# Comparison of biological properties among virulent and avirulent canine distemper virus strains



by

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# INTRODUCTION

Canine distemper virus (CDV) infection is a pantropic endemic and epidemic viral disease primarily of canidae and their close relatives. It is world-wide in distribution and manifests itself as an acute contagious disease with clinical signs of respiratory, gastrointestinal, and/or nervous manifestations.

Although the viral etiology was first demonstrated in 1905 (Carre, 1905), significant advances in understanding the biology of the canine distemper virus were not made until the virus was adapted to grow first in embryonated eggs and later in tissue culture (Gorham, 1960; Appel and Gillespie, 1972). These advances permitted the development of serological assays for monitoring the disease process and permitted the subsequent development of modified live vaccines.

The safety of modified live virus vaccines has been a concern since their inception. When canine distemper is diagnosed in a recently vaccinated dog, there is concern as to whether the infection was the result of a vaccine failure or a vaccine safety problem (Hartley, 1974; Krakowka et al., 1985; Cornwell et al., 1988). Some researchers (Krakowka et al., 1985) feel that the incidence of vaccine induced fatalities from canine distemper has increased in recent years because of the immunosuppressive effect of canine parvovirus.

In this study, a comparison of vaccine and field strains of CDV was made. Biological markers were established that could be used to distinguish vaccine virus from field virus in dogs with clinical

distemper. In addition, techniques for the isolation and propagation of field viruses were compared.

# LITERATURE REVIEW

## Physical Properties of Canine Distemper Virus

Canine distemper virus is an enveloped virus in the genus Morbillivirus within the family Paramyxoviridae. Measles and rinderpest virus are also members of this same genus (Imagawa, 1968; Appel and Gillespie, 1972; Fraser and Martin, 1978). The virus is a relatively large paramyxovirus and is composed of a pleomorphic envelope of host cell origin that surrounds an internal nucleic acid core. The virion varies from 100 to 300 nm in diameter. It is a single negative stranded RNA virus. The RNA has a molecular weight of approximately 6 X $10^6$ daltons (Martin and ter Meulen, 1976). Smaller defective RNA molecules are also present, especially in virions obtained by the passage of undiluted virus (Carter et al., 1973; Kiley et al., 1974; Underwood and Brown, 1974). Purified nucleocapsids of distemper contain 4-5% RNA (Waters and Bussell, 1974). Several size classes of viral RNA, some corresponding to replicative and transcriptive intermediates, have been detected in nuclear or cytoplasmic fractions from cells infected with distemper (Martin and ter Meulen, 1976). The virus has been banded in cesium chloride and potassium tartrate. The mean buoyant density of the virion in these substances is 1.230 to 1.233 g/ml.

The structure of the virus consists of six major polypeptides (Waterson et al., 1963; Waters and Bussell, 1973; Fraser and Martin, 1978; Hall et al., 1980; Rima, 1983). The nucleocapsid contains a single structural protein (NP) directly associated with the viral RNA. The size estimate for the NP protein of CDV is 58K (Campbell et al., 1980; Hall et

al., 1980; Orvell, 1980). A second core protein is associated with a phosphorylase enzyme and is known as the P protein. This protein is attached to the nucleocapsid and is a minor structural component with a molecular weight of 73K (Campbell et al., 1980; Hall et al., 1980; Orvell, 1980). The L protein, a very minor component of the virus, is the largest protein detected with a weight estimated to be between 160K and 200K. Stallcup et al. (1979) and Robbins et al. (1981a) have found this protein to be associated with purified nucleocapsid but others have not detected it in this complex (Robbins and Bussell, 1979; Tyrell et al., 1980; Robbins et al., 1981b).

The hemagglutination protein (H) is the major glycoprotein and can easily be detected as a 76K to 85K protein in CDV virions (Bussell et al., 1974; Campbell et al., 1980; Orvell, 1980). The H protein is responsible for viral adsorption to target cells. The smaller glycoprotein, the fusion protein (Fo), is a 41K unit which is rapidly degraded into two smaller subunits, a 27K F1 and a 14K F2. It is responsible for cell-to-cell fusion and is also referred to as the "fusion factor". The membrane or matrix protein (M) of CDV has been identified as the smallest virion protein with an apparent molecular weight of 34K (Campbell et al., 1980; Hall et al., 1980; Orvell, 1980). Nonstructural proteins in morbillivirus-infected cells were discovered by Wechsler and Fields (1978). They identified 2 proteins migrating between the H and P proteins of MV-infected cells. The functions of these proteins are not known.

Measles virus has also been studied extensively and contains analagous viral polypeptides (Fraser and Martin, 1978). There are several

differences, however. The H protein of measles virus will hemagglutinate certain primate species' erythrocytes, whereas the H protein of CDV does not hemagglutinate erythrocytes from any species. Although these viruses are serologically cross-reactive, there is a difference in the reactivity of antibody to the hemagglutinin-equivalent protein. Anti CDV antibody will precipitate only CDV hemagglutinin-equivalent protein. In contrast, anti measles virus antibody will precipitate both CDV-H and MV-H polypeptides.

Canine distemper virus is inactivated by heating for one hour at 55° C or 30 minutes at 60° C. The virus is labile at pH 3.0 and relatively stable at pH 4.5 to 9.0 (Kimes and Bussell, 1968). Ultraviolet light and lipid solvents are known to readily inactivate the viral infectivity. Lyophilized virus is fairly stable at room temperature, but not above 32° C (Piercy, 1961). The virus is stable to lyophilization; however, there is some loss of titer, usually about one $\log_{10}$, during the lyophilization process. Virus is inactivated by formalin or the photodynamic action of methylene blue. Virtually all of the commonly employed chemodisinfectant substances such as quaternary ammonium compounds, phenolic compounds, and sodium hypochlorite will inactivate the virus meaning that decontamination of the environment is quite easy.

## Clinical Features of Canine Distemper

Clinical signs of distemper vary depending on the virus strain, environmental conditions, host age, and immune status. It has been reported that more than 50 to 70% of CDV infections are subclinical (Greene, 1984). Mild forms of clinical illness are also common, with

signs including listlessness, decreased appetite, fever, and upper respiratory tract infections characterized by bilateral serous oculonasal discharge which can become mucopurulent, and by coughing and dyspnea.

Severe generalized distemper infection is the commonly recognized form of the disease. It can occur in dogs of any age but most commonly affects puppies, 12 to 16 weeks of age, that have lost their maternal antibodies or younger puppies that did not receive an adequate amount of maternal immunity. The initial febrile response in natural infections is usually unnoticed. The first sign of infection is a mild, serous-to-mucopurulent conjunctivitis, which is followed within a few days by a dry cough that rapidly becomes moist and productive. Lower respiratory sounds from the thorax increase and can be detected by auscultation. Depression and anorexia are followed by vomiting. Diarrhea subsequently develops, varying in consistency from brown fluid, to frank blood and mucous. Severe dehydration and emaciation can result from adipsia and fluid loss. Animals can die suddenly from systemic illness, but adequate therapy in many cases can reduce the mortality rate. Many of the acute signs of systemic CDV infection are attributable to secondary and/or concurrent infection with various secondary bacterial, mycotic, and viral pathogens (Appel and Gillespie, 1972; Gorham, 1960).

Neurologic signs of distemper infection usually begin 1 to 3 weeks after clinical signs begin to subside. Dogs can develop the neurologic signs without prior history of systemic disease, and there is no way to determine which animals will develop neurologic disorders. On an empirical basis, however, certain features of the systemic disease have

been described as being predictive of the incidence of neurologic sequelae. Pustular dermatitis in puppies is rarely associated with CNS disease, while dogs developing nasal and digital hyperkeratosis usually have various neurologic complications (Greene, 1984).

Neurologic complications of canine distemper are the most significant factors concerning prognosis and recovery from infection. Neurologic signs vary according to the area of the CNS involved. Increased sensitivity to touch and cervical rigidity can be found as a result of meningeal inflammation. Seizures, cerebellar and vestibular signs, sensory ataxia, and myoclonus are also common. Seizures can be of any type, depending upon the region of the forebrain that is damaged by the virus. The "chewing gum" type of seizures, classically described for CDV infection, occurs in dogs developing polioencephalomalacia of the temporal lobes. However, lesions from other causes in the same region can produce similar seizures (Greene, 1984).

