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Isolation of Canine Distemper Virus from Dogs with Chronic Neurological Diseases (40877)[1]

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Abstract. Successful isolation of CDV was accomplished from two out of six dogs diagnosed as ODE and two out of six dogs with chronic distemper encephalitis. Brain or bladder tissues obtained from diseased dogs and cultured *in vitro* yielded the cell-free virus. Cocultivation was not necessary for the transmission of the virus to susceptible ferrets. The brain from a dog with chronic distemper encephalitis, which had the earliest appearance of syncytium in culture, also showed CDV when triturated brain was assayed directly into ferrets. Direct isolation of CDV in ferrets was not possible from any other brain or tissues. CDV antigen was detected in brains of other dogs but viral isolation was not accomplished even after cocultivation, suggesting the presence of defective viruses. CDV antigen was not detected nor was virus isolated from kidney, lymph nodes, spleen, pituitary, lung, and liver of any dog. All dogs showed CDV neutralizing antibody in sera and CSF. The antibody was primarily IgG rather than IgM, suggesting remote and not recent infections. Within the ODE group or within the chronic distemper encephalitis group, the clinical manifestations, histopathology of brains, antibody, and IFA results were not measurably different whether CDV could be isolated or not.

Canine distemper virus (CDV) is closely related to human measles (MV) and bovine rinderpest viruses (1). The close relationship is evidenced not only by their antigenic and structural similarities but also by their clinical and pathological manifestations in their respective hosts. Although the striking symptoms of these diseases are largely respiratory and exanthematous, demyelinating encephalitis has been recognized in hosts naturally infected with CDV and MV. Persistent infections of the central nervous system have also been described with these viruses.

In man, MV has been implicated in acute measles encephalitis (2–4) and in subacute sclerosing panencephalitis (SSPE) (5, 6). Neurological involvement in dogs with CDV may manifest itself as an acute encephalitis, or it may take the form of a chronic demyelinating disease (7). Canine distemper encephalitis which ususally occurs in young dogs may occur weeks, months, and sometimes years after the clinical episode of systemic distemper. Demyelinating encephalitis occasionally develops without any preceding clinical illness in aged and immunized dogs, referred to as old-dog encephalitis (ODE) (8–10). ODE possibly represents in the dog a model for SSPE in man. CDV has been demonstrated in acute distemper encephalitis (11–13), however, the relationship of ODE to CDV is still poorly understood. Lincoln *et al.* (14) described two cases of ODE in which CDV viral antigens were detected in brain tissues by fluorescent antibody tests. Electron microscopic studies revealed paramyxovirus-like nucleocapsid structures in the intranuclear inclusions in nerve cells of one of the dogs with ODE (15). All attempts to isolate a viral agent from ODE cases in tissue culture or to transmit the disease to susceptible animals were unsuccessful (8, 14–16) until recently (17).

The present study describes in detail the successful isolation of infective CDV from two dogs diagnosed as ODE and two dogs

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with chronic distemper encephalitis. The isolations were accomplished by explant cultures *in vitro* of tissues derived from the diseased dogs. Cocultivation procedures were not necessary for viral isolation.

Materials and methods. Animals. The dogs in the study were brought to our attention by the Comparative Medicine Center of the Los Angeles County, Department of Health Services, with the collaboration of veterinarians in the Los Angeles metropolitan area. Of the 12 dogs included in this study, 6 were diagnosed as ODE and 6 as chronic distemper encephalitis. The following are the case descriptions of dogs from which CDV was isolated.

Case No. 2. A stray adult dog demonstrating convulsions and chomping of the jaws was brought to our laboratory for study. Histologic examination of tissue sections from this dog showed a severe nonsuppurative meningoencephalitis, with areas of demyelination in the cerebrum and cerebellum as well as the brain stem. There was perivascular lymphocytic infiltration, and glial nodule formation. Other tissues were relatively free of disease. Based on the age of the dog, clinical history, and histopathologic lesions described, this animal was considered to have had chronic distemper with severe neurologic lesions.

