VACCINATION OF RACCOONS (*Procyon lotor*) AGAINST CANINE DISTEMPER: AN EXPERIMENTAL STUDY

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

JEAN ARMAND PARÉ

In partial fulfilment of requirements

for the degree of

Doctor of Veterinary Science

August, 1997

ABSTRACT

VACCINATION OF RACCOONS (*Procyon lotor*) AGAINST CANINE DISTEMPER: AN EXPERIMENTAL STUDY

Jean A. Paré
University of Guelph, 1997

Advisor:
Ian K. Barker

A controlled vaccination trial was conducted using a commercial Vero cell line origin modified-live canine distemper virus (CDV) vaccine (Galaxy D®, Solvay Animal Health, Inc., Kitchener, Ontario, Canada) in 47 raccoon pups of known immune status.

All CDV antibody-negative pups developed detectable antibody titers within 2 weeks of vaccination. Eight CDV antibody-negative pups vaccinated once at 8 weeks of age and eight CDV antibody-negative pups vaccinated at 8, 12 and 16 weeks of age had similar titers at 20 weeks of age. Antibody-negative raccoons first vaccinated at 8 weeks of age mounted titers similar to those of raccoons first inoculated at 16 weeks of age. In eight unvaccinated pups, maternal antibody titers waned to negligible levels by 20 weeks of age. The half-life of maternal antibodies was

estimated at 10.55 days. When eight raccoons with maternal antibodies were vaccinated at 8, 12 and 16 weeks of age, only the third vaccination resulted in increased antibody titers. At 20 weeks of age, pups which initially had maternal antibodies to CDV had lower titers than CDV antibody-negative pups given the same vaccination protocol. When challenged with a virulent raccoon-origin CDV that caused clinical disease 29 and 30 days post-inoculation in three out of four controls and lesions of canine distemper (CD) in all four, 16 vaccinated animals showed no clinical signs of distemper over a follow-up period of 42 days and were free of lesions of CD at necropsy. Results of this study suggest that vaccination using this modified-live virus product in raccoon pups was safe and efficacious, and yielded protection from clinical disease. A vaccination schedule consisting of serial inoculations at 8, 12, and 16 weeks of age is recommended.

Acknowledgements

Funding for this project was generously granted in full by the Endangered Species Fund of the Metro Toronto Zoo and my salary was provided by the Zoological Society of Metropolitan Toronto. I am thankful to all members of my supervisory committee, Drs. Ian K. Barker, Graham J. Crawshaw, Scott A. McEwen, and Roger P. Johnson, for the scientific guidance that they provided. Doctor Max J.G. Appel of Cornell University kindly donated the challenge virus. Scott Taylor, of the Ontario Ministry of Natural Resources rabies research station in Midhurst, Ontario, was instrumental in obtaining a sufficient number of raccoon litters. Emma Hamilton contributed precious and reliable help in the weekly handling of raccoons. Experimental raccoons received attentive and excellent care from the personnel of the Isolation Unit at the Ontario Veterinary College (Dave Bridle, Tony Cengija, Jackie Rombeek, and Angela Pinsonneault). Doctor Susy Carman, Tom Synnott, and Mioara Antochi from OMAFRA VLS Mammalian Virology performed the canine distemper serum virus-neutralizing antibody tests. I also wish to thank Cheryl Pause for statistical counseling, and Barbara Jefferson, Carol Lee Ernst, Dr. Pascal Michel, and Dr. Stéphane Lair, for their assistance. Veterinary Medical Diets Incorporated (Guelph, Ontario,

Canada) generously donated 1,000 kg of cat food.

I dedicate this thesis to my beloved parents, Pierrette and Roland Paré.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Canine distemper (CD) is a contagious viral disease with a high case fatality rate that has been traditionally associated with the domestic dog (*Canis familiaris*) and other species of canids. Canine distemper virus (CDV) belongs to the genus *Morbillivirus* in the family Paramyxoviridae (Fenner, 1976; Pringle, 1992). The clinical course of the disease is characterized by any combination of upper and lower respiratory, gastrointestinal, cutaneous, and neurologic signs (Appel and Gillespie, 1972; Appel, 1987). In addition to canids, many other wild terrestrial carnivore species are susceptible to CDV (Appel and Gillespie, 1972; Budd, 1981; Montali et al., 1987; Appel and Montali, 1994; Haas et al., 1996). As a consequence, zoological institutions have long been confronted with the task of minimizing risks of transmission of CD from infected free-ranging animals to their collections. Outbreaks of the disease in zoos were described as early as the 1920s at the Philadelphia Zoo (Fox, 1923) and since have been reported regularly in other zoo or park settings (Armstrong and Anthony, 1942; Goss, 1948; Sedgwick and Young, 1968; Kotani et al., 1989; Appel et al., 1994).

The Metropolitan Toronto Zoo (MTZ) hosts many

endangered species of carnivores managed under Species Survival Plan (SSP) guidelines and recommendations, including black-footed ferrets (*Mustela nigripes*), red or lesser pandas (*Ailurus fulgens fulgens, A. f. styani*), Siberian tigers (*Panthera tigris altaica*), snow leopards (*Panthera uncia*), and a Chinese leopard (*Panthera pardus japonensis*). Canine distemper infection has been documented in all these species or subspecies (Mickwitz, 1968; Bush and Roberts, 1977; Parihar and Chakravarty, 1980; Gould, 1983; Williams et al., 1988; Fix et al., 1989; Kotani et al., 1989; Sikarskie et al., 1991; Eulenberger et al., 1993; Appel et al., 1994). As is the case with many zoos, the MTZ is prime habitat for wildlife. The MTZ site consists of 288 hectares of woods, valleys and waterways, creating a natural setting for pavilions and spacious outdoor exhibits. A perimeter fence does not prevent wild animals from accessing the site. Contact between free-ranging and captive animals is therefore unavoidable.

Many difficulties are encountered when trying to protect collections of captive carnivores against CD. The first concern is CD prophylaxis in exotic species, which is problematic. For example, black-footed ferrets and red pandas are exquisitely susceptible to CD, and to fatal vaccine-induced disease from modified-live virus (MLV)

vaccines (Erken and Jacobi, 1972; Bush et al., 1976; Carpenter et al., 1976; Itakura et al., 1979; Montali et al., 1994).