## Pathogenesis

The pathogenesis of canine distemper has been extensively studied, but certain features, such as the mechanism by which the virus produces encephalitis, are the center of current controversy. In natural exposure, the virus spreads between dogs by aerosol droplets and contacts epithelium of the upper respiratory tract. Within 24 hours, the virus replicates in tissue macrophages and spreads by local lymphatics to tonsils and bronchial lymph nodes (Appel, 1969). Following a local burst of virus production in these sites, the virus is then spread by lymphatics and blood to distant lymphoreticular tissues. This viremia occurs anywhere

from 2 to 4 days after initial infection. CDV-infected mononuclear cells are found in other lymphoid organs such as the bone marrow, thymus, and spleen. By days 4 to 6 post infection, virus protein can be detected by fluorescent antibody staining within lymphoid follicles in the spleen, lamina propria of the stomach, small intestine, mesenteric lymph nodes, and Kupffer's cells in the liver. Widespread virus proliferation in lymphoid organs corresponds to an initial rise in body temperature and leukopenia. The leukopenia is primarily a lymphopenia, caused by viral damage to lymphoid tissues, affecting both T and B cells (Krakowka et al., 1980).

Further spread of CDV to epithelial and CNS tissues on days 8 to 9 post infection probably occurs hematogenously as a cell-associated and plasma phase viremia and depends on the dog's humoral and cell-mediated immune status. Fourteen days after infection, animals with distemper virus antibody titers of greater than 1:100 clear the virus from most tissues and show no clinical signs (Appel, 1969; Appel et al., 1982). In vitro, specific CDV antibody has been effective in neutralizing extracellular CDV antibody as well as in inhibiting its intercellular spread (Ho and Babiuk, 1979a).

Dogs with delayed production of antibody undergo virus spread to epithelial tissues 9 to 14 days after infection. Clinical signs that develop may eventually resolve as antibody titers increase and virus is cleared from most body tissues. However, virus may persist in neurons and integument, such as foot pads, for extended periods. Spread and persistence of virus in these tissues may be responsible for delayed CNS

signs and digital hyperkeratosis (hard pads) that occur in some dogs (Greene, 1984).

Dogs with poor immune response undergo virus spread to many tissues by days 9 to 14 post infection. These tissues include skin, exocrine and endocrine glands, and epithelium of the gastrointestinal, respiratory, and genitourinary systems. The clinical signs in these dogs are dramatic and severe, and virus persists in their tissues until death.

Neurological forms of CDV infection can be divided into acute or chronic encephalitis. The acute form is characterized by virtually any combination of neurological signs but most commonly seen are petit mal or grand mal seizures. These convulsive episodes occur with increasing frequency over time. The neurological signs in these cases include disorders attributable to cranial nerve damage, meningitis, and signs attributable to diffuse cerebral disease, i.e., confusion and head pressing. A cerebellar form in which incoordination and instability may be seen, and a spinal cord form in which paralysis or paresis is a predominant sign, may also occur. Some dogs are photophobic and a few may become blind because of CDV-induced retinal and optic nerve damage. A characteristic neurologic sign is hyperkinesia or chorea which may persist into convalescence.

The chronic form of encephalitis associated with canine distemper occurs after apparent recovery from infection. Chronic encephalitis is characterized histopathologically by severe and nonselective demyelination and perivascular mononuclear cell infiltration. As with acute CDV encephalitis, the pathogenesis of demyelination in the chronic form is not

understood. The effect of antimyelin antibodies has recently been studied in dogs with chronic encephalitis, and they may induce or contribute to the demyelination process (Krakowka et al., 1973; Krakowka et al., 1981; Koestner et al., 1974; Vandevelde et al., 1982a). The CNS signs can be extremely varied and are difficult to diagnose as attributable to CDV infection without histopathologic examination. The reasons for this are the dogs are no longer viremic and the isolation of the virus in leukocytes, excretions, or secretions is not possible. Dogs affected with chronic encephalitis usually have increased CDV-specific antibody in the cerebral spinal fluid and serum. Specific immunoglobulin can be demonstrated both intracellularly and extracellularly within lesions in the CNS (Krakowka and Koestner, 1976; Vandevelde et al., 1981, 1982a, 1982b).

A major nonneural manifestation of CDV infection in dogs is CDV-associated immunosuppression characterized by depletion of T and B lymphocytes (Krakowka et al., 1975; Krakowka et al., 1980). In animals with secondary bacterial or viral infections, normally nonpathogenic organisms may become lethal due to the immunosuppression induced by CDV. The most likely mechanism that permits infection by secondary invading organisms is via a direct or indirect viral effect on the host immune system. This immunomodulating effect is a significant and important component of the disease. Lymphopenia has been noted as a hematological finding (McCullough et al., 1974), and CDV associated immunosuppression has been documented using a variety of in vitro and in vivo assays (Krakowka et al., 1975; Krakowka et al., 1980; Krakowka, 1982).

As a consequence of direct viral infection in either lymphoid cells and/or macrophages, lymphocytes from CDV-infected dogs are rendered incapable of producing effective in vitro and in vivo immune responses. Early virolytic effects of CDV on the lymphoid system and macrophages suppress established normal host defenses. Immunosuppression is not simply due to a direct virolytic effect, since immunosuppression persists long after virus can no longer be readily demonstrated in lymphoreticular tissues (Krakowka et al., 1975).

Although attenuated vaccine viruses have not been shown to be immunosuppressive to the same degree (Schultz, 1976), Potgieter et al. (1980) reported the occurrence of enteritis and neutropenia in 3 of 3 dogs given canine parvovirus (CPV) one week after being vaccinated with canine distemper and infectious canine hepatitis vaccine; one dog died. Dogs given CPV but not vaccinated previously with the combination vaccine remained well. The reverse situation has also occurred when dogs given distemper vaccine while ill with presumed CPV enteritis have subsequently died with confirmed distemper (Jezyk, 1980).

A controversy exists concerning the immunosuppressive effects seen with multivalent canine vaccines containing both CDV and CPV. Ritter (1983a, 1983b) posed the question of possible safety problems with vaccine-induced CD due to immunosuppression caused by CPV contained in combination modified live-virus (MLV) vaccine. He stated that the combined vaccine induced lymphopenia at day 5-7 post vaccination. Gill (1983) and Beckenhauer (1983) contradicted this statement by reporting that lymphocyte counts did not drop after vaccination with parvovirus

alone or declined slightly but remained within the normal range. Their conclusion was drawn from a study involving a total of 3 test animals. In testing conducted in over 20 dogs at the National Veterinary Services Laboratories, canine origin parvovirus vaccines routinely caused a lymphopenia after vaccination (Evans, 1982).

## Species Susceptibility

Canine distemper virus is an infectious disease of several members of the order Carnivora. A number of families have been shown to be susceptible to CDV, and prominent among them are the Canidae such as dogs, dingos, and foxes; the Procyonidae which includes raccoons, kinkajous, and lesser pandas; the Mustelidae which includes ferrets, mink, skunks, and badgers; and the Hyaenidae which includes the hyena (Krakowka et al., 1985).

No adequately documented case of naturally acquired infection with CDV in cats has been published. Experimentally, newborns, 6 to 8-week-old kittens, and adult cats were infected with the Snyder Hill strain (Appel et al., 1974). The cats experienced limited replication of the virus, but did not develop clinical disease nor was virus shed. The authors concluded that the cat is unlikely to be affected with CDV under natural conditions. An inclusion body encephalitis attributed to CDV was described in tigers (Gould and Fenner, 1983; Blythe et al., 1983). Although distemper virus was not isolated from these animals, at least one animal showed a rising titer to CDV during infection. Formalin-fixed brain tissue from both animals stained positive for CDV antigen using an indirect immunofluorescence procedure (Krakowka et al., 1985).

Of the common laboratory animal species, the most work has been done in mice with murine-adapted CDV (Appel and Gillespie, 1972; Gorham, 1960). Adaptation of virus to mice is best accomplished by serial intracerebral inoculation of suckling mice with viral suspensions (Gilden et al., 1981). In most cases, the infection is characterized as a monophasic acute encephalopathy with mild meningitis and focal to multifocal areas or confluent zones of necrosis within the brain (Gilden et al., 1981). This phenomenon is age-dependent and adult mice seem to be resistant to replication of even murine-adapted virus, whereas weanling mice show an intermediate pattern of clinical disease (Lyons et al., 1980). Approximately 40% of weanling mice inoculated with murine passaged-CDV die acutely. The remaining convalescent animals may live for long periods of time. The consequences of this infection are a neurologic syndrome and an obesity syndrome in surviving affected animals (Bernard et al., 1983; Lyons et al., 1982). The strain of mice influenced the severity of disease and resistant strains of weanling mice developed a slowly evolving encephalitis at 13 to 17 months post-infection rather than the acute fulminant form noted in susceptible strains of mice (Bernard et al., 1983).