Case No. 8. A 7-year-old, 9-kg male terrier appeared blind. There was motor incoordination manifested by walking in circles. The animal was unable to rise 5 days later but would lie on its side and paddle intermittently with the legs while extending the head and neck. There was a central ulcer on the left cornea and a mature cataract in the other eye. The dog had received no vaccinations in recent years, but had been immunized with CD vaccine during the first year of life. The spinal cord had a mild demyelination with neuronal swelling, and there were small foci of lymphoid cells in the perivascular spaces with additional foci of gliosis. The brain sections contained severe diffuse spongiform areas of demyelination and there was extensive neuronal degeneration, gliosis, perivascular mononuclear cell infiltration, and focal necrosis of the neuropil. Spongiform degeneration was especially severe. Demyelinating lesions, as well as perivascular cuffing and gliosis, were present in the sections of the cerebrum as well as the cerebellum and brain stem. Lesions in the other tissues were minimal and included pulmonary pneumoconiosis, mild pulmonary fibrosis, and mild glomerulonephritis in the kidneys. The age of the dog, in conjunction with the clinical history, and the histopathologic changes described, lead to the conclusion that this animal had an advanced case of old-dog encephalitis.

Case No. 9. A 17-kg male German shepherd cross was estimated to be 2 years old. The dog had been vaccinated for distemper 1 month prior to the onset of signs. The animal had a history of progressive posterior paralysis which developed over a period of a month. It had received a recent bilateral pectineal tenotomy. Tissue sections were found to contain severe diffuse nonsuppurative meningoencephalitis. The meninges contained a mild lymphocytic infiltrate. Brain lesions were most severe in the brain stem, cerebellum, and mid-brain. Severe spongiform demyelination in the cerebellum and brain stem was evident as well as prominent mononuclear infiltration of the perivascular spaces. Neuronal degeneration was severe and multiple focal areas of gliosis were present. Other necropsy tissues did not contain any significant lesions. Considering the age of the dog, the clinical history of progressive paralysis without fever or other clinical signs, and the histopathologic lesions of the nervous system, this animal was considered to have had chronic distemper with severe neurologic involvement.

Case No. 10. A 6-year-old, 6-kg, spayed female miniature poodle was vaccinated against distemper as a puppy and revaccinated annually. The animal experienced progressive locomotor disturbances including wobbling, stumbling, and tremors over a period of a month. The dog would hold its feet in a wide stance when encouraged to stand, and would make a few exploratory movements, but otherwise appeared disinterested and slept most of the time. The animal had good body condition

but with pannus formation over both corneas. The brain and cord sections from the dog showed the presence of a severe diffuse nonsuppurative encephalitis with spongiform demyelination affecting most areas of the brain. There was marked neuronal degeneration, extensive lymphoid perivascular cuffing, and glial nodule formation. No evidence of inclusion bodies was found in the brain or other tissues. The other tissues were free of disease. The age of the dog, the case history, progressive locomotor signs, apparent blindness, loss of equilibrium, tremors, lack of coordination, and the histopathologic lesions in the brain and cord indicate that this animal had old-dog encephalitis.

Female ferrets. Female ferrets 8–11 weeks old, susceptible to CDV, were obtained from Marshall Research, North Rose, N.Y. Transmission of CDV to ferrets was tested by intraperitoneal injection of 1 ml of 10% tissue suspensions prepared in balanced salt solution and clarified by centrifugation in the cold or of 1 ml of cell-free culture supernatant from explants of tissues. Ferrets, which did not succumb with CDV upon inoculation with the test material, were always challenged with the ferret-adapted CDV (Distemperoid, Fromm Laboratories, Inc., Grafton, Wis.) to insure susceptibility.