The killed CDV vaccine currently used in North America, a beta propiolactone-inactivated Onderstepoort strain virus vaccine with Quil A as adjuvant, is experimental and may become unavailable in the near future. It is not ideal in that it requires sequential inoculations and still provides only short-duration humoral immunity, and no cell-mediated immunity (Gillespie, 1965; Appel et al., 1984; Greene, 1990). In red pandas, the humoral response to killed vaccines was shown to be inconsistent, as many individuals failed to develop a titer (Montali et al., 1983; Montali et al., 1994). Natural CD has occurred in animals vaccinated multiple times with a killed vaccine (Sedgwick and Young, 1968; Sikarskie et al., 1991). Nevertheless, at the present time, the killed preparation that is currently made available to institutions holding black-footed ferrets and pandas remains the safest means of vaccinating species with a high risk of vaccine-induced distemper.

Vaccine technology is a fast-evolving field, and new avenues are being explored. Immune-stimulating complex (ISCOM) vaccines consist of immunogenic proteins incorporated in an open, cage-like structure resulting from

the interaction of saponins with cholesterol and phospholipid, simulating a cell membrane. Such vaccines have already proved safe and efficacious in immunizing selected species against morbilliviruses (De Vries et al., 1988; Visser et al., 1989; Visser et al., 1992). Recombinant technology has led to CDV-vaccinia virus and CDV-canary pox virus, in which genes coding for specific CDV antigenic membrane proteins have been inserted. Vaccines containing such viruses, able to replicate and express CDV antigens on their surface but unable to cause disease, may become commercially available in the future (Wild et al., 1993; Chappuis, 1995; Stephensen et al., 1997).

Whatever the means of vaccination of susceptible carnivores in a captive collection, efforts should also be directed at minimizing risk of exposure and pressure of infection. Canine distemper has long been enzootic in the common or North American raccoon (*Procyon lotor*) in many parts of the continent (Helmboldt and Jungherr, 1955; Robinson et al., 1957; Habermann et al., 1958; Parker et al., 1961; Karstad and Budd, 1964; Jamison et al., 1973; Hoff et al., 1974; Monson and Stone, 1976; Budd, 1981; Maurer and Nielsen, 1981; Evans, 1984; Hamir et al., 1992; Laperle, 1993; Roscoe, 1993). While coyotes (*Canis latrans*), foxes (*Vulpes fulva*) and skunks (*Mephitis mephitis*) may all

develop disease and transmit CD, raccoons have been incriminated or suspected as the probable source of infection in several zoo outbreaks (Sedgwick and Young, 1968; Fix et al., 1989; Sikarskie et al., 1991; Appel et al., 1994). Wild raccoons are abundant on the MTZ site and CD epidemics occurred within that population in 1981 (Cranfield et al., 1984), 1986 (Rosatte et al., 1991), and 1992 (Schubert-Kuehner, 1995).

Trapping and euthanasia of raccoons as a means of mitigating risks to zoo animals raises an ethical dilemma. Furthermore, it merely creates a vacated niche, open to immigration of susceptible or potentially infected raccoons from the areas surrounding the zoo. Wild raccoons from the MTZ site are therefore trapped, vasectomized or hysterectomized, dewormed, vaccinated with a MLV vaccine, tattooed, ear-tagged, and released, with the aim of creating a "barrier" population of resident non-breeding, healthy and CD-immune raccoons on site.

However, such vaccinated raccoons have been found infected with canine distemper, sometimes as little as 3 months after release. Doubts concerning the efficacy of MLV vaccines in raccoons grew stronger with anecdotal accounts of distemper in vaccinated raccoons in rehabilitation establishments (C. Mason, personal communication), and in a research facility (S. Taylor, personal communication).

Although a key species in the dynamics of canine distemper in North America, only a single study of the efficacy of MLV CD vaccination in raccoons and of their ability to survive subsequent challenge could be found in the literature (Evans, 1984). This study documented pre- and post-challenge serum CDV neutralizing antibody titers in ten vaccinated young raccoons, but results need to be interpreted with caution as the protocol presented several weaknesses. Furthermore, the vaccine used in that study is not available anymore. There are no data concerning maternal antibodies in young raccoons, nor are there any data from controlled vaccination trials using currently available commercial MLV CD vaccines. Such information is needed to provide guidance for a rational approach to the problem of wild raccoon management.

This thesis documents a controlled vaccination trial using a commercial MLV CD vaccine in raccoon pups, and subsequent challenge with a raccoon isolate of canine distemper virus. The main objectives were: 1) to determine, quantify and compare the humoral response of young raccoons to single and multiple sequential doses of a commercial MLV CD vaccine; 2) to determine whether age at first vaccination influences the immune response to vaccination in young raccoons; 3) to determine the maternal antibody decay curve in raccoon pups; 4) to determine whether maternal antibodies

interfere with immunization in raccoon pups; 5) to evaluate the protection against clinical disease afforded by vaccination, by means of a challenge study.

LITERATURE REVIEW

1. Canine Distemper

1.1: Etiology

Carré, in 1905, first demonstrated that CD was caused by a virus. Canine distemper virus has since been classified in the genus *Morbillivirus* of the family Paramyxoviridae (Fenner, 1976; Pringle, 1992). It is closely related to the measles, rinderpest, peste des petits ruminants, phocine distemper, and dolphin and porpoise morbilliviruses (Appel and Gillespie, 1972; Appel, 1987; Visser et al., 1993).

Canine distemper virus is a non-segmented, single-stranded, enveloped RNA virus of negative polarity. It has a spherical or sometimes filamentous morphology with a diameter ranging from 100 to 700 nm. The nucleocapsid is surrounded by a lipoprotein envelope with a membrane protein on the inside and two glycoproteins (H and F) of antigenic significance on the outside (Appel, 1987; Kingsbury, 1991). The H (hemagglutinin) protein is responsible for attachment of the virus to the target host cell's membrane and the F (fusion) protein is responsible for the fusion of the viral and plasma membranes, leading to liberation of the viral genetic machinery in the cytoplasm (Wild et al., 1995).