In hamsters, the neurovirulence potential is dependent on the plaque type of the virus. Cosby et al. (1981) have inoculated the large and small plaque variants of the Onderstepoort strain CD virus into hamsters and have shown that the small plaque variant of tissue culture-adapted CDV is neurovirulent for the hamster, whereas the large plaque variant is not.

Since CDV is closely related to MV, it has been suggested that CDV may be infectious for primates. It is known that primates are susceptible to experimental inoculation with virulent CDV (Yamanouchi et al., 1977) and that central nervous system lesions produced mimicked those caused by MV infection in either primates or man. It has been suggested that CDV is involved in the etiology of multiple sclerosis (MS), a debilitating central nervous system demyelinating disease. However, serological and epidemiologic studies have not supported this hypothesis (Cook and Dowling, 1977; Appel et al., 1981; Burridge, 1978). Canine distemper virus has never been recovered from patients with MS.

## Virus Cultivation In Vitro

Canine distemper virus is a difficult virus to propagate in vitro. However, once the virus is adapted to tissue culture, it can be transmitted further to other cell culture systems, and readily propagated thereafter (Cabasso et al., 1959; Rockborn, 1958). The most reliable method for in vitro growth of virulent CDV is by use of a macrophage system. Appel and colleagues have shown that virulent virus will readily infect primary cultures of canine pulmonary macrophages (Appel and Jones, 1967). Similar findings were reported using ferret origin peritoneal macrophage cultures (Whetstone et al., 1981). The easiest way to transfer infection from macrophages to other cells is by performing co-cultivations of infected cultures with standard tissue culture cell lines (Confer et al., 1975a; Bui et al., 1982). A second cell culture type shown to be susceptible to virulent virus inoculation is a bovine proliferative cell culture system reported by Metzler et al. (1980a, 1980b, 1981). They

showed that inoculation of bovine fibroblastic cells obtained from long term cultures of peripheral blood macrophages with tissue suspensions containing virulent CDV resulted in the recovery of virulent virus from a persistent noncytolytic infection within the fibroblastic cells. Subsequent study has shown that virus from these bovine fibroblast cultures could be transferred to other continuous cell lines by co-cultivation methods (Confer et al., 1975a). A third culture system, primary canine bladder epithelium, has also been used to isolate virulent CDV (Bui et al., 1982).

Canine distemper virus has been propagated in many different cell culture systems including cells of avian, mustelid, canine, human, feline, and simian origin (Appel and Gillespie, 1972). Virus readily adsorbs to target cell monolayers. Peak adsorption occurs within 1 hour and is essentially complete by 4 hours (Appel and Gillespie, 1972). Investigators have shown that free infectious virus is released into the supernatant between 24 to 36 hours after inoculation (Confer et al., 1975a). Peak viral titers in supernatants are generally observed between 3 to 5 days after infection.

Canine distemper virus can produce cytopathic effects (CPE) when inoculated onto several lines of cultured cells. The most obvious CPE is the formation of multinucleated giant cells where fusion is mediated between adjacent infected cells by the envelope membrane associated Fo protein. Coincident with the formation of giant cells is the appearance of intracytoplasmic and intranuclear eosinophilic inclusion bodies (Krakowka et al., 1985). These inclusion bodies can be easily seen when

the culture monolayers are stained with hematoxylin and eosin. Accompanying syncytia formation, the host cells form cytoplasmic strands and eventually lyse. Viral cytopathology is more obvious and more dramatic in young, actively growing cell cultures. The extent and duration of viral CPE depends on the composition of the original viral inoculum. For example viral fluids have been shown to contain more than one plaque type (Krakowka et al., 1985), and the ratio of the plaque types may affect overall CPE. Plaque variants can affect the size of the multinucleated giant cell produced.

There are viral variants of CDV that replicate without an overt viral CPE leading to a persistent infection (Metzler et al., 1980b; ter Meulen and Carter, 1982; ter Meulen and Martin, 1976; Narang, 1982; Krakowka et al., 1985). Persistently infected cells will contain cytoplasmic and in some instances, nuclear inclusion bodies, but monolayers will lack other manifestations of viral CPE. The morphological appearance and growth characteristics for these infected cells are virtually identical to those in uninfected control cultures.

## Vaccination

Immunity to CDV infection is considered long term, and lasting immunity and immunologic homogeneity of the virus have made disease prevention possible through vaccination. Humoral immunity is involved in host defenses since passive administration of serum antibody has been beneficial in preventing distemper (Peacock, 1966). Passively administered globulin was used extensively prior to the development of effective vaccines, but inadequate standardization of potency and

interference with modified live virus vaccines contraindicated its continued use. Naturally acquired passive immunity blocks both infection and adequate immunization in the early post partum period (Appel and Gillespie, 1972; Gorham, 1960). Three per cent of antibody transfer occurs in utero and 97% in the colostrum, resulting in an initial titer in new pups that is usually equal to 77% of that in the bitch. Maternal antibody to distemper has a half-life of 8.4 days, and typically these antibodies will decline to below detectable levels by 10-12 weeks of age (Gillespie et al., 1958; Baker et al., 1959). Without the ingestion of colostrum, offspring of immune bitches with titers of from 200-500 are protected for approximately one week (Appel and Gillespie, 1972). Bitches with titers of greater than 1:1000 may pass on proportionately more antibodies in utero, protecting colostrum-deprived puppies from CDV infection for 3 to 4 weeks (Krakowka et al., 1978).

Puppies vaccinated with MLV vaccine cannot produce appropriate immune responses until maternal antibody decreases below a level of 1:20 (Baker et al., 1959). Antibody measurements have been made in puppies to determine the age at which they can be successfully immunized. This information has been used to create nomograms based on the bitch's titer that can be used to predict the optimal time of vaccination in puppies. Pups with a maternal antibody titer of 1:100 are considered resistant to disease and immunization with MLV vaccines is futile. For this reason, initial vaccination with CD vaccine may not induce a protective antibody titer, and a second and sometimes third vaccination at 3 to 4 week intervals is recommended to produce a lasting serum antibody

concentration. Yearly boosters are recommended for this disease despite the relatively long-lived immunity afforded by vaccination.

Vaccines produced for canine distemper have been continually improved with respect to level and duration of immunity. The first inactivated vaccines were used in the 1920s and were derived from virus-infected ferret tissue homogenates that were inactivated with formalin (Laidlaw and Dunkin, 1928). These vaccines produced poor immunity in dogs, required multiple injections, and caused tissue reactions because of the presence of foreign protein. Ott et al. (1959) developed an adjuvanted formalin inactivated vaccine which produced higher antibody titers than the non-adjuvanted vaccine after 2 or 3 vaccinations, but immunity did not last longer than 3 months.

The development of a ferret-passaged live virus for the control of distemper in foxes and dogs was first reported by Watson (1939). Ferret passaging was the first method used to attenuate the virus, and the first commercially available MLV vaccine was Green's Distemperoid (Green and Swale, 1939). Only partial attenuation was achieved, and clinical signs were sometimes noted 1 to 2 weeks after vaccination. Haig adapted Green's ferret origin distemper virus to the avian chorioallantoic membrane and, after further attenuation on these membranes, produced the Onderstepoort vaccine strain of canine distemper virus vaccine (Haig, 1948). Cabasso and Cox successfully adapted a field distemper virus to the egg, and introduced the Lederle strain of egg-adapted distemper virus vaccine (Cabasso and Cox, 1949). This strain was introduced as the first modified live egg-cultivated vaccine. Following vaccination, egg adapted MLV

multiplies transiently in the lymphoid system, generating an immune response. The virus is not spread to epithelial tissues and virus excretion does not occur (Krakowka and Koestner, 1976). For this reason, vaccine-induced immunity with this type of virus is never as great or as long-lasting as the immune response occurring after natural exposure.

Distemper virus was adapted to cell culture in the late 1950s through the work of Rockborn (1958), Cabasso et al., (1959), Vantsis (1959), and others. By 1960, the first cell culture origin distemper vaccines were introduced (Sinha et al., 1960; York et al., 1960; Baker, 1966). The newer cell culture products produce immunity in a similar manner to that of the egg-adapted MLV products, but also have the advantage of containing less foreign antigenic material while being more immunogenic. They also protect dogs at an earlier age, even in the presence of high maternal antibody titers (Kahn and Rubic, 1979).