Cell culture. The nutrient medium for cell culture was modified Eagle's medium, enriched with increased concentrations of amino acids, vitamins, and glucose and supplemented with 20% newborn calf serum and 10% tryptose phosphate (18). At the time of the necropsy, pieces of tissues were immediately placed in the cell culture medium and delivered to the laboratory for explant preparation. Tissues were also frozen at −70° for future use. Except for the bladder epithelium, the squashed cover slip technique was used for all tissues. The tissues were minced finely in a 60-mm petri plate and aliquots were distributed to five to seven additional plates. A 25-mm square No. 2 cover slip was placed over the minced tissues in each plate and the cover slip firmly pressed down to squash the tissues. For brain tissue explants, samples were obtained from various parts of the brain, mixed, minced together, distributed to additional plates, and cover slips were placed. Five milliliters of nutrient medium were added to each plate and cultures were placed in a humidified incubator which was maintained at 37° and pH of 7.3 by a continuous flow of CO_2 in air. The medium was changed twice weekly. Approximately 3 months after cultures were initiated, the concentration of the newborn calf serum in the medium was decreased to 5%.

For bladder epithelium cultures, epithelial cells were scraped off the bladder with a scalpel, suspended in growth medium, and distributed into 60-mm petri plates. By the following day, epithelial cells were attached to the bottom of plates and had commenced to divide.

Isolation of CDV by cocultivation techniques (5, 6) was attempted by the use of continuous canine kidney cell lines, MDCK (ATCC,CCL-34) and DK (kindly supplied by Dr. A. H. Fieldsteel, Stanford Research Institute Menlo Park, Calif.).

Neutralization procedure. Serum and CSF neutralizing antibody titers against CDV were determined by a microtiter technique as described by Appel and Robson (19). In this test, Vero cells and the Onderstepoort strain of CDV which had been adapted to Vero cells (kindly supplied by Dr. M. Appel, Cornell University, Ithaca, N.Y.) were used. Specific neutralization of CDV isolates was also performed by the microtiter technique (19). Serum and CSF samples were adsorbed with *Staphylococcus aureus,* Cowan strain containing protein A to remove immunoglobulin G (20, 21). The starter culture for Cowan strain of *Staphylococcus aureus* was supplied by Dr. J. Cherry, UCLA Hospital and Clinic, Los Angeles, California.

Immunofluorescent procedure. The indirect fluorescent antibody (IFA) method was employed for detection of CDV antigen in tissues. Serum of dogs hyperimmunized with CDV and fluorescein-conjugated rabbit antiserum against canine γ-globulin were used. Immunofluorescent examination consisted of smear preparations of various tissues fixed in cold acetone.

Results. The squashed cover slip technique described in this report provided an excellent method for the growth of cells from the following tissues: brain, lymph nodes, kidney, lung, pituitary, liver, and spleen. In most instances, cell outgrowth and migration from beneath the cover slip was observed in a few days after culture and maximal growth was seen in 3–4 weeks. Cultures have been maintained for over 6 months with the continued presence of intact cells. On a few occasions, viable cell cultures were maintained for over a year. Bladder epithelial cells grew vigorously and could be passaged by trypsinization with 0.25% trypsin in balanced salt solution. The jejunum and trachea did not produce cellular outgrowth by either the squashed cover slip procedure or the scraping method.

Squashed cover slip culture of the brain from a dog in which CDV was not isolated is seen in Fig. 1a. Figure 1b shows the presence of multinucleated giant cells in the brain culture from dog No. 2, in which CDV was isolated when the cell-free supernatant was inoculated into ferrets. Table I summarizes the presence of CDV antigen in brains of dogs and isolation of infectious CDV from tissues of dogs with chronic neurological diseases. Successful isolation of CDV was accomplished from two dogs diagnosed as ODE and two dogs with chronic distemper encephalitis. Specific CDV antigen was detected by IFA techniques in brains of all dogs from which infectious CDV was isolated. Brain tissues obtained from chronic distemper encephalitis dogs Nos. 2 and 9, ODE dog No. 10, and cultured *in vitro* yielded CDV. Multinucleated giant cells were noted 18–30 days after culture. Cocultivation was not needed for passage of the virus into susceptible ferrets. The ferrets succumbed with typical distemper infection 14–21 days after injection. The explant culture of the brain from dog No. 2 with chronic distemper encephalitis, which resulted in the earliest appearance of multinucleated giant cells (18 days), also showed infectious CDV when the frozen brain was triturated and assayed directly into ferrets. Direct isolation of CDV in ferrets was not possible with other frozen brains. Interestingly, CDV was passed into ferrets from bladder epithelial culture of ODE dog No. 8, but not from the brain culture even though CDV antigen was detected in the brain by IFA. Direct inoculation of frozen bladder epithelium homogenates into ferrets did not yield CDV. Explant cultures of spleen, kidney, lymph node, pituitary, lung, and liver from the dogs did not result in any observable syncytium nor was CDV isolated.

