Isolation of Viruses from Primary Dog Cell Cultures and the Occurrence of Viral Antibody in Donor Animals (34518)

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The viral flora of canine tissues was reviewed recently by Gillespie (1). Twelve viruses were listed as occurring in dogs, and it was observed that known canine viruses are generally pathogenic. The four agents which seem to occur with the highest frequency are infectious canine hepatitis virus (ICHV), canine distemper virus (CDV), reovirus type I, and canine herpesvirus (1). SV-5 has recently been associated with respiratory disease in dogs (2, 3). Although these viruses can be isolated from the fluids or exudates of diseased dogs, little information is available concerning their incidence in tissues. Since viral vaccines are produced in primary canine cell cultures, we wanted to determine the type and incidence of viruses in the kidneys and livers of dogs. We selected stray dogs from a wide geographical area for this purpose, many in fair to poor health, because we hoped to isolate the largest possible number and the widest possible variety of viruses. Such information could be helpful in devising methods to exclude canine viruses from cells used in production of vaccines.

We presented a preliminary report describing our findings with 26 different lots of canine kidney cells, which were observed for about 21 days and then examined by fluorescent microscopy for virus infection (4). The following summarizes further observations on virus isolation and viral antibody patterns in 55 other dogs.

Materials and Methods. The purpose of this study was to cultivate primary kidney and liver cells from a large number of individual dogs, incubate and observe the cultures for 4–5 weeks, then examine them for viruses by immunofluorescence and by electron microscopy. Series I, which consisted of 25 dogs, was handled as described in a previous report (4). A single kidney was removed aseptically from each dog after the anesthetized dog was exsanguinated. Serum was separated and stored at —20° C until needed. Cultures were held for 4–5 weeks before preparation of secondary cultures in plastic dishes, fixation, and examination by fluorescent or electron microscopy. Series II, consisting of 30 dogs, was handled as follows: primary cell cultures were prepared from kidney and liver tissues, incubated up to 5 weeks, the cells scraped from the container surface and the suspensions stored frozen at—70° until they could be studied further. Stored materials were then thawed, examined in the electron microscope, and inoculated onto canine embryo cell monolayers (see *Cell cultures*). Inoculated cells were incubated for 5–7 days, then fixed and stained by the indirect Coons method, using (a) dog sera known to contain antibody against CDV, ICHV, and reovirus; (b) pooled canine gamma globulin (Globulon, kindly provided by the Pitman-Moore Company); and (c) the dog's own serum.

Physical condition of dogs. The dogs in series I were nonconditioned, random source dogs acquired immediately prior to the collection of tissues and serum from a USDA

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licensed animal vendor. Their physical condition was considered to be fair to poor, since they had recently undergone the stresses of capture, impoundment and transportation. The dogs in series II were in poor physical condition. They were selected from a larger group of dogs undergoing quarantine and conditioning at the NIH Animal Center, Poolesville, Maryland. All were ill at the time of collection. Approximately half of the series II dogs received live, attenuated CDV-ICHV vaccine 2 to 3 weeks prior to collection. The records of these inoculations were available to us. The dogs were acquired from two sources, but the majority came from a vendor located in Pennsylvania. They originated from a geographic area from Michigan to sourthern Virginia, from the east coast to Ohio and Tennessee.

Cell cultures. Primary cell cultures were prepared in 32-oz glass bottles or 8-oz Falcon flasks (plastic). Cells were initially cultured in Eagle's No. 2 medium with 10% fetal calf serum (virus-free) and neomycin (20 μg/ml). After forming confluent sheets, cells were maintained on Eagle's basal medium with 1–2% fetal calf serum. Canine embryo cells were prepared from whole dog embryos and used in their 5th–15th passages. These cells were found free of viruses when examined by immunofluorescent techniques (4) and electron microscopy.

Immunofluorescence. The methods we used have been described before (4). Anticanine globulin was prepared in a burro by repeated subcutaneous injections of $(NH_4)_2SO_4$ precipitated globulin mixed with complete Freund's adjuvant. We conjugated the anticanine globulin with fluorescein isothiocyanate and purified the conjugate by gel filtration.

Electron microscopy. We used a modification of the Sharp method (5) adapted to negative staining, which has been described in previous reports (6, 7).