Canine distemper and human measles viruses are antigenically related, and experimental infection of dogs with measles virus protected them from subsequent infection with CDV (Gillespie and Karzon, 1960). Measles vaccine virus produces a self-limiting infection in the lymphoid system of dogs similar to that of MLV-CDV vaccines (Greene, 1984). Measles vaccination offers the advantage of protection in young puppies with high concentrations of maternal antibodies to distemper (Wilson et al., 1974, Wilson et al., 1976). As mentioned previously, the measles vaccine virus can stimulate an immune response in the presence of CD antibodies. Cell-mediated immunity induced by the measles virus is thought to be the primary factor involved in the protective response (Brown, 1975; Gerber

and Marron 1976; Krakowka et al., 1978). In addition, cross-reacting antibody, viral interference, and interferon mechanisms may be involved (Ho and Babiuk, 1979b).

Immunity to distemper acquired from measles vaccination is not only transient but weaker than that derived from a successful vaccination with MLV distemper vaccine (Strating, 1975; Norrby and Appel, 1980). Comparison of distemper-vaccinated and measles-vaccinated dogs shows that the latter are not as well protected against aerosol challenge. These dogs develop the initial febrile response but do not exhibit the diphasic temperature response or other clinical signs typical of CD infection (Ott, 1970; Strating, 1975). Dogs vaccinated with MV are not protected when challenged intracerebrally with virulent distemper virus although MLV-CD vaccines will routinely protect against challenge by this route (Standard Requirements, 1974).

## Biological Markers of Virulence

The spread of canine distemper virus is associated with the infection of macrophages (Coffin and Liu, 1957; Cornwell et al., 1965) which act to disseminate virus in the early stages of infection. Poste (1971) described differences in the growth of virulent and attenuated strains of CDV in alveolar and peritoneal macrophages from both dogs and ferrets. The virulent strain was from a field case, but it was used at two passage levels. The first passage level was after adaptation to growth in canine cells. The second passage level was after additional adaptation to ferret cell culture. The attenuated strain was a vaccine strain (designated CDV/BW) that was capable of growth in both dog and ferret cell cultures.

Poste noted that while the two passage levels of the virulent strain and the attenuated strain grew in all macrophage types, the CPE produced by the virulent strains were unique in alveolar macrophages. In these cells, the nuclei in the polykaryocytes were arranged randomly and many of the nuclei were highly pleomorphic and grossly distorted. Most of the polykaryocytes contained nuclei of widely different sizes (anisokaryocytosis). The distribution of the chromosomes in the infected cells were abnormal with evidence of chromosomal fragmentation or pulverization. Although the attenuated strain grew to comparable titers in alveolar macrophages, similar nuclear damages was not detected with the vaccine strain.

Replication of the dog kidney cell adapted Rockborn vaccine strain of CDV in dog alveolar macrophages was examined by Appel (1978). He noted that by serially passaging the virus in dogs (intravenous inoculation followed by isolation of the virus from cervical lymph nodes surgically removed 5 days later) the virus increased in virulence. Accompanying this increase in virulence was a shift in the relative ability of the virus to grow in alveolar macrophages versus canine kidney cells. The avirulent virus grew well in kidney cells but poorly in macrophages while the virulent virus grew well in macrophages but poorly in kidney cells. Appel assumed that a virus population of increased virulence emerged during passage in dogs and this population, which preferentially replicated better in macrophages, overcame the attenuated virus population which grew better in kidney cells. He stated that virulence in CDV appears to be linked to the ability of virus to infect and replicate in dog macrophages,

but the Rockborn strain was the only one studied. Appel did not address any differences in nuclear cytopathology between the virulent and attenuated passage levels of the Rockborn strain.

The relative titers obtainable in chicken embryos, dog kidney cells, Vero cells, and ferret peritoneal macrophages were determined by Whetstone et al. (1981) for 8 isolates from vaccines and 2 preparations of the virulent Snyder Hill strain. The nontissue culture and nonegg adapted virulent Snyder Hill strain grew better in ferret macrophages than in the other systems tested. However, there was no appreciable difference in the titers of the attenuated strains obtained in macrophages compared to the titers obtained in the system to which they were adapted except for the Vero adapted Lederle strain and chicken fibroblast adapted Baker strain which titered higher in ferret peritoneal macrophages. In no instance did a vaccine strain demonstrate a higher infectivity in the kidney cell cultures than in macrophages. This observation contradicts the findings of Appel (1978), but peritoneal macrophages were used in the study by Whetstone et al. (1981), and alveolar macrophages were used in the study by Appel (1978). This difference in the source of macrophages may be important since Poste (1971) only noted differences in nuclear cytopathology with virulent strains in alveolar and not peritoneal macrophages. The cytopathology seen with the strains in the various tissue culture systems was not described in detail by Whetstone et al. (1981). The authors concluded that ferret peritoneal macrophages were an acceptable alternative to canine alveolar macrophages for the isolation of

virulent CDV, but the Snyder Hill strain was the only strain utilized in the study.

Confer et al. (1975a) demonstrated differences between virulent and attenuated CDV strains by their relative ability to grow in cell cultures. They found that after the virulent R252 and Snyder Hill strains were adapted to grow in Vero cells by subpassaging 5 times, the strains produced eosinophilic nuclear inclusions 7 days postinfection in Vero cells stained with May Grunwald-Giemsa stain. Distinct fluorescent nuclear bodies were also seen with the virulent strains in cells stained 7 days postinfection with fluorescence labeled CDV antibody. In a later study (Confer et al., 1975b), the nuclear bodies were examined by electron microscopy and found to be aggregates of nucleocapsid-like structures. The only attenuated strain examined was the Onderstepoort strain, and although it produced polykaryocytes and exhibited cytoplasmic fluorescence that was typical of those seen with the virulent strains, it did not produce nuclear inclusions or fluorescing nuclear bodies. Nuclear aggregates were also not observed when the cells were examined electron microscopically (Confer et al., 1975b). This study and the one with alveolar macrophages (Poste, 1971) indicate that nuclear cytopathology and not the permissiveness of cells for viral replication, may be a marker for virulence.

In other studies conducted with CDV in Vero cells, Cosby et al. (1981, 1983) found that the Onderstepoort strain produced both large and small plaques on Vero cells. By picking plaques of different sizes, they were able to obtain cultures that would produce either large

or small plaques exclusively. Weanling hamsters inoculated intracerebrally with the large plaque virus developed an acute neurological illness characterized by ataxia, convulsions, and paralysis typical of an acute encephalitis. Animals inoculated with the small plaque virus failed to produce any clinical signs of illness for the first 3 months postinoculation but did develop a general deterioration in condition characterized by weight loss and increased susceptibility to infection over the next 9 months. Differences in the nuclear cytopathology caused by the 2 isolates were not described, and the neurovirulence for animals other than hamsters was not investigated by the authors.

Hirayama et al. (1986) compared the biological and molecular properties of 7 attenuated and 3 virulent strains of CDV. They found that all the strains would produce plaques in Vero cells, but while the mean diameters of the plaques were reported and varied with the strains, the authors did not state if both large or small plaques were observed. Four of the 7 attenuated strains had mean plaque sizes of >0.7 mm, and these strains were the only ones that were neurovirulent in suckling mice. Of the three attenuated strains and three virulent strains that had a mean plaque size of 0.4 mm, only the Onderstepoort strain was neurovirulent in suckling mice.

While Hirayama et al. (1986) felt that a correlation between plaque size in Vero cells and neurovirulence in mice was suggested by the data, they believed that the capacity of the strains to form pocks on the chorioallantoic membrane (CAM) of 7-day-old chicken embryos was a better

indicator of virulence in dogs. All the virulent strains showed low pock-forming capacity compared to relatively high capacities for the attenuated strains. However, all the vaccine strains had been passaged at least 40 times in eggs and the virulent strains had never been passaged in eggs.

## Molecular Markers of Virulence

Cosby et al. (1981) demonstrated that a large plaque variant of the Onderstepoort vaccine strain of CDV was virulent for hamsters when inoculated intracerebrally while a small plaque variant was not. Polyacrylamide gel electrophoresis of radiolabeled polypeptides and glycoproteins indicated no difference in the size of the proteins produced from persistent cultures of the large and small plaque variants. In a similar study, Axthelm et al. (1987) could not detect differences in viral proteins from a ferret virulent strain and a ferret avirulent strain of CDV. The proteins were separated by polyacrylamide gel electrophoresis and stained by the Western (immunoblot) technique.

Differences in viral proteins produced by the Onderstepoort strain, a strain isolated from a dog with chronic encephalitis, and two strains isolated from dogs with old dog encephalitis were investigated by Shapshak et al. (1982). They noted that the nucleocapsid protein (NP) of the strains isolated from dogs with chronic neurological disease were of a lower molecular weight than the NP from the Onderstepoort strain. One-dimensional peptide maps following protease digestion demonstrated a unique pattern for the Onderstepoort strain and for the strain from the dog with chronic encephalitis. The patterns for the 2 strains causing old

dog encephalitis were the same. Differences were also observed in peptide maps obtained with the H, Fo, and F1 polypeptides but the differences did not correlate with virulence. No differences were seen in the digests of the M polypeptide.