The viral isolates have been identified unequivocally as CDV by additional passages into susceptible ferrets using 10% spleen suspensions and by specific neutralization of the cell culture-passaged isolates with the NIH Research Reference Serum, canine distemper antiserum (ferret, Cat. No. V329-501-555). Prior to the microtiter neutralization tests, the isolates were adapted to continuous canine kidney and Vero cells.

In Table II, the total CDV neutralizing antibody in sera of dogs with chronic neurological diseases ranged from 1:16 to greater than 1:128. Neutralizing antibody was also present in spinal fluids of these dogs in titers ranging from undiluted to 1:8. Adsorption studies with *Staphylococcus aureus,* Cowan strain indicated that the bulk of the neutralizing antibody in both the serum and CSF was IgG and not IgM or other immunoglobulins, suggesting remote and not recent infection in these four dogs.

In addition to the four dogs described above, four other dogs diagnosed as ODE and four other dogs with chronic distemper encephalitis were studied similarly. CDV isolation was not accomplished from the brain or any other tissues of these additional dogs even after cocultivation with continuous dog kidney cell lines. However, neutralizing antibody and immunofluorescent results were not measurably different in these dogs as in dogs where successful isolation of CDV was accomplished.

Discussion. These studies report the isolation of infectious CDV from dogs diagnosed as ODE and chronic distemper encephalitis. Isolation was accomplished by cultivation *in vitro* of brain or bladder tissues obtained from the diseased animals. Previously, investigators have reported the

