	13/49	A26/61	Throat	<20 (13)	20 (21)
		Hemad.	Throat, tonsil, trachea-bronchi-lung pool†	< 4 (13)	<4 (21)
		Herpes	Tonsil, bronchi-trachea-lung pool†	< 1 (13)	<1 (21)
D007	5/51	A26/61	Throat†	<20 (5)	320 (21)
		ICH	Tonsil	<20 (5)	80 (21)
		Hemad.	Lungs	< 4 (5)	<4 (21)
D016	8/37	Hemad.	Throat, trachea-bronchi-lung pool, spleen, kidney	< 4 (8)	<4 (21)
D033‡	5/39	Herpes	Tonsil, trachea-bronchi-lung pool, spleen	< 1 (5)	<1 (21)
		Hemad.	Tonsil, liver, kidney	< 4 (5)	<4 (21)
		ICH	Kidney	1280 (5)	1280 (21)

* Day 0 is day of arrival.
† Agent reisolated.
‡ Giant cells present in lungs consistent with a diagnosis of canine distemper.

from throat swab specimens. In 5 instances the same agent was recovered from the acute throat specimen and from the post-mortem tissues after an interval of several weeks (Table I). Multiple isolations were obtained from the post-mortem tissues of 5 dogs. From 4 dogs, 2 agents were recovered; the fifth dog yielded 3 agents. From most of the dogs, the same agent was isolated from more than one tissue specimen; from 2 dogs, the viruses were recovered from other than respiratory tract tissues. With one exception, a rise in antibody titer against the canine adenoviruses (ICH and A26/67) was demonstrated in serum specimens from fatally-infected dogs from which these isolates were obtained. However, no rise in antibody titers to the hemadsorbing agent or the canine herpes virus was demonstrated in dogs which provided these isolates.

Identification of the hemadsorbing agent. The hemadsorbing isolate, C958, used as a reference strain was obtained from the lung of a dog that died with respiratory disease. The primary DK cell isolate was purified by 3 successive passages of terminal dilutions of infected DK cell cultures. Antisera prepared against this isolate neutralized each hemadsorbing isolate. Amniotic fluid from infected embryonating eggs agglutinated guinea pig, human 0 and chicken red blood cells at 4°C, room temperature and 37°C. The virus was completely inactivated by chloroform; the titer fell from 6.5 $\log_{10}$ to less than 1.0 $\log_{10}$ in the treated preparation. The growth of C958 virus and control RNA virus (Sendai) was not suppressed by treatment with IUDR, whereas the control DNA virus (ICH) was inhibited 100-fold. The virus preparation could be passed through a 450 mμ millipore filter with little or no decrease in titer, however, when filtered through a 300 mμ and 220 mμ filter there was a 100-and 10,000-fold drop in titer, respectively. The virus did not pass a 100 mμ filter. Its size was estimated to be 102 mμ(8). The agent was tentatively placed in the myxovirus group on the basis of these characteristics.

Parainfluenza virus antibody has been

TABLE II. Identification of the C958 Isolate.

Antiserum *vs* (strain)	1/Neutralization antibody titer to Homologous virus	C958 virus
Paraflu 1 (HA-2)	16	<4
(Sendai)	256	<4
" 2 (Greer)	256	<4
" 3 (HA-1)	256	<4
(SF-4)	1024	4
SV-5	256	1024
Mumps	256	4
Newcastle disease	256	4
Rabies	>20000	<4
Canine distemper	1280	<4
C958*	256	
Canine gamma globulin†	—	64

* C958 antisera—did not neutralize any of above viruses except SV-5, titer 1:64.
† Pitman-Moore.

found in dogs(9). Therefore, cross neutralization tests were carried out between the C958 isolate and members of the parainfluenza subgroup of myxoviruses. A marked reciprocal neutralization was obtained between C958 and SV-5 viruses with little or no cross reactions to the other viruses tested (Table II). Accordingly, the C958 virus was placed in the SV-5 group of myxoviruses. It is of interest that commercial canine gamma globulin contained a significant neutralizing antibody titer (1:64) to the C958 virus.