Canine distemper virus is labile outside the host. Its

half-life in the environment is 1 to 3 hours at 37°C, 2 hours at 21°C, and 9 to 11 days at 4°C (Appel et al., 1981). It is inactivated by chloroform, ether, formalin, phenol, hypochlorite and quaternary ammonium compounds, among other agents. It is destroyed rapidly by heat and radiation, and it is light-sensitive.

Only one serotype of CDV is presently recognized, although a variety of biotypes or strains exist that may vary in pathogenicity and tissue tropism (Shapshak et al., 1982; Summers et al., 1984; Oervell et al., 1985; Harder et al., 1996).

Canine distemper virus has a worldwide distribution (Appel, 1987; Chappuis, 1994).

1.2 **Host range:**

Canine distemper virus has a broad host range. Natural infection with CDV occurs mostly in terrestrial carnivores (order Carnivora, suborder Fissipeda). All species of the Canidae, Mustelidae and Procyonidae are considered susceptible (Appel and Gillespie, 1972; Budd, 1981; Montali et al., 1987). Domestic cats (*Felis catus*) do not show signs of CD infection although the virus will replicate in the lungs and associated lymphoid tissue (Appel et al., 1974). However, the susceptibility of the larger species (*Panthera*

sp.) of the family Felidae to CD disease is now well established (Appel et al., 1994; Harder et al., 1995; Roelke-Parker et al., 1996).

The susceptibility of the Viverridae was questioned (Montali et al., 1987) in spite of reports of CD in binturongs (*Arctictis binturong*) (Armstrong and Anthony, 1942; Goss, 1948). However, documented natural infection in a masked palm civet (*Paguma larvata*) (Machida et al., 1992) attests to the susceptibility of at least some species of viverrids. Natural infection and disease in the Hyaenidae has only recently been documented conclusively (Alexander et al., 1995; Haas et al., 1996).

The perinatal death of three polar bear (*Ursus maritimus*) and one spectacled bear (*Tremarctos ornatus*) cubs in a zoo constitutes the only report of CD in the Ursidae (Schönbauer et al., 1984). Detection of high serum antibody titers in American bears, probably *Ursus americanus* (Chappuis, 1994), and free-ranging Marsican brown bears (*Ursus arctos marsicanus*) in Italy (Marsilio et al., 1997), suggests that the virus circulates in these populations of ursids. Canine distemper has been reported to occur in the giant panda (*Ailuropoda melanoleuca*) (Qiu and Mainka, 1993; Mainka et al., 1994), which is now considered by most taxonomists to belong to the family Ursidae (Wozencraft,

1993). The same author discusses the taxonomy of the red panda, an animal traditionally placed in the family Procyonidae, and, summarizing the ongoing controversy, also classifies the highly CD-susceptible red panda in the subfamily Ailurinae within the family Ursidae.

In addition to terrestrial carnivores, natural CDV infection and disease has occurred in species as unrelated as Lake Baikal seals (*Phoca sibirica*) (Grachev et al., 1989; Mamaev et al., 1996), Japanese macaques (*Macaca fuscata*) (Yoshikawa et al., 1989), and collared peccaries or javelinas (*Tayassu tajacu*) (Appel et al., 1991). While the virus will replicate in lymphoid tissue of pigs (*Sus scrofa*), infection is inapparent (Appel et al., 1974).

Human (*Homo sapiens*) volunteers have developed transient asymptomatic viremia (Nicolle, 1931) and one developed disease (Appel et al., 1981) following experimental CD infection.

Raccoons are procyonids and distemper in this species will be reviewed separately at the end of this section.

1.3 **Modalities of transmission**

Virus shedding, which may begin as soon as 7 days post exposure, occurs in all body excretions of acutely infected dogs (Appel, 1987). Nasal exudate and saliva contained

infectious virus 5 days after exposure in mink (*Mustela vison*) and domestic ferrets (*Mustela putorius furo*) and persisted until death in the latter (Gorham and Brandly, 1953). Animals with more chronic disease may also still transmit the virus (Appel, 1987). Transmission is believed to occur primarily through direct contact or by aerosol at close range (Laidlaw and Dunkin, 1926), since the virus does not survive long in the environment (Gorham and Brandly, 1953; Gorham, 1966). A sick ferret may transmit disease to another across a distance of 5 feet (Gorham and Brandly, 1953).

1.4 Pathogenesis:

The pathogenesis of CD has been studied mostly in the dog (Appel, 1969), and in the ferret and mink (Gorham and Brandly, 1953; Crook et al., 1958). It appears to be fairly similar across these species.

Appel (1987) provided an extensive review of the pathogenesis of CDV infection in dogs. Following inhalation, the virus replicates in cells of lymphatic tissues in the respiratory tract. The virus is then carried by migrating macrophages and lymphocytes to all lymphatic tissues of the animal by 7 days post exposure. The time of onset and the degree of the humoral and cell-mediated immune responses of

the infected animal will then determine whether it will recover, die, or develop subacute disease with persistent infection.

Failure of the immune system to respond quickly will allow a second viremia via CDV-infected macrophages and lymphocytes, infecting most tissues, including epithelia of the alimentary, respiratory, and urogenital tracts. Exocrine and endocrine glands, and the central nervous system (CNS) are also targeted. In dogs, disseminated infection may lead to death within 2 to 4 weeks post infection. Occasionally, a delayed onset of immune response may lead to subacute disease, often encephalitis, or to persistent infection. In the latter case, some dogs may eventually recover. Dogs, and presumably individuals from other species, that survive the disease no longer shed the virus and are likely immune for life (Appel, 1987).

In ferrets and mink, virus was present in the nasal tissues, lung, spleen and blood as early as the second day post infection (Crook et al., 1958). The pathogenesis in susceptible exotic carnivore species has not been extensively studied but is believed to be similar to that in dogs, ferrets and mink (Budd, 1981; Evans, 1984; Montali et al., 1987).