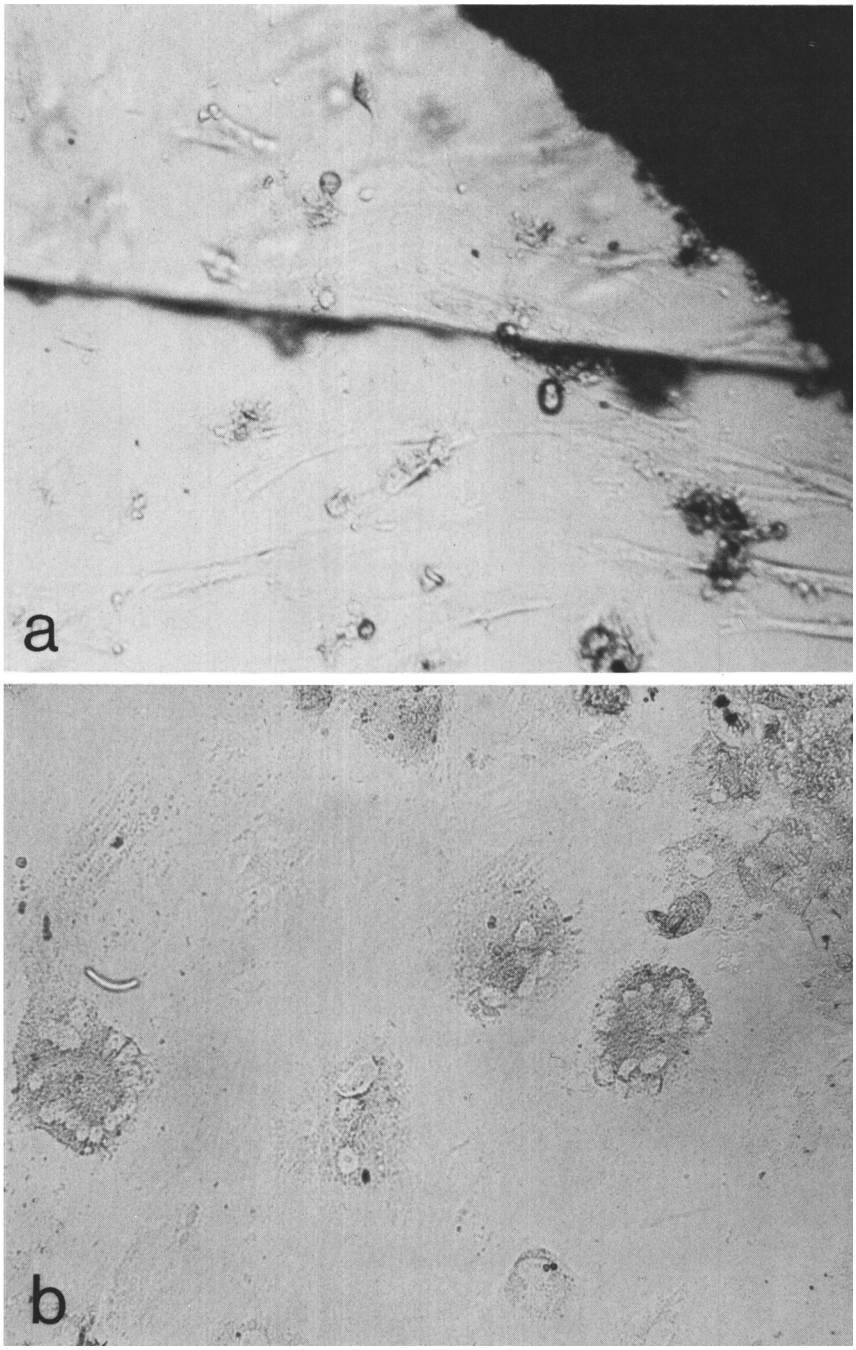

FIG. 1. Explant cultures of dog brains: (a) Culture of a brain from a dog in which CDV was not isolated. The dark line in the middle of the photograph represents the edge of the cover slip. Note the cellular outgrowth from the dark explant. Photograph of living cells, 31 days in culture (68×). (b) Culture of a brain from dog No. 2 showing multinucleated giant cells and syncytia, 40 days in culture (135×).

TABLE I. ISOLATION OF CDV AND PRESENCE OF ANTIGEN IN BRAIN OF DOGS WITH CHRONIC NEUROLOGICAL DISEASES

Dog No.	Diagnosis	CDV antigen in brain by IFA	Tissue virus isolated	Direct isolation into ferrets	Day syncytia observed in culture (days)	Passage in ferrets from culture supernatant
2	Chronic distemper encephalitis	+	Brain	+	18	+
9	Chronic distemper encephalitis	+	Brain	0	30	+
8	ODE	+	Bladder	0	180	+
10	(ODE)	+	Brain	0	28	+

unsuccessful attempts to isolate an agent by inoculation of brain suspensions or brain cell suspensions into cell cultures or to transmit the disease to susceptible animals (8, 14–16). In the present report, attempts to isolate the CDV from ODE dogs by inoculation of tissue suspensions into susceptible ferrets likewise failed to yield the virus. Successful isolation occurred only when tissues from ODE dogs were cultured *in vitro* for long durations. At least two possibilities exist to explain these results. First, attempts to isolate virus by inoculation of tissue suspensions into ferrets were unsuccessful since any virus in these suspensions could have been neutralized by the host antibody present. Explant cultures of the tissue effectively removed the dog antibody allowing for successful recovery of CDV from the cultures. On the other hand, there is also the possibility that the persistent virus in the ODE dogs is maintained in a subviral state and the cultivation *in vitro* allowed for the reactivation of the virus. A similar phenomenon has been observed in the latency of herpes simplex virus in the spinal ganglia of experimentally infected mice (22, 23). During the latent periods, infectious herpes simplex virus could not be detected in cell-free homogenates of ganglia, whereas infectious virus was produced when the ganglia were explanted in organ cultures *in vitro* (22).