Identification of viruses. ICHV isolates in nearly every case were presumptively identified first by electron microscopy, on the basis of their characteristic adenovirus morphology [cubical symmetry, 252 capsomeres; (8)]. Confirmation was obtained on every isolate by neutralization with specific antiserum, which we prepared in rabbits against an American Type Culture Collection ICHV strain (density gradient purified). Distemper virus was identified by neutralization with anti-CDV serum obtained from the Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland. The herpesvirus isolate was identified by electron microscopy, on the basis of its being enveloped and having a cubical symmetry nucleocapsid characteristic of the herpesvirus group [162 capsomeres; (9)]. The isolate was neutralized $>$ 95% with anticanine herpesvirus serum, prepared by Dr. Leonard Binn, against canine herpesvirus.

Antibody titrations. Complement fixation (CF) titrations were done by the standard microtiter method (10). Using the chessboard dilution technique, endpoints recorded as those dilutions giving 3-4+ reactions. Neutralization titrations were done by reacting various dilutions of serum against concentrations of virus sufficient to give numbers of fluorescing foci or microplaques that would permit convenient reading (infected cells between 0.05% and 5% of the total). Concentration ranges of viruses used were: ICHV, $10^{4.0}$–$10^{4.5}$/0.2 ml; CDV, $10^{2.0}$–$10^{2.5}$/0.2 ml; herpesvirus, $10^{1.5}$–$10^{2.0}$/0.2 ml. Equal volumes of appropriate serum and virus dilutions were mixed and incubated at 25°, 0.2-ml volumes adsorbed for 1 hr on canine embryo cell monolayers contained in plastic petri plates. Inoculum was distributed over the surface of cell monolayers by frequent movement in a mechanical device. Then tissue culture fluid was replaced (without removing serum-virus mixture), and cultures were incubated at 36° for the following periods of time: ICHV, 40–48 hr; herpesvirus, 40–48 hr; CDV, 5 days. Cultures containing ICHV and CDV were fixed and stained by the indirect Coons method to reveal infected cell foci or microplaques. Herpesvirus infected cultures were examined at low magnification with a scanning or an inverted microscope and the microplaques

counted. Serum neutralization endpoints were those dilutions of serum giving 80% or greater reduction in infected cell foci or microplaques, compared with cultures inoculated with virus only. The reovirus type I used as CF antigen was obtained from the American Type Culture Collection.

Dog embryo cell cultures. For subculture of new virus isolates, we used a line of cells derived from primary cultures of dog embryos. This line of cells could be passaged at least 15 generations; was sensitive to ICHV, CDV, canine herpesvirus, and reovirus type I; and was free of adventitious viruses, on examination by fluorescent and electron microscopy. These cells were used in all neutralization experiments for the propagation of viruses.

Reference viruses. Canine herpesvirus, strain D004, ICHV, and reovirus type I were obtained from the American Type Culture Collection. CDV, the Lederle avirulent strain, was obtained from the Research Reference Reagents Branch, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Maryland.

Results. Isolation of viruses. Tables I and III show the viruses isolated from the first series of 25 dogs, which were sacrificed during October and November of 1967. ICHV was isolated from 2 and CDV from 10 of the 25 dogs. Positive distemper cultures were initially recognized by fluorescent microscopy. ICHV was easily recognized by its characteristic cytopathic effects (CPE) and characteristic morphology in the electron microscope. Four other kidney cell cultures, on secondary culture, showed fluorescence with several sera (by the indirect Coons reaction), and each of these cultures produced slight-to-moderate CPE when whole cells were passaged into fresh, virus-free dog embryo cell cultures. The CPE did not resemble that caused by ICHV or herpesvirus. These were not neutralized by antidistemper virus serum; no recognizable virus structures were seen on examination in the electron microscope. Thus, only ICHV and CDV have been identified in series I. The four cultures designated "x" in Table I will require further study to determine the significance of the CPE and whether viruses are present or not.

TABLE I. Isolation of Viruses from Primary Dog Kidney Cell Cultures and the Occurrence of Viral Antibody in the Donor Animals; Series I.

Dog no.	Virus isolated	Antibody present vs.: ICHV	Distemper virus	Reovirus
1	Distemper	+	−	+
2	Distemper	+	−	−
3	"x"[a]	−	−	AC[b]
4	−	+	+	+
5	−	−	−	AC
6	"x"	+	+	+
7	−	+	+	+
8	−	−	+	AC
9	Distemper	+	−	−
10	Distemper	−	+	−
11	"x"	+	+	−
12	−	+	+	+
13	Distemper	−	−	AC
14	Distemper	−	−	−
15	Distemper	−	−	+
16	−	−	+	+
17	Distemper	+	+	+
18	−	+	−	−
19	−	−	−	+
20	"x"	−	−	+
21	−	+	+	+
22	Distemper	+	+	+
23	ICHV	+	−	AC
24	Distemper	−	+	−
25	ICHV	−	−	AC

[a] "x" designates cultures which did not contain identifiable agents but which showed some CPE and/or immunofluorescence; further study will be required to determine its significance.