1.5 Clinical signs

Clinical signs of CD have been reviewed by Appel and Gillespie (1972), Budd (1981), and Appel (1987). Pyrexia, usually paralleling viremia, may be associated clinically with apparent lethargy and/or anorexia. Other clinical signs reflect the spread of the virus to epithelial surfaces. Respiratory signs include catarrhal to purulent oculonasal discharge; encrustation, thickening or swelling of the muzzle; sneezing, coughing, and dyspnea. Gastrointestinal signs include anorexia, vomiting and diarrhea. Cutaneous signs encompass erythema, pustules, crusts, and marked hyperkeratosis of the footpads (hard pad). A wide range of neurologic signs, from depression to convulsions, and including aberrant behavior (e.g. lack of fear toward humans), may be observed in animals infected with CDV.

Animals with CD may exhibit any combination of these signs, concurrently or sequentially, or may simply be found dead. Signs may vary even among litter mates exposed to the same source of virus (Appel and Gillespie, 1972). Host factors (e.g. age, body condition, immune status) and strain of virus have been proposed as explanations for variations in the clinical picture. Signs and severity of disease also differ among species: at one end of the range are

subclinical infections in domestic cats and pigs (Appel et al., 1974) and at the other are domestic and black-footed ferrets, in which infection carries a high case fatality rate, approaching 100% (Davidson, 1986).

1.6 Gross and histologic lesions:

The pathology of CD has been reviewed (Appel and Gillespie, 1972; Budd, 1981; Appel, 1987; Montali et al., 1987, Dungworth, 1993). Animals dying of CD either may be in good flesh or in poor body condition depending on the duration and severity of illness. External macroscopic findings may include the cutaneous signs described above, serous or mucopurulent blepharoconjunctivitis and rhinitis, diarrhea and sometimes icterus. The only consistent internal gross finding in the dog is that of thymic atrophy. Enamel hypoplasia and other dental anomalies have been reported. Other findings may include pulmonary consolidation, and enteritis, that may be hemorrhagic.

Microscopic lesions may be found in most tissues but are predominantly observed in lymphatic structures, lungs, internal and external epithelial surfaces, and central nervous system. Intranuclear and intracytoplasmic eosinophilic inclusion bodies may be seen in neurons, glial cells, lymphoreticular cells and in epithelial cells of

bronchioles, alveoli, gastric glands, biliary and pancreatic ducts, renal pelves and the bladder. Syncytia or giant cells may also be seen (Appel and Gillespie, 1972; Dungworth, 1993). Both inclusion bodies and giant cells often accompany the lesions described below and are key to establishing a diagnosis of CD.

In the lymphoid tissues, lymphocyte depletion in both T- and B-dependent areas, with swelling and proliferation of reticular cells in acute cases, and regenerative hyperplasia later in the course of disease, may be present. In the lungs, interstitial pneumonia, bronchiolitis, bronchitis, and bronchiolar epithelial sloughing or hyperplasia are common findings.

Lesions of the central nervous system may be remarkably mild even in the presence of severe neurologic signs. They range from minimal non-suppurative cell infiltrates to severe disseminated non-suppurative meningoencephalitis with demyelination.

Canine distemper is known to induce immunosuppression (Krakowka et al., 1975; Mangi et al., 1976; Kauffman et al., 1982; Muneer et al., 1988; Greene, 1990). Secondary or concurrent infections are not uncommon. There are numerous reports of infection with CDV, concurrent with other viral (Diters and Nielsen, 1978; Fix et al., 1989; Hamir and Rupprecht, 1990), bacterial (Jakowski and Wyand, 1971;

Wojcinski and Barker, 1986), and protozoal infections (Cranfield et al., 1984; Stoffregen and Dubey, 1991; Thulin et al., 1992).

Recently, CDV infection has been linked with juvenile cellulitis and metaphyseal bone lesions in young growing dogs (Baumgärtner et al., 1995; Malik et al., 1995).

1.7 **Diagnosis:**

Helpful diagnostic tests in the live diseased animal are few, and have been reviewed by Appel (1987) and by Greene and Appel (1990). The case history and clinical signs may be suggestive. Demonstration, by neurologic examination, of multifocal lesions in the CNS supports a tentative diagnosis of CDV encephalitis. Changes in hematologic and serum biochemistry parameters, if present, are non-specific. Viral inclusion bodies in the erythrocytes and leukocytes of blood smears or in bone marrow aspirates are highly suggestive of CD. Cytology from conjunctival, preputial or other mucosal surface scrapings may allow detection of inclusion bodies in epithelial cells. Immunofluorescence or immunohistochemistry to demonstrate CD viral antigen may be performed on such scrapings. Radiography may help in ruling out some potential differential diagnoses.

In cases with neurologic manifestations, cerebrospinal

fluid (CSF) analysis and electroencephalography can also strengthen a diagnosis of CDV infection. Detection of antibody in the CSF is diagnostic if the blood-brain barrier is intact. Detection of CDV-specific IgM in the serum indicates recent infection or vaccination (Appel, 1987). Recently, a reverse transcription-PCR test to detect CDV nucleoprotein gene in canine peripheral blood mononuclear cells has been found to be a fast and sensitive supplementary method for CD diagnosis (Shin et al., 1995).

The diagnosis of CD in post-mortem material is easier, in spite of the usually mild and non-specific gross lesions. Immunofluorescence to detect viral antigen can be done on tissue impression smears. Demonstration by routine histopathology of typical inclusion bodies, especially in brain, lungs, and bladder epithelium is reliable. Immunofluorescence or immunohistochemistry to detect viral antigen in formalin-fixed tissues are other options. Electron microscopy may disclose the presence of virions in infected cells. Virus isolation may also be attempted in acute or subacute cases but is more time-consuming. Virus is most successfully isolated in primary lymphocyte or pulmonary macrophage cell cultures.

1.8 Treatment and prophylaxis:

There are no antiviral drugs effective against CDV; therefore, treatment is non-specific. Supportive therapy with antibiotics, fluids, and symptomatic treatment may be attempted but is usually unrewarding (Shell, 1990). Injections of canine anti-CDV serum have been used both in diseased animals and in animals at risk during epidemics, but their usefulness has not been clearly demonstrated (Budd, 1981). Reports on the use of compounds such as diethyl ether (Womer, 1973), isoprinosine (Glasgow and Galasso, 1972), and closantel (de la Torre, 1989) in the treatment of distemper are inconclusive. Acupuncture has been proposed as an adjunct to vaccination in order to enhance cellular immunity (Sciesinski, 1990).