TABLE II. CDV NEUTRALIZING ANTIBODY IN SERUM AND CSF BEFORE AND AFTER ADSORPTION WITH STAPH A

	Serum		CSF	
Dog No.	Total	After Staph A	Total	After Staph A
2	NA[a]	NA	1:2	0
9	≥1:128	1:3	1:2	0
8	1:16	1:2	1:1	—
10	1:16	1:2	1:8	1:2

[a] NA = not available.

In one of the two dogs described with chronic distemper encephalitis (Case No. 2), direct transmission of CDV was accomplished from the brain suspension into susceptible ferrets. Interestingly, this brain when cultured *in vitro* also demonstrated giant cell formation at the earliest date (18 days). Explant culture of the brain from the other dog with chronic distemper encephalitis was necessary before isolation was successful. Similar success in isolating CDV from dogs with prolonged mild focal demyelinating distemper encephalitis was reported recently (16).

Recovery of the infectious CDV in explant culture was accomplished without having to use the techniques of cocultivation which were necessary in SSPE (5, 6). This may indicate that the mechanism of viral persistence in ODE differs from SSPE and that CDV can undergo complete replication in tissues of ODE dogs in contrast to SSPE in which measles virus replication appears to be abortive. These findings were also substantiated by the recent observations of Hall *et al.* (24) when they reported that by immunoprecipitation techniques dogs with ODE and chronic distemper encephalitis had high levels of antibody to all

CDV structural polypeptides, indicating that all of these polypeptides were synthesized in the dogs during the course of the disease. The animals which were referred to in the paper of Hall *et al.* (24) are the same as those studied in the present report. The antibody reactivity pattern for structural polypeptides was quite similar in dogs whether CDV was recovered successfully or not. In contrast, patients with SSPE seem to lack antibody to the virus membrane (M) protein (24–27) and cocultivation of brain explants with permissive cells is required for virus isolation (5, 6).

Since CDV is so closely related to measles virus (1), it has been thought that chronic neurological diseases produced by the two viruses in their respective natural hosts would also be similar and that similar mechanism of persistence and pathogenesis would be involved. One would have then expected that canine ODE might represent a model for human SSPE. From the studies described above, it would appear that the mechanism of persistence is different in these two diseases. Considering that there is a wide spectrum of central nervous system diseases caused by CDV (acute encephalitis, subacute encephalitis, chronic disseminated meningoencephalitis, and ODE) (7, 28), there is a strong possibility that the few dogs studied in this report do not represent the exact model for SSPE. Since the frequency of ODE cases (as described in this paper) appears to be much higher than SSPE, one might postulate that a rarer form of ODE may exist which may have the mechanism of viral persistence similar to SSPE. A continued search for such an animal model would be warranted and is currently underway.

The persistence of infectious CDV in the bladder epithelium of the dog No. 8 diagnosed as ODE has fascinating implications. Several possibilities exist: 1. Persistence of virus can occur in a variety of tissues. 2. Bladder epithelium is known to be permissive to CDV (29) and by mechanisms yet unknown but similar to the brain, persistent infection can occur. 3. Accidental infection of the permissive bladder epithelial cells by infected leukocytes may have occurred during cultivation *in vitro*. Measles virus has been isolated from lymph nodes of patients with SSPE (30) and CDV has been described in the circulating leukocytes of dogs with canine distemper (31, 32). However, culture of a lymph node from the dog did not yield CDV. Further studies are necessary to determine which of the possibilities exists.

To our knowledge, this is the first report of the isolation of infectious CDV from dogs diagnosed as ODE. Persistence of CDV in tissues rather than recent infections would be a plausible explanation, since the bulk of the neutralizing antibody in both sera and CSF was IgG rather than IgM and since transmission of the virus to susceptible ferrets could not be accomplished by direct inoculation of tissue suspensions.

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