[b] AC = anticomplementary.

Tables II and III show the results obtained on the second series of 30 dogs. Primary cell cultures were prepared during February, March, and April of 1969. Eight of the 30 animals yielded ICHV; herpesvirus was isolated from one individual. Three of the primary cell cultures showed initial CPE, but electron microscopy did not reveal ICHV, CDV, or herpesvirus. Attempts at passaging these cultures into embryonic dog cell cultures have been unsuccessful and fluorescent microscopy findings inconclusive. These cul-

TABLE II. Isolation of Viruses from Primary Dog Kidney Cell Cultures and the Occurrence of Viral Antibody in the Donor Animals; Series II.

		Antibody present vs.:			
Dog no.	Virus isolated	ICHV	Distemper virus	Herpes virus	Reovirus
1	ICHV	+	+	−	+
2	−	+	+	−	+
3	ICHV	+	+	−	+
4	−	+	+	−	+
5	Herpes	n.d.[a]	+	−	+
6	−	+	+	−	+
7	−	+	−	−	+
8	−	−	+	−	+
10	−	+	+	−	+
11	ICHV	+	+	−	+
12	−	+	+	−	+
13	ICHV	−	+	−	+
15		−	+	−	+
17	"x"[b]	+	+	−	+
18	ICHV	+	+	−	+
19	"x"	+	−	−	+
20	−	−	−	−	−
21	−	n.d.	+	−	+
22	"x"	−	+	−	+
23	ICHV	+	−	−	+
24	−	+	+	−	−
25	−	n.d.	+	−	+
26	−	+	+	−	+
27	−	+	+	−	+
28	ICHV	+	−	−	+
29	−	+	+	−	+
30	ICHV	n.d.	+	−	−
31	−	n.d.	+	−	+
32	−	+	+	−	+
33	−	+	+	−	+

[a] n.d. = not done.

[b] "x" designates cultures which did not contain identifiable agents but which showed some CPE and/or immunofluorescence; further study will be required to determine its significance.

tures, like the four in series I, will require further study to determine the significance of the initial CPE.

In addition to the primary kidney cell cultures, primary liver cell cultures were prepared from each of the 30 dogs in series II. These cultures yielded only three ICHV isolates, which is in contrast to eight ICHV

TABLE III. Isolation of Viruses from Primary Dog Kidney Cell Cultures.

Experimental series	No. dogs	ICHV	Distemper virus	Herpes virus	Reovirus
I	25	2	10	0	0
II	30	8	0	1	0
Total	55	10 (18%)	10 (18%)	1 (2%)	0

TABLE IV. Viral Antibody Present in Dogs Used for Production of Primary Kidney Cell Cultures.

Experimental series no.	No. dogs	Antibody present in dogs whose cultures were:							
		ICHV		Distemper virus		Herpes virus		Reovirus	
		Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
I	25[a]	(52%) 12/23	(50%) 1/2	(53%) 8/15	(40%) 4/10		(No positive cultures)	(63%) 12/19	(No positive cultures)
II	30[b]	(78%) 14/18	(86%) 6/7	(83%) 25/30	(No positive cultures)	(0%) 0/29	(0%) 0/1	(90%) 27/30	(No positive cultures)

[a] Six of these sera were anticomplementary.
[b] Five of these sera were not tested for ICHV.

isolates and one herpesvirus isolate obtained from primary kidney cell cultures. In each case where ICHV was isolated from the liver, ICHV was also isolated from the kidneys.

Occurrence of viral antibody in donor animals. Tables I, II, and IV show the results of antibody titrations on each of the 55 dogs used for primary kidney cell production.

(1) *ICHV antibody.* Thirteen of the 25 dog sera from series I (52%) contained neutralizing antibody. In series II, 20 of 25 sera (80%) contained neutralizing antibody. Table IV shows that antibody incidence was about the same in dogs yielding and not yielding ICHV.

Sera from series II were screened at a dilution of 1:5 for ICHV antibody, by both CF and neutralization methods. Only 12 of 30 sera were positive by CF, whereas 20 of 25 were positive by the neutralization test. No CF positives were neutralization negative, which suggested to us that the neutralization test might be more sensitive for ICHV antibody detection. Therefore the sensitivities of the CF and neutralization antibody titration methods were compared, and the results are shown in Table V. Three sera containing

TABLE V. Relative Sensitivity of Complement Fixation (CF) and Neutralization Titrations of Naturally Occurring ICHV Antibody.