Control of distemper is achieved mainly through vaccination (Appel and Gillespie, 1972; Budd, 1981; Appel, 1987; Chappuis, 1995). Both killed (inactivated) and modified-live (attenuated) CDV vaccines have been used in dogs, fur-bearing animals and zoo animals.

In dogs, killed vaccines do not confer protection against CDV infection but do protect against disease (Gillespie, 1965; Appel et al., 1984). At least two sequential inoculations are needed to induce neutralizing and complement-fixing antibody production. Killed CDV

vaccines do not induce cell-mediated immunity (CMI) and confer short-duration protection (Appel et al., 1984; Greene, 1990).

In species other than the dog, protection imparted by killed vaccines is inconsistent. Various inactivated vaccines were commonly used in zoos until the report, in 1968, of a devastating CD outbreak in exotic carnivores that had been vaccinated with a killed virus vaccine (Sedgwick and Young, 1968). Red pandas failed to develop an adequate titer following sequential inoculations of two different inactivated vaccines prepared from a Rockborn and an Onderstepoort strain of CDV, respectively (Montali et al., 1983). The death of a vaccinated red panda from CD (Sikarskie et al., 1991) further underlines the unreliability of killed vaccines. However, since there are a number of reports of MLV (including avianized CDV vaccines) vaccine-induced distemper in red pandas (Erken and Jacobi, 1972; Bush et al., 1976; Itakura et al., 1979; Montali et al., 1994) and black-footed ferrets (Carpenter et al., 1976), an adjuvanted (Quil A) beta propiolactone-inactivated Onderstepoort strain CDV vaccine remains the current recommendation for CD prophylaxis in these species (Montali et al., 1994). This vaccine did elicit substantial CDV neutralizing antibody titers in black-footed ferrets

(Williams et al., 1988) but it is not a commercial item and production may cease in the near future. It is strictly and selectively distributed by the maker to institutions holding these species.

The CD-MLV vaccines available today are made using the Onderstepoort strain of CDV, initially propagated in chicken embryo (hence the term chicken embryo origin, or CEO)(Haig, 1956), or the Rockborn strain, originally developed on canine cell lines (Rockborn, 1960). In Canada, two vaccines using Onderstepoort strain virus, grown in Vero cell lines (Galaxy D®, Solvay Animal Health, Inc., Kitchener, Ontario) or in Pro-cell Stable Cell line (Progard Puppy-DPv®, Intervet Canada Ltd., Whitby, Ontario), are listed in the Fifth Edition (1997) of the Compendium of Veterinary Products (CVP) published by the Canadian Animal Health Institute (North American Compendiums Ltd., Hensall, Ontario). A third Onderstepoort strain vaccine (Fervac-D®, United Vaccines, Madison, Wisconsin) is available in the United States. The CVP lists two manufacturers of canine cell line origin MLV CD vaccines. Availability of various types of vaccines is in a constant state of flux and companies are reluctant to share information regarding the components of the vaccine they produce.

MLV vaccines produce long lasting immunity and stimulate both CMI and humoral immunity (Greene, 1990). In

dogs, virus neutralizing, cytotoxic and complement fixing antibodies can be detected in the serum 6 to 10 days post vaccination (Appel, 1987). Virus neutralizing antibodies remain high for at least one year. Virus-specific CMI reaches maximal levels between 7 to 10 days post vaccination (Krakowka and Wallace, 1979; Shek et al., 1980). Protection against virulent challenge lasts many years (Appel and Gillespie, 1972).

Both CEO and canine cell line origin vaccines are considered efficient and safe in dogs. Conversely, the canine cell line origin vaccine is too virulent for use in domestic ferrets and in some exotic carnivores (Budd, 1981; Montali et al., 1983; Montali et al., 1994) resulting in an unacceptable rate of vaccine-induced distemper. In most of these species, the CEO MLV vaccine has proven to be safe and its use is preferred to that of the less immunogenic killed vaccine (Montali et al., 1983; Montali et al., 1994). The latter is to be used in species in which even avianized MLV vaccines have caused vaccine-induced disease (red pandas, black-footed ferrets), and in species in which the safety of attenuated live vaccines has not been demonstrated.

Vaccination protocols are dictated by the likelihood of passive transfer of maternal antibodies from the dam to the offspring. Maternal antibodies are acquired in part from the

immune bitch *in utero,* but chiefly through colostral intake. This also holds true in other carnivores. Passive immunity is thereby conferred to the progeny (Appel and Gillespie, 1972; Appel, 1987; Greene, 1990). In the dog (Gillespie et al., 1958) and domestic ferrets (Appel and Harris, 1988) maternal antibodies to CDV have a half-life of 8.4 and 9.4 days respectively, and will interfere with active immunization until they fall to a serum concentration sufficiently low that vaccine virus replication can occur (Gillespie et al., 1958; Appel and Gillespie, 1972).

Some dogs may still have enough maternal antibodies at 14 weeks of age to prevent successful immunization (Gillespie et al., 1958; Baker et al., 1959). In ferret kits, maternal antibodies may interfere with antibody formation in response to vaccination for up to 47 days after birth (Ott and Gorham, 1955; Appel and Harris, 1988). Some mink kits from hyperimmune dams failed to respond when vaccinated at less than 10 weeks of age (Gorham et al., 1962).

In puppies that possess maternal antibodies against CDV, heterotypic vaccination with measles virus vaccine, alone or in combination with CDV, will induce some active immunity (Appel et al., 1984). Puppies appear to be immunocompetent by the first day of age, so that they may

seroconvert early in response to vaccination in the absence of maternal antibodies (Appel and Gillespie, 1972; Greene, 1990). Ferret kits may produce antibodies to CDV as early as 8 days after birth (Ott and Gorham, 1955; Ott et al., 1965).