In contrast to the higher molecular weight of the NP protein of the Onderstepoort strain reported by Shapshak et al. (1982), Hirayama et al. (1986) could not detect any appreciable differences in the molecular weights of the viral proteins from 7 avirulent strains (including Onderstepoort) and 3 virulent strains. The slight differences in electrophoretic mobility that were noted among the strains did not correlate with virulence.

Differences in the NP, P, F, and H proteins of the Convac, Onderstepoort, and Rockborn vaccine strains of CDV were evaluated by Orvell et al. (1985) with the use of 149 monoclonal antibodies. Each strain had a few unique antigenic sites. Variation was found in four, one, and three different antigenic sites of the NP, P, and H proteins respectively. No antigenic differences could be demonstrated between the three attenuated strains of CDV using the 10 monoclonal antibodies that reacted to the F protein.

Varsanyi et al. (1987) sequenced the F1 protein of the Onderstepoort and Convac vaccine strains of CDV. The two sequences were identical at the N-terminal region except for one amino acid. The sequence showed a high degree of homology with the previously determined N-terminal sequence of the F1 polypeptide of measles virus (Varsanyi et al., 1985) and moderate homology with the corresponding sequence of 5 other

paramyxoviruses (Richardson et al., 1980; Hsu and Choppin, 1984; Blumberg et al., 1985; Paterson et al., 1984; Server et al., 1985; Spriggs et al., 1986).

## EXPERIMENTAL DESIGN

Based on the previous studies, it is unlikely that the analysis of the molecular weights of the proteins of CDV will reveal differences that can be correlated to virulence. Complex studies that have utilized monoclonal reactivities and protein digest mapping have demonstrated considerable variation among the vaccine strains indicating that a marker common to all vaccine or all field strains would be difficult to determine. Sequencing of the fusion protein, the most likely source of a virulence marker, has indicated a high degree of conservatism in this protein for different CDV strains and for paramyxoviruses as a whole. Because of the unlikelihood of finding a molecular marker of virulence by existing techniques, this project concentrated on the conflicting findings on biological markers to determine which markers, if any, are consistent with a large sampling of field and vaccine strains of CDV.

This study examined three biological properties of CDV to determine if any of the three will consistently differentiate field from vaccine strains. These included: (1) comparison of the ability of the strains to infect macrophages and epithelial cells; (2) evidence of significant cytopathologic effect in alveolar and peritoneal macrophages and Vero cells; and (3) the ability of the strains to produce pocks on the chorioallantoic membrane of chicken eggs.

Four vaccine strains and 5 field strains of CDV were used in the experiment. Four of the 5 field strains were isolated from clinical cases of canine distemper. The fifth isolate was from a dog showing signs of

old dog encephalitis. None of the field strains had been passaged in tissue culture.

Evidence of cytopathology including giant cell formation in canine alveolar and ferret peritoneal macrophages and intranuclear inclusion bodies in Vero cells were determined by staining the cells with May Grunwald-Giemsa stain at 7 days post inoculation. In addition, the field isolates of CD were also titered in ferret alveolar macrophages to determine the most susceptible cell for virus isolation. Titer comparisons of strains in macrophages, Vero cells, and primary canine kidney cells were determined by the fluorescent antibody technique. In eggs, the inoculation of the chorioallantoic membranes of chick embryos was used to determine the pock forming titer of the CDV strains.

# MATERIALS AND METHODS

## Viruses

Four canine distemper virus strains used in the study were obtained from commercially available vaccines. They included a canine kidney cell line origin Rockborn strain, an African green monkey kidney cell line (Vero) origin Lederle strain, a chicken fibroblast origin (CFO) Lederle strain, and a canine kidney cell line origin Snyder Hill strain. The actual source of the vaccine viruses will not be given because of the possibility of disclosing proprietary information. Five field isolates used in the study were obtained directly from dogs. They included the Snyder Hill-NVSL strain[1] in a 20% canine brain suspension and A75-17, A76-9, and A76-21 strains in canine lymphatic tissue suspensions[2] obtained from dogs with clinical distemper. The fifth virus was strain A76-30 in a canine lymphatic tissue suspension isolated from a case of old dog encephalitis.[2] This information is summarized in Table 1.

The vaccine viruses were isolated from multivalent vaccines by blocking the other viruses with the appropriate antisera and then culturing in primary canine kidney cells (Rockborn strain), a canine kidney cell line (Snyder Hill strain), or Vero cells (Lederle strains) for 8 days. After freezing and thawing once, the viruses were dispensed and titrated in the tissue culture systems described below. They were also tested for extraneous canine adenovirus and canine parainfluenza virus by neutralizing the canine distemper virus with monospecific antiserum and

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[1] Obtained from T. O. Bunn, Ames, Iowa.

[2] Obtained from M. J. G. Appel, Ithaca, New York.

Table 1. Canine distemper virus strains

| Strain | Source | Substrate |
|---|---|---|
| Rockborn | Vaccine | Canine kidney cell |
| Snyder Hill | Vaccine | Canine kidney cell |
| Lederle | Vaccine | Vero cell |
| Lederle | Vaccine | Chicken fibroblast |
| Snyder Hill-NVSL | Field | Canine nervous tissue |
| A75-17 | Field | Canine lymphatic tissue |
| A76-9 | Field | Canine lymphatic tissue |
| A76-21 | Field | Canine lymphatic tissue |
| A76-30 | Field[a] | Canine lymphatic tissue |

[a]Obtained from a case of old dog encephalitis.

inoculating the virus-serum mixture onto primary canine kidney cells. The cells were examined for cytopathogenic effect at 10 days. All cultures were negative for extraneous vaccine viruses.

## Replication in Tissue Culture

The ability of the CD strains to replicate in various culture systems was determined by culturing the viruses in primary canine kidney cells, Vero cells, ferret peritoneal macrophages, and canine alveolar macrophages.

### Kidney cells

The primary canine kidney cells and Vero cells were trypsinized from stationary 150 $cm^2$ tissue culture flasks and planted onto 8-chamber slides[1] for the detection of virus growth by the fluorescent antibody technique. Cells were planted at 150,000 cells/ml, 0.4 ml/well, and inoculated the same day as planted with 0.1 ml of virus per well.

The medium used for all tissue culture procedures was Eagle's minimum essential medium with Earle's salts, L-glutamine, and nonessential amino acids.[2] Seven percent fetal bovine serum (FBS), gentamicin (25 mcg/ml), penicillin (25 IU/ml), and streptomycin (100 mcg/ml) were added. The same medium, without fetal bovine serum, was used as diluent in all virus titration procedures.

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[1] Lab-Tek Products, Naperville, Illinois.

[2] F-15, Grand Island Biological Company, Grand Island, N. Y.

Macrophages

Ferret peritoneal macrophages were collected after inoculating a ferret with 3 ml of sterile mineral oil intraperitoneally 4 days prior to harvest. At day 4, the animal was anaesthetized with 0.75 ml ketamine hydrochloride,[1] and the ventral abdomen was shaved and cleansed. Approximately 200 ml of the medium described above (without FBS) and containing 10 units/ml heparin were then injected into the peritoneal cavity at a point on the midline and about 5 cm below the sternum.

The abdomen was massaged for one minute, and the fluid was removed under suction with a 14-gauge catheter attached to a 60 ml syringe. The catheter was inserted into the peritoneal cavity at a point on the posterior abdomen lateral to the midline. Of the 200 ml of medium injected, approximately 150 ml were retrieved. The cell suspension was then centrifuged at 200 x g for 10 minutes. The supernatant was decanted, and the packed cells obtained from one ferret were resuspended into 40 ml of tissue culture medium with 15% FBS to obtain a concentration of between 2 and 4 x $10^6$ cells/ml. The macrophages were planted into 8-chamber slides, 0.4 ml/well, and incubated at 37° C in a 5% $CO_2$ atmosphere. At 24 hours, the medium was changed and the slides were inoculated with 0.1 ml/well of CD virus.

Canine alveolar macrophages were collected from dogs euthanatized with T-61.[2] The thoracic cavity was opened and the lungs and trachea

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[1] Ketaset, Bristol Laboratories Inc., Syracuse, New York.

[2] National Laboratories Corp., Somerville, New Jersey.

removed. A 14-gauge cannula was inserted into a major bronchus of the dog and secured with string. Sixty milliliters of tissue culture medium were injected into the lungs and removed with a 60 ml syringe. This lavage procedure was performed twice.