Serum no.	Titer		Fold difference
	CF	Neutralization	
12	135	3,645	27
25	135	10,935	81
29	15	1,215	81

ICHV antibody gave 27- to 81-fold (average $\geqq$ 60-fold) higher titers by the neutralization method.

(2) *CDV neutralizing antibody.* Twelve of the 25 dog sera in series I (48%) contained neutralizing antibody against CDV. In series II (from which no CDV was isolated), 25 of 30 dog sera (83%) contained CDV antibody. As can be seen in Table I, four of the 10 dogs yielding CDV had CDV antibody; the remaining six had no detectable CDV antibody. Thus, no distinctive antibody pattern could be associated with dogs yielding CDV.

TABLE VI. Relationship of Vaccination to Isolation of Viruses and Presence of Antibody.

	Total no. dogs	ICHV isolated	ICHV antibody present
Received vaccine	16	(31%) 5/16	(80%) 12/15
No. vaccine	14	(21%) 3/14	(82%) 9/11

(3) *Herpesvirus neutralizing antibody.* None of the 30 dog sera tested in series II contained detectable neutralizing antibody against canine herpesvirus. This is of particular significance in the case of dog No. 5 (see Table II), whose cell cultures yielded a herpesvirus.

(4) *Reovirus CF antibody.* Twelve of 19 usable sera (63%) in series I and 27 of 30 sera in series II (90%) gave positive CF reactions. No reoviruses were detected in any of the 55 primary kidney cell culture lots.

Relationship of vaccination to the isolation of viruses and the presence of antibody. In dog series II, approximately half of the animals had been vaccinated with live attenuated ICHV and CDV vaccine several days before sacrifice (see Materials and Methods). Table VI shows the pattern of viruses isolated in vaccinated and unvaccinated animals. It can be seen that ICHV was isolated in about equal numbers in the two groups.

Attempts to recover CDV directly from kidney homogenates. Three of the dog kidneys, from which CDV was readily isolated in primary cell cultures, were removed from —70°C storage for further study. Homogenates of kidney tissue were prepared aseptically by grinding weighed pieces in a mortar, then adding sufficient tissue culture fluid to give a 10% suspension. These homogenates were clarified by 2,000 rpm centrifugation for about 10 min, the supernatant fluids removed and inoculated onto CDV-sensitive dog embryo cell cultures. Inoculum was removed after 1 hr and cultures observed for development of CDV. A known CDV strain was inoculated similarly on replicate cell cultures as a positive control. Control cultures showed obvious CDV microplaques by immunofluorescence, within 7 days. However, no evidence of virus replication was observable in cultures inoculated with tissue homogenates, even after 2 weeks incubation. Thus, CDV could be readily isolated from these three kidneys by propagation of living cells in primary cultures, but not by extracting the tissues.

Discussion. One of the purposes of this study was to determine which viral agents are most likely to be found in dog tissues serving as a source of primary cell cultures. We isolated ten ICHV's, ten CDV's, and one herpesvirus from kidney cell cultures. Thus, 21 of 55 kidney cell cultures (38%) contained identifiable viruses.

Of 30 liver cell cultures examined, only 3 (10%) contained detectable viruses (all were ICHV). In each case where liver cultures were virus-infected, the kidney cultures were infected with the same agent. The fact that fewer liver cultures were virus-contaminated than kidney cultures suggests that canine liver tissue might be considered as a source of virus-free cells.

There was an obvious difference between the virus flora observed in series I and series II, since ten distemper isolates were obtained in series I, and none were observed in series II. Although the dogs in the two series were obtained from a similar source, they were collected at different times of the year. Series I dogs were obtained during the early fall of 1967, whereas those in series II were collected in the late winter and early spring of 1969. Rockborn (11) found in one study that CDV incidence rose in the fall months and declined in the winter and spring months. Although symptoms of distemper were not recognized in series I dogs, perhaps seasonal variation in the incidence of CDV could account for our observations. CDV was present the kidneys of these dogs during the season that Rockborn found was a period of increased disease.

There was also a difference between the incidence of viral antibody in the two series. Thirteen (52%) of 25 sera from series I contained no CDV antibody, whereas only 5 (17%) of 30 sera from series II were CDV

antibody negative. This is consistent with the above suggestion that series I dogs might have been taken during a season when acute CDV infection was increasing (associated with lower antibody incidence and series II dogs were taken when the population had just passed a seasonal peak of acute infection (associated with higher antibody incidence).