Recommendations for vaccination of exotic carnivores against CDV have been published (Montali et al., 1983; Fowler, 1986; Jacobson et al., 1988; Bittle, 1993; Montali et al., 1994) and constantly evolve in light of new information. Canids, mustelids and procyonids in which the use of commercial canine cell line origin MLV CD vaccines has proved unsafe generally should be given a commercial CEO MLV vaccine, if available. Notable exceptions are black-footed ferrets and red pandas in which only killed vaccines are deemed safe. In these two species, a protocol of two to three inoculations annually is recommended (Montali et al., 1994). The need for vaccination of viverrids and hyaenids is more controversial but it has been carried out using CEO MLV vaccines (Montali et al., 1994). Recommendations for felids are likely to be established in the future in light of the recent emergence of CD in this family. Vaccination of ursids is seldom practiced, except for the giant panda, in which a killed vaccine is recommended (Montali et al., 1994). Because of the lack of information concerning duration of maternal immunity in practically all exotic species, the manufacturer's guidelines for domestic animals are usually

extrapolated to the species to be vaccinated.

Aerosol vaccination has been used on furbearing animal farms with success (Morris et al., 1954; Hagen and Gorham, 1970, Gorska and Gorski, 1983a, Gorska and Gorski, 1983b). In addition to stimulating systemic immunity, aerosol vaccination has the advantage of promoting local immunity. In the face of an outbreak in an establishment, it is also practical in being fast, and in requiring minimal handling of the animals (Budd, 1981).

In addition to immunization of animals from a collection, quarantine and sanitation measures are an integral part of CD prophylaxis. The American Zoo and Aquarium Association (AZA) recommends a minimum of 30 days of quarantine for any new animal upon arrival to an AZA-accredited zoological institution, prior to introduction into a collection (Miller, 1995).

1.9 Distemper in raccoons:

The North American raccoon (*Procyon lotor*) is the type species of the type genus of the family Procyonidae, in the order Carnivora (Nowak, 1991). Other procyonids are the coatis (*Nasua* and *Nasuella* sp.), ringtails or cacomistles (*Bassariscus* sp.), kinkajous (*Potos* sp.), olingos (*Bassaricyon* sp.) and, controversially (Wozencraft, 1993),

the red panda. Raccoons, coatis, and ringtails are more closely related and form the subfamily Procyoninae (Wozencraft, 1993). The North American raccoon is found across southern Canada, from Nova Scotia to British Columbia, throughout the United States except for portions of the Rockies, and south through Mexico to Panama (Kaufmann, 1982). It has been introduced into France, Germany, and republics of the former USSR, where it has become established (Kaufmann, 1982). South of Panama, on the South American continent, it is replaced by the crab-eating raccoon (*P. cancrivorus*). The other five recognized raccoon species (*P. insularis, P. maynardi, P. pygmaeus, P. minor,* and *P. gloveralleni*) are insular forms in the Antilles and islands off the coast of Mexico (Nowak, 1991).

The North American raccoon is a stocky, small to medium size plantigrade mammal that weighs between 3.6 and 9 kg (Kaufmann, 1982). Males are larger than females, and average body weight increases with the latitude (Nowak, 1991). The general coloration is gray with a black mask and five to ten black rings on the tail.

Raccoons are found in a variety of habitats such as woodland, marshes, farmland and urban areas (Sanderson, 1987). They will den in trees, in burrows and other various ground shelters, and in attics, chimneys and walls of human

homes. Winter den locations may change within or between years, and differ from the whelping den that the mother will choose in the spring. In addition to denning, raccoons use sleeping sites that may be different every day (Kaufmann, 1982). This illustrates the movement of individuals within their home range and their need, or propensity, to constantly seek out new shelters. In doing so, they are well served by their adaptability and dexterity. Raccoons rank high on the intelligence scale of wild mammals (Sanderson, 1988) and demonstrate a well-developed ability to learn, memorize, and to pass on learned behaviors to succeeding generations (Davis, 1907; Sanderson, 1988). They often can gain access into human-built structures, denning sites unaccessible to other species.

Population densities vary with habitat. In a suburb in Ohio, density was estimated at 68.7 raccoons per km^2. In one study in Toronto, raccoon densities ranged from 56 per km^2 to 4 per km^2 going from forest-park to field habitat, respectively (Rosatte et al., 1991). In another study in Scarborough, a suburb of Toronto where the MTZ is located, an estimate of 10 per km^2 was made (Schubert-Kuehner, 1995). All of the above facts serve to stress the difficulties that need to be surmounted when trying to achieve control of the raccoon population on the zoo site and minimize contact with the collection.

Signs suggestive of canine distemper in raccoons were first described early in the century (Fox, 1922) and led to the assumption that this species was susceptible to CD. This became an established fact with the publication of confirmed reports of CDV infection in raccoons (Helmboldt and Jungherr, 1955; Kilham et *al.*, 1956). Robinson et al.(1957) documented an epizootic of CD in northwestern Indiana. Since then, there have been numerous reports of epizootics and areas of CD endemicity in raccoons across North America (Habermann et al., 1958; Parker et al., 1961; Jamison et al., 1973; Hoff et al., 1974; Monson and Stone, 1976; Maurer and Nielsen, 1981; Evans, 1984; Potgieter and Patton, 1984; Sikarskie et al., 1991; Hamir et al., 1992; Laperle, 1993; Roscoe, 1993; Appel et al., 1994), including Ontario (Karstad and Budd, 1964; Cranfield et al., 1984; Wojcinski and Barker, 1986; Rosatte et al., 1991; Schubert-Kuehner, 1995). In Toronto, in 1985, the prevalence of raccoons with serum antibodies to CDV was estimated at 61%. In Scarborough, the raccoon population increased by 40% between 1987 and 1989, possibly due to replenishing of the population following an outbreak of CD in 1986 (Rosatte et al., 1991). Incidence of CD in Scarborough raccoons, estimated from carcasses collected by the city's public animal control agency, ranged from 6.6% in 1992, to 0.6% in

1994, with a peak of 35.7% for October of 1992 (Schubert-Kuehner, 1995). Prevalence of CDV-antibody positive raccoons when pooled over the same years in that study, was estimated at 49.2%.

The pathogenesis of CD in experimentally infected raccoons appears to parallel that in the dog (Evans, 1984). Transmission occurred when sick animals were allowed free contact with susceptible raccoons for a few hours (Evans, 1984). Fomites probably play a minor role in transmission, given the labile nature of the virus outside the host (Appel and Gillespie, 1972; Appel, 1987). Season may influence the incidence of CD as many reported outbreaks have occurred in winter or in the colder months (Habermann et al., 1958; Monson and Stone, 1976; Hamir et al., 1992; Roscoe, 1993; Laperle, 1993). This may reflect increased opportunity for intraspecies contact during mating season or, conceivably, the cold may allow for longer survival of the virus outside the host (Appel, 1987), or both. Conversely, some epidemics have occurred, or have peaked in the warmer months of the year (Hoff et al., 1974; Evans, 1984), coinciding with dispersal of juvenile raccoons, and hence increased contact opportunity.