The cell suspension obtained was then centrifuged at 200 x g for 10 minutes. The supernatant was decanted and the packed cells resuspended at $10^6$ cells/ml with tissue culture medium containing 15% FBS. The macrophages were planted onto 8-chamber slides, 0.4 ml/well and incubated at 37° C in a 5% $CO_2$ atmosphere. At 24 hours, the medium was changed and the slides were inoculated with 0.1 ml/well of virus.

Virus assay in tissue culture

Each cell type used was inoculated with ten-fold dilutions of each virus, $10^{-1}$ through $10^{-4}$, 4 wells/dilution. The chamber slides were then incubated at 37° C in a 5% $CO_2$ atmosphere for 6 days. At the conclusion of the incubation period, the slides were fixed in acetone and stained with fluorescein tagged canine origin canine distemper conjugate. Cells were examined for typical fluorescence using an Orthoplan[1] microscope equipped with a 200-watt mercury light source and an incident-light illuminator. Each well that contained at least one fluorescing cell was considered positive, and virus titers were determined by the Spearman-Karber method as refined by Finney (1971). Using this system, the lowest detectable titer was 1.7 $\log_{10}$ $TCID_{50}$ /ml (1 of 4 wells positive at the $10^{-1}$ dilution). When no virus was detected at the $10^{-1}$ dilution, the titer was calculated

[1] E. Leitz Inc., Rockleigh, New Jersey.

as $\leq 1.5$ $\log_{10}$ $TCID_{50}$/ml since virus could be present in one or more wells at the $10^0$ dilution.

## Cytopathologic Effect

After the slides were examined for fluorescence, they were then stained with May-Grunwald Giemsa stain and examined for cytopathology by light microscopy. The rubber gaskets attached to the slides were removed, and the slides were stained for 15 minutes at room temperature with May-Grunwald stain.[1] The stain was removed by inverting the slides. The slides were then stained for 20 minutes with Giemsa stain[2] diluted 1:15 in distilled water. The stain was removed by inverting the slides and rinsing them in distilled water for 10 to 20 seconds. The slides were air dried, and paraffin oil and coverslips were applied.

## Pock Formation in Eggs

Seven-day old embryonated chicken eggs were used to titrate the CD viruses. The CAM was dropped by punching a hole in the air sac and then punching another hole in the side of the egg. Dilutions of the viruses were made in the tissue culture medium described previously, $10^{-1}$ through $10^{-4}$, and 0.1 ml of virus was inoculated into 4 eggs per dilution. The holes were sealed with glue, and the eggs were incubated at 35-37° C for 6 days. At the end of the incubation period, the eggs were opened and the CAMs examined for plaques typical of CD (white to grayish-white plaque

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[1] Harleco, Gibbstown, New Jersey.

[2] Fisher Scientific, Orangeburg, New York.

surrounded by an edematous area). The number of CAMs in each dilution showing plaques was recorded as the number of positive responses, and the 50% endpoint of infection was calculated by the same method used for the tissue culture titrations.

### Infectivity of Field Isolates in Macrophages

To determine if ferret alveolar macrophages would be an acceptable cell for the isolation of field strains of CD, the ability of the 5 field isolates from dogs to infect ferret alveolar macrophages was compared to the titers obtained for the viruses in canine alveolar macrophages and ferret peritoneal macrophages. The ferret alveolar macrophages were obtained and cultured using the techniques described for canine alveolar macrophages except that the cannula was inserted into the trachea and both lungs were washed twice.

# RESULTS AND DISCUSSION

## Replication in Tissue Culture

The titers of the vaccine strains in tissue culture as determined by the fluorescent antibody technique are found in Table 2. As a group, the vaccine strains titered significantly lower ($p < 0.05$) in canine alveolar macrophages than in primary canine kidney cells or Vero cells. Individually, the Rockborn and Lederle CFO strains titered lower in both canine alveolar macrophages and ferret peritoneal macrophages than in epithelial cells (primary canine kidney cells and Vero cells). There was no appreciable difference in the titers obtained for the Snyder Hill strain and Lederle Vero cell origin (VCO) strain in macrophages and epithelial cells. Of interest is the finding that the Lederle CFO strain replicated in both primary canine kidney cells and Vero cells. The titers obtained in these two systems were 4.7 and 5.0 $\log_{10}$ tissue culture infective $dose_{50}$ ($TCID_{50}$)/ml respectively. The titer in eggs was 3.5 $\log_{10}$ egg infective $dose_{50}$/ml obtained when the virus was titered in eggs.

All 4 of the field isolates from clinical canine distemper cases replicated in canine alveolar macrophages but no viral replication could be detected by the fluorescent antibody technique in primary canine kidney cells or Vero cells (Table 3). These findings were statistically significantly ($p < 0.01$). Strain A76-30, obtained from a clinical case of old dog encephalitis was an exception. Titers of 2.5 $\log_{10}$ $TCID_{50}$/ml in primary canine kidney cells and 3.7 $\log_{10}$ $TCID_{50}$/ml in Vero cells were obtained.

Appel (1978) took an avirulent canine kidney cell adapted Rockborn strain and passaged the virus in dogs to increase its virulence. He then compared the relative ability of the attenuated virus and the virulent virus to replicate in canine alveolar macrophages and canine kidney cells. The avirulent CD virus titered higher in canine kidney cells than in macrophages, while the virulent CD virus titered higher in macrophages than in canine kidney cells.

Appel's results with the Rockborn strain were confirmed in this study for all strains tested. All avirulent vaccine strains titered significantly higher ($p < 0.05$) in epithelial cells than in canine alveolar macrophages. The Rockborn, Snyder Hill, and Lederle CFO strains titered higher in primary canine kidney cells. The Vero adapted Lederle strain had a titer of 3.5 $\log_{10}$ $TCID_{50}$/ml in canine macrophages and primary canine kidney cells, but it had a titer of 4.5 $\log_{10}$ $TCID_{50}$/ml in Vero cells.

All field isolates from clinical CD cases could be detected by the fluorescent antibody technique in canine alveolar macrophages but not in primary canine kidney cells. This is consistent with Appel's finding that virulent Rockborn virus replicated to a higher titer in alveolar macrophages than primary canine kidney cells. Strain A76-30 obtained from a clinical case of old dog encephalitis grew in epithelial cells, but the titer was higher in canine alveolar macrophages. Growth in epithelial cells indicated that this strain may have been of vaccine origin. The fact that it now titered lower in epithelial cells than in macrophages after at least two passages in dogs is consistent with what Appel found with the Rockborn strain after it was passaged in dogs. Unlike the 4

Table 2. Viral titers[a] of canine distemper vaccine strains in tissue culture

| Strain | Canine alveolar macrophage | Ferret peritoneal macrophage | Primary canine kidney | Vero |
|---|---|---|---|---|
| Rockborn | 2.7 | 3.0 | 4.5 | 3.7 |
| Snyder Hill | 3.7 | 4.5 | 5.0 | 4.5 |
| Lederle VCO[b] | 3.5 | 5.0 | 3.5 | 4.5 |
| Lederle CFO[c] | 2.7 | 3.0 | 4.7 | 5.0 |

[a]Titers are expressed in $\log_{10}$ $TCID_{50}$/ml.

[b]VCO = Vero cell origin.

[c]CFO = Chicken fibroblast origin.

Table 3. Viral titers[a] of canine distemper field strains in tissue culture

| Strain | Canine alveolar macrophage | Ferret peritoneal macrophage | Primary canine kidney | Vero |
|---|---|---|---|---|
| Snyder Hill-NVSL | 5.0 | 4.3 | ≤1.5 | ≤1.5 |
| A75-17 | 4.3 | 2.3 | ≤1.5 | ≤1.5 |
| A76-9 | 4.3 | 3.0 | ≤1.5 | ≤1.5 |
| A76-21 | 3.0 | 1.7 | ≤1.5 | ≤1.5 |
| A76-30 | 4.5 | 5.0 | 2.5 | 3.7 |

[a]Titers are expressed in $\log_{10}$ $TCID_{50}$/ml.

field strains from clinical CD, strain A76-30 was not virulent for dogs when administered intravenously (Appel, 1989).

Whetstone et al. (1981) tested the virulent Snyder Hill strain in various cell systems and reported that it achieved the highest titer in ferret peritoneal macrophages. In the present study, the virulent Snyder Hill-NVSL strain along with 4 other field isolates were cultured in the macrophages and epithelial cells listed in Table 3. The results were consistent with those of Whetstone et al. (1981) in that the field strains from clinical cases of canine distemper would replicate only in canine alveolar or ferret peritoneal macrophages.