Surprisingly, relatively few different kinds of viruses have been found in the tissues of dogs, compared with those of simians (1, 12). We encountered only three identifiable agents: ICHV, CDV, and herpesvirus. Each of these can be detected readily by appropriate techniques. ICHV and herpesvirus produce rapid, characteristic CPE (limiting dilutions of these two viruses can be recognized in 2-3 weeks). CDV can be recognized by fluorescent microscopy within about 7 days, at which time single foci of infection usually involve 10–100 cells. However, the fact that each of these viruses may remain latent or masked in tissues requires that cell cultures be held for periods longer than those required for simply detecting minimal concentrations of mature virions. The total time required (a) to unmask a virus and (b) to allow growth to a point where the sensitivity level of the detection method is reached may be variable.

Twelve of 30 dog sera were CF positive with ICHV antigen, and each of these sera was also positive by neutralization test. The CF reaction should detect antibody to any canine adenovirus [it is a group specific reaction; (13-15)]; the neutralization reaction detects antibody specific to each adenovirus type (13–15). Therefore, if any of the 12 dog sera had been CF positive and neutralization negative, it would have indicated experience with an adenovirus type different from our reference strain of ICHV. Such was not the case, which suggests that only one type of adenovirus infected these dogs. This deduction is strengthened by the fact that each of ten adenovirus isolates we recovered were more than 80% neutralized by anti-ICHV serum.

In our study, ICHV and distemper virus isolates were obtained from dogs with or without neutralizing antibody. Thus, these two viruses can come into equilibrium with host cells in such a way as to exist for significant periods of time in the presence or absence of detectable neutralizing antibody, and without causing obvious diseases. We have no good explanation as to why ICHV and CDV can be isolated from dogs which do not have measurable antibody, unless they were suffering inapparent acute infections. Acute infections with attenuated viruses would probably produce few recognizable symptoms.

No reoviruses were isolated, although the serologic evidence indicates that a high proportion of the animals had experienced infection. Apparently, reovirus rarely infects the kidneys or livers of dogs, or, if they do, the virus is not retained in recoverable form for very long periods of time.

CDV could not be isolated from cell-free homogenates of kidneys from which the virus was isolated in primary cell cultures. If latency is defined as a state of infection without apparent disease, CDV could be said to have been latent in the kidneys of these dogs. Further, the virus appears to have been masked, *i.e.*, not detectable by ordinary extraction (which involved cell lysis) and planting on sensitive cells. Possibly the rigors of cell lysis during tissue extraction inactivated mature virions, if they were present. It can be concluded, however, that detection of CDV in the kidneys of these dogs by inoculation of tissue extracts was inadequate and that observation of cell cultures derived from the kidneys was required for detection.

One of the most reasonable approaches to the production of virus-free primary canine cell cultures is to begin and maintain dog colonies which are free of known canine viruses. Insuring that colonies are free of viral agents involves periodic serologic monitoring of animals for antibody against known agents. Table V indicates that the virus neutralization test is about 60 times more sensitive than the CF test for detecting ICHV antibody. Therefore, the neutralization test would be the method of choice for serologic monitoring of dog colonies for evidence of

ICHV infection. Since (a) some dogs might suffer inapparent infections with attenuated viruses, and (b) animals having viruses in their organs don't necessarily have detectable viral antibody, it would probably be desirable to test sera from all animals in a colony periodically to determine whether nonpathogenic virus strains have been introduced.

Summary. Fifty-five different lots of primary dog kidney cell cultures and 30 lots of primary liver cell cultures (derived from stray dogs) were incubated for 4–5 weeks and examined for viruses. Ten ICHV's, ten CDV's, and one herpesvirus were isolated from kidney cultures; three ICHV's were isolated from liver cultures. Thirty-eight % of kidney cultures and 10% of liver cultures contained viruses. CDV was isolated only from dogs studied in early fall; no CDV was isolated from dogs studied in late winter and early spring. No characteristic antibody pattern was found in dogs yielding or not yielding viruses, since ICHV and CDV were isolated from dogs with and without antibody. Cell-free homogenates of CDV-infected kidneys were inadequate for demonstrating the virus, whereas primary cell cultures from these kidneys readily yielded virus. There was evidence from direct isolations and from antibody reactions that no adenovirus other than ICHV infected the dogs studied. ICHV antibody detection was about 60 times more sensitive by neutralization tests than by CF.

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