In one study, 418 of 541 (77%) of raccoons diagnosed with CD infection at necropsy were under 15 months of age

(Evans, 1984), but raccoons of all age may contract CD. Gender does not seem to influence susceptibility of raccoons to CDV infection (Schubert-Kuehner, 1995).

Evans (1984) reviewed the clinicopathologic features of CDV infection in raccoons reported in the literature and summarized necropsy findings in 541 raccoons diagnosed with CD. Clinical signs and lesions of CD in raccoons are similar to those observed in dogs, with the exception of the jaundice and hyperbilirubinemia that was prominent in sick raccoons during one epizootic (Kilham and Herman, 1954; Kilham et al., 1956), and believed to be associated with a different strain of CDV (Kilham et al., 1956). Icterus was also noted by Cranfield et al (1984), and by Evans (1984) in several raccoons. Evans postulated that viral-induced damage to biliary epithelium was involved in the development of icterus.

Neurologic signs are commonly reported in free-ranging raccoons (Robinson et al., 1957; Habermann et al., 1958; Karstad and Budd, 1964; Hoff et al., 1974; Monson and Stone, 1976; Maurer and Nielsen, 1981; Budd, 1981; Evans, 1984; Roscoe, 1993). Loss of shyness in many neurologically affected raccoons leads to increased odds of coming to human attention. Furthermore, the similarity of the clinical picture with that of rabies, a reportable disease, may lead to increased reporting of raccoons with neurologic, as

opposed to upper respiratory signs. These factors may result in overestimation of the prevalence of neurologic disease in CDV-infected raccoons. Such signs range from lack of fear towards humans to convulsions. Sick raccoons may appear blind.

Conjunctivitis accompanied by ocular and nasal mucopurulent discharge is commonly observed. Skin lesions consist of alopecia and pustular dermatitis. Self mutilation, sometimes occurring soon after a seizure, has been observed in experimentally infected raccoons (Evans, 1984). Gastrointestinal disturbances, manifested as diarrhea, may be present. Diarrhea was exacerbated when concomitant intestinal cryptosporidiosis was present (Evans, 1984). Cystitis with pyuria was a frequent finding in one report (Monson and Stone, 1976). Body condition ranges from good to emaciated. This may reflect duration of sickness in a given animal. Evans (1984) reported that duration of clinical illness in 14 experimentally infected raccoons averaged 10.4 days, but two raccoons died on the day that clinical signs were first noticed. In another experiment, two infected raccoons were overtly ill for 5 and 9 days respectively prior to death (Hoff et al., 1974). In another report, duration of illness ranged from 2 to 10 days (Kilham et al., 1956).

Histopathologic changes are similar to those seen in the dog (Evans, 1984). Depletion of white pulp in lymphoid structures; hyperplasia and/or necrosis of cells on epithelial surfaces; intracytoplasmic and intranuclear acidophilic inclusion bodies; and syncytia, or giant cells are typical findings. In the CNS, lesions vary in severity (Habermann et al., 1958; Hoff et al., 1974; Maurer and Nielsen, 1981; Evans, 1984; Potgieter and Patton, 1984; Hamir and Rupprecht, 1990). They may be minimal. A non-suppurative meningoencephalitis with or without demyelination may be observed. Inclusion bodies may be present in neurons and glial cells. Degeneration and mineralization of the testis was described in male raccoons dying from CD (Hamir et al., 1992).

Incubation periods of CD in raccoons ranged from 12 to 20 days following contact exposure (Evans, 1984), and 8 to 20 days following intraperitoneal (IP) inoculation (Kilham et al., 1956). In another experiment, inoculation of virulent CDV, via unspecified routes, caused clinical signs in 9 to 14 days (Robinson et al., 1957). Intramuscular (IM) and IP inoculation in two raccoons resulted in incubation periods of 11 and 30 days, respectively (Hoff et al., 1974). The proportion of infected raccoons that recover has been estimated at 42% following experimental contact challenge (Evans, 1984), and 50% following parenteral challenge

(Kilham and Herman, 1954). The presence of a high prevalence of apparently healthy seropositive wild raccoons in serologic surveys appears to support this allegation (Parker et al., 1961; Jamison et al., 1973; Hoff et al., 1974; Rosatte et al., 1991; Schubert-Kuehner, 1995). The literature certainly suggests that raccoons are highly susceptible to infection with CDV but that a proportion of animals may recover, with or without clinical signs.

In the first report of vaccination of raccoons against canine distemper, Kilham et al. (1956) used an avianized distemper virus that successfully protected five raccoons from intraperitoneal virulent challenge, while two of the three controls died. An egg-adapted CDV vaccine similarly protected all six raccoons from challenge by unspecified routes of inoculation while five of six unvaccinated controls developed the disease (Robinson et al., 1957). However, definitive conclusions cannot be drawn from these studies since the immune status of the experimental raccoons prior to vaccination or challenge was not determined in any of these trials. The accounts of raccoons surviving IP challenge with virulent CDV warrant careful interpretation for the same reason.

Ten young raccoons, vaccinated with a CEO vaccine (Fromm D®, Fromm Laboratories, Madison, Wisconsin, USA), were challenged when allowed free contact with an infected

raccoon (Evans, 1984). Only one of ten challenged animals exhibited transient clinical signs of CD, and all were clinically healthy and free of lesions of CD at the end of a post-challenge observation period of 40 days. In that study, the protocol is unclear as to the vaccination schedule, the immune status of pups prior to vaccination, and the duration of the challenge exposure of the experimental raccoons to the sick raccoon as well as the degree of interaction between these animals. The protocol also fails to indicate whether or not serology of serum samples were run simultaneously, so that pre- and post-challenge titers cannot be interpreted. While such a challenge method is as accurate as possible in mimicking natural exposure in an experimental setting, it will result in an uneven duration and magnitude of challenge among infected animals, and therefore yield data that warrant careful interpretation. Nevertheless, in that study, the vaccine did seem to protect challenged raccoons from clinical CD disease.