The virus titers of all vaccine strains tested in the study by Whetstone et al. (1981) obtained in ferret peritoneal macrophages were equal to or higher than the titers obtained in canine kidney cells or eggs. The data presented in Table 2 show that the Rockborn strain and the Lederle CFO strain achieved a higher titer in primary canine kidney cells than in ferret peritoneal macrophages ($p < 0.05$). Consequently, the ferret peritoneal macrophage system is not the most sensitive test system for titrating all vaccine strains of CD virus.

As concluded previously, the ability of a CD strain to grow in primary canine kidney cells or Vero cells appears to be a marker for differentiating field strains from vaccine strains. Vaccine strains will grow in epithelial cells while virulent field strains will not.

## Cytopathologic Effect

### Inclusion body formation

The 9 CD strains were inoculated into Vero cell cultures to determine if nuclear changes in those cells could be used to differentiate vaccine strains from field strains. The cultures were incubated for 6 days and then stained with fluorescein tagged CD antiserum. After the slides were examined for virus by the fluorescent antibody technique, they were stained with May-Grunwald Giemsa stain and examined for inclusion bodies. There was no evidence of viral replication of the 4 field isolates from clinical cases of canine distemper when the cells were examined by either the fluorescent antibody technique or May-Grunwald Giemsa stain.

Confer et al. (1975a) reported that virulent CD strains produced eosinophilic nuclear inclusion bodies in Vero cells while the avirulent Onderstepoort strain did not. However, the virulent strains used by Confer et al. (1975a) were not direct field isolates. They had been adapted to tissue culture and had been passaged in Vero cells at least 5 times. The isolates used in the present study were obtained directly from canine tissue and had not been passaged in cell culture.

Based on the findings of the present study, it can be concluded that the ability to induce nuclear inclusion bodies in Vero cells is not an acceptable marker for virulent field viruses since these strains will not replicate sufficiently in Vero cells to form nuclear inclusion bodies without adaptation.

Giant cell formation

Two distinct types of giant cells were observed in macrophage cultures inoculated with CDV. The first type was one in which the nuclei in the polykaryocytes were randomly arranged, highly pleomorphic and grossly distorted (Figure 1). The second type was one in which the nuclei were uniform in size and positioned to form a ring around the outer edge of the giant cell which resembled a clockface (Figure 2).

All the strains except the Rockborn and Snyder Hill vaccine strains produced giant cells in canine alveolar macrophages. The giant cell morphology observed is described in Table 4. Only the Lederle VCO vaccine strain and the Snyder Hill-NVSL and A76-17 field strains produced giant cells in ferret peritoneal macrophages. There was no correlation between the type of giant cell observed and the source of the strain. The giant cell type observed was not consistent with a specific strain.

Poste (1971) described a giant cell in canine alveolar macrophages infected with virulent CDV that was identical to the irregular giant cell seen in this study. Ferret peritoneal macrophages infected with the same virus produced the circular type giant cell similar to Figure 2. Only the circular type giant cell was described by Poste in canine alveolar macrophages and ferret peritoneal macrophages infected with the vaccine strain CDV/BW.

Although Poste felt that there may be some significance to his finding that only virulent virus would produce the irregular inclusion bodies in canine alveolar macrophages, there was no correlation between giant cell morphology and virulence with the larger number of strains used

Figure 1. Irregular giant cell formation in canine alveolar macrophages infected with the CDV field isolate Snyder Hill and stained with May-Grunwald Giemsa, x 400

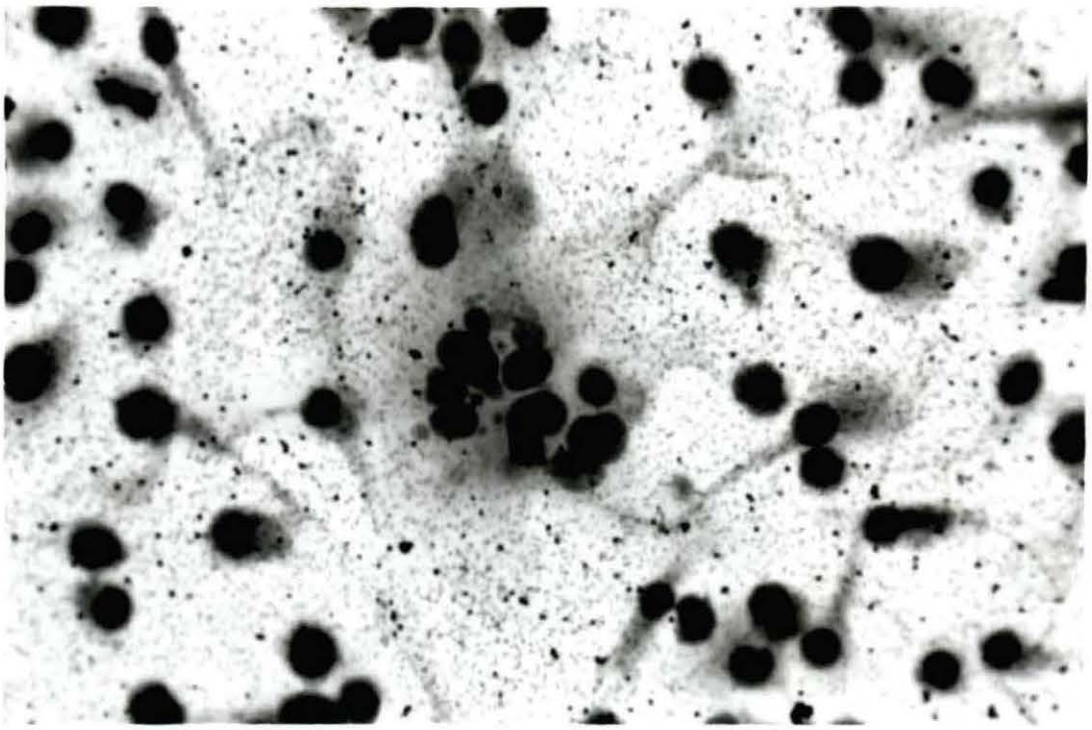

Figure 2. Circular giant cell formation in canine alveolar macrophages infected with CDV field isolate A76-21 and stained with May-Grunwald Giemsa, x 400

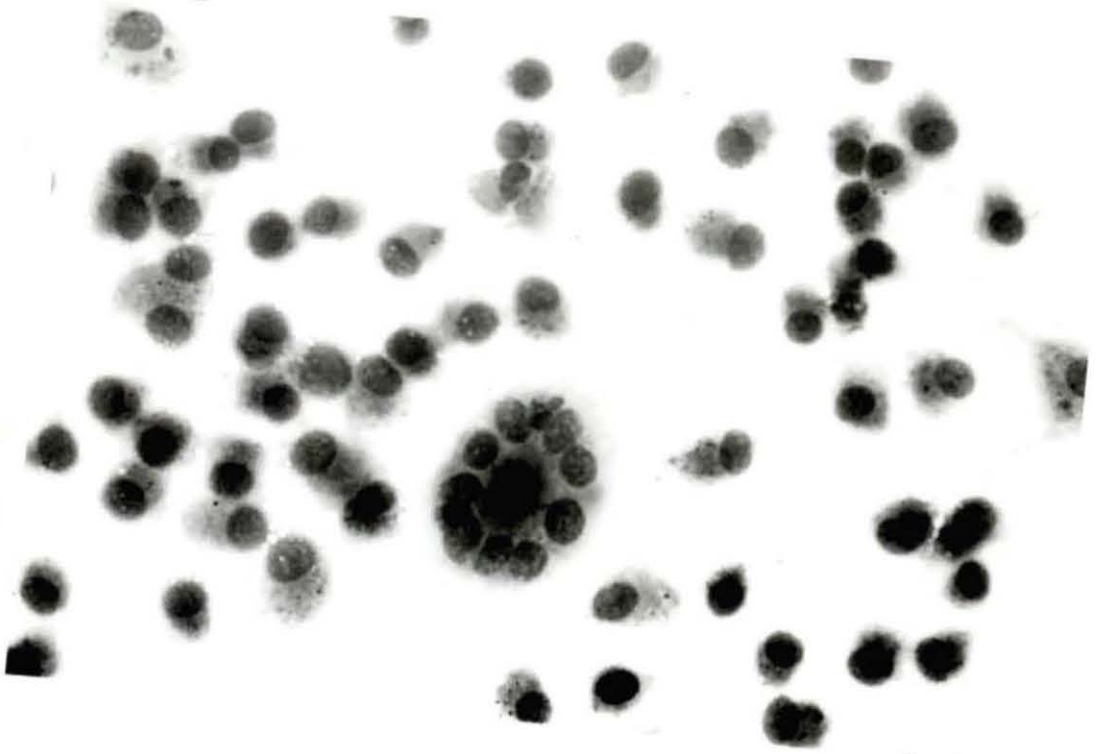

Table 4. Comparison of vaccine and field strains by giant cell morphology[a] in macrophages infected with canine distemper virus

| Strain | Source | Canine alveolar macrophage | Ferret peritoneal macrophage |
|---|---|---|---|
| Rockborn | Vaccine | None | None |
| Snyder Hill | Vaccine | None | None |
| Lederle VCO[b] | Vaccine | Irregular | Irregular |
| Lederle CFO[c] | Vaccine | Irregular | None |
| Snyder Hill-NVSL | Field | Both | Circular |
| A75-17 | Field | Irregular | Irregular |
| A76-9 | Field | Circular | None |
| A76-21 | Field | Circular | None |
| A76-30 | Field | Both | None |

[a]Irregular = nuclei in the giant cells were randomly arranged, highly pleomorphic and grossly distorted; circular = nuclei in the giant cells were uniform in size and positioned to form a ring around the outer edge of the cell; both = irregular and circular giant cells.