Since SV-5 is a common contaminant of monkey kidney cell cultures, it was deemed necessary to verify the recovery of this virus from dogs. For this purpose paired serum specimens of 10 dogs developing antibody rises to the C958 virus were tested against a wide spectrum of parainfluenza viruses in neutralization and HI tests (Table III). The dogs developed rises in antibody titer to both the C958 and SV-5 viruses and only 1 of 10 animals showed a rise in titer to parainfluenza 3 virus. No rises in titer were demonstrated to other viruses.

TABLE III. Specificity of Antibody Response in Dogs Developing Rises in Titer to the C958 Myxovirus.

Serological test	No. of dogs tested	No. of dogs with rise in antibody titer to the indicated virus: Paraflu 1 HA-2	Paraflu 1 Sendai	Paraflu 2 Greer	Paraflu 3 HA-1	Paraflu 3 SF-4	Mumps (Enders)	Newcastle disease virus	SV-5	C958 (D008*)
Neutralization	10	0	0	0	0	1	0	0	10	10
Hemagglutination-inhibition	10	0	0	0	1	1	0	0	10	9

* The D008 parainfluenza SV-5 like virus isolate was employed in the hemagglutination-inhibition test.

Discussion. The assembly of large numbers of men or animals into a new environment has often resulted in epidemics of severe respiratory disease. Investigations to find the etiological agent have been vastly aided by the development of tissue culture techniques. It has been shown from such studies that a number of dissimilar agents may cause clinically similar diseases. For example, myxoviruses, herpes viruses and enteroviruses have been recovered from horses with respiratory disease(10).

The dogs in this study have been derived from multiple environments and maintained in a common facility under conditions that would favor the transmission of respiratory agents. Therefore, the recovery of multiple

agents from dogs with respiratory disease is not surprising and was analogous with respiratory disease episodes seen in military recruits. Following the inoculation of cell cultures a 35% rate of virus recovery was made from throat specimens (20 of 57 dogs) and a 70% (7 of 10 dogs) rate from post-mortem specimens. The primary DK cell culture was the most important cell culture system employed in achieving this percentage of isolations. Moreover, each of the 3 groups of viruses were recovered in primary DK cells.

The number of multiple agents recovered from a given dog, particularly the dogs which succumbed to respiratory disease, makes it extremely difficult to assess the etiological significance of each agent in the signs of disease observed. Moreover, the role of CDV cannot be overlooked as histopathologic evidence of CDV was observed and antibody titers against this agent increased in many of the dogs (Binn, Lazar & Cross—unpublished results). The persistence of canine adenovirus and the SV-5-like virus in these dogs was consistent with known properties of these viruses. Adenoviruses were originally recovered as "latent" agents(11) and SV-5 is known to persist in primates for long periods(12).

Although the presence of SV-5 viruses often reflects "contamination" of monkey kidney cell cultures, in this study there was substantial evidence that the isolated SV-5 viruses came from the dogs. Repeated recoveries of SV-5 like viruses were made from the same specimen in primary DK, whereas, there was no evidence of hemadsorption in control uninoculated cultures. Rises in antibody titers to the isolated virus were demonstrable in sera from these dogs employing the neutralization test. Moreover, reactions in such tests were specific for the isolated virus and SV-5 but not for parainfluenza 1, 2, 3, mumps and Newcastle disease viruses.

SV-5-like viruses have been recovered from man(13,14,15). The isolation of this virus from dogs poses questions on the origin of this "simian" agent and the role of the dog in the epizootiology of the virus.

Further studies are required to determine the distribution, epizootiology, veterinary and public health significance of the isolated viruses in dogs. Studies on the pathogenicity of the isolated viruses for dogs are in progress.

Summary. Viruses were recovered from throat specimens of 21 of 53 dogs with respiratory disease and from the post-mortem tissues of 7 of 10 dogs. Many of these dogs were infected with more than one agent. The agents were 2 canine adenoviruses, a canine herpes virus and a SV-5-like virus. Rises in antibody titer to the isolated viruses were demonstrated in dogs which survived. The antibody response to the SV-5-like virus was specific to the isolated virus and SV-5 and not to other members of the parainfluenza group.

The authors wish to thank Dr. A. D. Alexander for many helpful suggestions, Dr. C. Biggers for histopathologic examinations and D. Withers and J. Herman for technical assistance.

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Received May 17, 1967. P.S.E.B.M., 1967, v126.