[b]VCO = Vero cell origin.

[c]CFO = Chicken fibroblast origin.

in the present study. It can be concluded, therefore, that giant cell morphology in canine alveolar macrophages is not a reliable marker to distinguish vaccine strains from field strains.

## Pock Formation

None of the 5 field strains used in the study would form pocks on CAMs of 7-day old embryonated chicken eggs. However, 3 of the 4 vaccine strains (Rockborn, Snyder Hill, Lederle VCO) would also not form pocks. Only the Lederle CFO strain produced pocks on CAMs.

Hirayama et al. (1986) reported that attenuated canine distemper strains readily formed pocks on the chorioallantoic membranes while virulent CD strains did not. However, of the 7 attenuated strains that Hirayama et al. (1986) tested, all had been passaged in eggs at least 25 times. None of the 3 virulent strains tested by Hirayama and colleagues had been passaged in eggs. Consequently, the ability to form pocks on CAMs appears to be related to a history of passage in eggs and not to whether the strain was a vaccine or a field isolate.

## Infectivity of Field Isolates in Macrophages

The 5 field isolates were titered in canine alveolar macrophages, ferret alveolar macrophages, and ferret peritoneal macrophages to determine which cell system was the most sensitive for the isolation of field strains. The results, shown in Table 5, demonstrate a higher infectivity in canine alveolar macrophages (mean of 4.22 $\log_{10}$ $TCID_{50}$/ml) than ferret alveolar macrophages (mean of 2.42 $\log_{10}$ $TCID_{50}$/ml) or ferret peritoneal macrophages (mean of 3.26 $\log_{10}$ $TCID_{50}$/ml). The Snyder Hill-NVSL

strain titered 5.0 $\log_{10}$ $TCID_{50}$/ml in the canine macrophages and 4.3 $\log_{10}$ $TCID_{50}$/ml in ferret macrophages. This difference of only .7 $\log_{10}$ $TCID_{50}$/ml may be due to the fact that it had been passaged previously in ferrets. The A76-30 isolate obtained a titer of 5.0 $\log_{10}$ $TCID_{50}$/ml in ferret peritoneal macrophages. This high titer is consistent with the previous results with the vaccine strains (Table 2) and indicate that this avirulent isolate may actually be of vaccine origin.

The fluorescent antibody staining of field strains A75-17, A76-9, and A76-30 was not as bright as the staining of the Snyder Hill-NVSL and A76-30 field strains. This weak fluorescence may be explained by the fact that the canine distemper antibody used to prepare the fluorescein conjugate was obtained from dogs administered vaccine strains and challenged with the virulent Snyder Hill-NVSL strain. Antigenic differences between the field strains and Snyder Hill-NVSL might result in a weaker fluorescence for the field strains because of a decrease in specific antigen-antibody binding.

One reason for assaying the field strains in canine alveolar macrophages, ferret alveolar macrophages, and ferret peritoneal macrophages was to determine if the cell type (alveolar vs. peritoneal) or the species of origin (canine vs. ferret) was contributing to the differences seen in the infectivity of the field strains in canine alveolar and ferret peritoneal macrophages. The results in Table 5 illustrate that the viral titers of the field isolates obtained by the fluorescent antibody technique were significantly higher ($p < 0.05$) in

Table 5. Viral titers[a] of field strains of canine distemper virus in various macrophage systems

| Strain | Canine alveolar macrophage | Ferret alveolar macrophage | Ferret peritoneal macrophage |
|---|---|---|---|
| Snyder Hill-NVSL | 5.0 | 4.0 | 4.3 |
| A75-17 | 4.3 | 1.7 | 2.3 |
| A76-9 | 4.3 | 1.7 | 3.0 |
| A76-21 | 3.0 | 1.7 | 1.7 |
| A76-30 | 4.5 | 3.0 | 5.0 |

[a]Titers are expressed in $\log_{10}$ $TCID_{50}$/ml.

canine alveolar cells than in ferret macrophages of either alveolar or peritoneal origin. The difference in titers between ferret alveolar and ferret peritoneal macrophages was not significant ($p > 0.05$). Therefore, the species of origin of the cells was the principal factor affecting viral replication.

Whetstone et al. (1981) concluded that ferret peritoneal macrophages could be used for isolating field virus and for the detection of CDV in tissues not suitable for frozen sections. This conclusion was based on information obtained with only the Snyder Hill-NVSL strain. As mentioned previously, canine alveolar macrophages were more sensitive to ferret peritoneal macrophages for the detection of the 4 field isolates from dogs with clinical distemper. Therefore, the use of fluorescent antibody techniques and ferret peritoneal macrophages for the isolation of CDV may not be the preferred test system. The preferred test system would use canine alveolar macrophages to culture the isolate. The fluorescein tagged CD conjugate used should be prepared from a pool of sera from dogs infected with different field isolates. The use of a pool of sera may result in more intense staining if antigenic diversity is indeed the cause of the dim fluorescence seen when the field isolates were stained with conjugate prepared from vaccine strains.

## SUMMARY

In this study, three methods of differentiating CDV field strains from vaccine strains were evaluated. They were comparisons of the ability of the strains to infect macrophages and epithelial cells as determined by the fluorescent antibody technique, evidence of giant cell formation in macrophages and nuclear inclusion bodies in Vero cells, and the ability of the strains to produce pocks on the chorioallantoic membrane of embryonated chicken eggs.

Significant cytopathologic effect in macrophages did not correlate with strain origin. The field strains would not replicate in Vero cells so cytopathologic differences in this cell type could not be determined. The ability of the strains to form pocks on CAMs did not correlate with virulence because the only isolate that formed pocks was a vaccine strain that had been passaged in eggs. The most reliable test method compared the ability of a strain to infect macrophages and epithelial cells. Using the fluorescent antibody technique, vaccine strains replicated in epithelial cells and macrophages where as the field strains isolated from clinical cases of canine distemper would only replicate at a detectable level ($10^{-1}$) in macrophages.

Based on the findings and conclusions of this study, the following procedure is proposed for determining whether a case of clinical distemper in a recently vaccinated dog was due to vaccine failure or caused by the vaccine itself. Tissues from the dog are titered in either canine alveolar macrophages or ferret peritoneal macrophages and in an epithelial cell such as canine kidney cells or Vero cells. After incubating for 7

days, the cells are examined for specific viral fluorescence by the fluorescent antibody technique. If the isolate is of vaccine origin, it will be detected by the fluorescent antibody technique in both the macrophages and the epithelial cells. If the isolate is a field strain, it will only be detected in the macrophages.

When the 9 strains utilized in this study were evaluated by the proposed technique, 4 of 4 vaccine strains grew in both cell types and 4 of 4 virulent field strains would only grow on macrophages. The ninth strain, A76-30, obtained from a dog with old dog encephalitis grew in both types of cells. The ability of A76-30 to replicate in epithelial cells suggests that it was of vaccine origin rather than a true field strain capable of producing clinical distemper.

When comparing canine alveolar, ferret alveolar, and ferret peritoneal macrophages, the most sensitive cell type for isolating field isolates was found to be canine alveolar macrophages. However, it is necessary to sacrifice a dog to obtain these cells. Since multiple harvests of peritoneal macrophages can be obtained from a ferret, ferret peritoneal macrophages may be preferred by some investigators wishing to distinguish vaccine virus induced canine distemper from field virus distemper.

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