The use of CEO vaccines for CD prophylaxis in raccoons, with no adverse effect, has also been documented by several other authors (Sedgwick and Young, 1968; Miller, 1971). Avianized MLV vaccines are the current recommendation for raccoons (Montali et al., 1983; Jacobson et al., 1988; Bittle, 1993).

There are no convincing reports of vaccine-induced CD

in raccoons, except for one case in which Galaxy D® was incriminated (R.H. Evans, personal communication). However, raccoons at the MTZ have long been vaccinated with the Galaxy® line products with no adverse effect. Bush and Roberts (1977) contend that raccoons are susceptible to vaccine-induced distemper but fail to substantiate their statement. While vaccine-induced distemper has occurred in both the red panda (Erken and Jacobi, 1972; Bush et al., 1976; Itakura et al., 1979; Montali et al., 1994) and the kinkajou (*Potos flavus*)(Kazacos et al., 1981) which are procyonids, the issue in vaccinating raccoons would appear to relate to efficacy more than to safety. However, systematic experiments testing the safety of the various strains of attenuated CD virus used in vaccines have not been carried out.

A recent study has suggested that vaccination of raccoons as part of a trap-vaccine-release (TVR) program reduced the incidence of clinical distemper during an epizootic in Scarborough, Ontario (Schubert-Kuehner, 1995). Mistakenly, the author reports the use of an inactivated CD vaccine, referring to an avianized MLV vaccine (Fromm-D®, Solvay Animal Health, Inc., Kitchener, Ontario). Nevertheless, based on documented CD in vaccinated animals, there is accumulated circumstantial evidence that

vaccination with commercial canine MLV vaccines does not confer adequate protection against CDV infection in raccoons (**Appendix I**).

MATERIALS AND METHODS

1. Vaccination trial

1.1 Objectives

The objectives were to: 1) document the antibody response of raccoons following vaccination with a MLV CD vaccine; 2) compare the antibody response following single as opposed to multiple sequential vaccinations; 3) compare the ability of eight- and sixteen-week-old raccoons to respond to a single dose of MLV CD vaccine; 4) determine the decay curve of maternal antibodies in young raccoons; 5) evaluate the effect of maternal antibodies on the vaccine-induced antibody response in raccoons.

The outcome measured was a detectable humoral immune response over time, expressed as serum virus-neutralizing antibody titer at weekly intervals.

1.2 Experimental design

The design was that of a clinical prophylactic trial using 47 eight-week-old raccoons of known immune status as experimental units assessing the efficacy of a commercial MLV CD vaccine in terms of eliciting an antibody response. Each raccoon was randomly allocated to one of six groups:

A) Seronegative, unvaccinated controls (n=7)

B) Seronegative, vaccinated at 8, 12 and 16 weeks of age

(n=8)

C) Seronegative, vaccinated once, at 8 weeks of age (n=8)

D) Seronegative, vaccinated once, at 16 weeks of age (n=8)

E) Seropositive, unvaccinated controls (n=8)

F) Seropositive, vaccinated at 8, 12 and 16 weeks of age (n=8)

Seronegativity and seropositivity were defined as the absence and presence of detectable serum CDV neutralizing antibodies (≥ 1:2), respectively, in eight-week-old animals.

Blood was collected weekly from all raccoons from 8 to 20 weeks of age (12 weeks post-vaccination (PV)) except for raccoons of Group D, which were followed until they 24 weeks of age (8 weeks PV).

1.3 Experimental animals

1.3.1 Source

Between May 9th and June 19th 1996, 18 litters of raccoons were collected from the wild around Barrie, Arthur, and Scarborough, in the province of Ontario, Canada. Litter size ranged from one to six pups. From these, 65 pups (28 males, 37 females) were selected for the trial. The criteria for admissibility were that the pups were between 4 and 7 weeks of age when collected, and clinically healthy.

1.3.2 Age determination

Raccoon pups were assigned to groups over a 7 week period, formally entering the study when they reached 8 weeks of age. The following guidelines were used for aging raccoon pups.

Raccoons are born with hair. The back, initially sparsely furred, is well covered by 1 week of age. The mask is fully haired at 2 weeks of age, and the tail rings at three. The eyes and ear canals are closed at birth, and both usually open after 18 to 24 days. Pups less than 3 weeks of age squirm actively and chitter but cannot support their weight with their legs. They begin walking in the fourth to sixth week, and by the end of the seventh week, can walk, run and climb (Hamilton, 1936; Montgomery, 1968; Montgomery, 1969).

Raccoon pups were often ambulatory when first examined. At that point, age estimation was based chiefly on pattern of dental eruption (Montgomery, 1964): in 4-week-old raccoon pups the deciduous first, second, and third incisors and the canine teeth are in place; at 6 weeks of age the deciduous second, third and fourth premolars are in place; the deciduous first premolars and permanent first incisors are in place at 8 weeks of age.

1.3.3 Health, housing and feeding

Weak pups, or pups with ocular or nasal discharge were rejected. Pups that were judged healthy, and fit the age criterion, were transported to the Mammal Research Wing (MRW) of the MTZ Animal Health Center where they were housed with litter mates, in either stainless steel dog cages, or large newly-constructed pens, depending on their age, ambulatory status and maturity.

Some of the younger pups were bottle or syringe fed initially with KMR® milk replacer (Pet-Ag, Inc., Hampshire, Illinois), progressively thickened with rice cereal for babies (Rice Cereal®, H.J. Heinz Company of Canada Ltd., North York, Ontario). Once they were eating from a dish, they were quickly weaned, first onto a beef-based carnivore mix prepared at the MTZ, then onto commercial dry cat food. Most older pups relished the carnivore mix and eagerly accepted it from the start. All were weaned prior to their transfer to the Ontario Veterinary College's Isolation Unit (OVCIU) at 8 weeks of age. At the latter institution, animals were housed in pairs, in spacious stainless steel dog or primate cages. At both facilities, great care was given to the provision of toys, wading pools, boxes and tunnels, for environmental enrichment.

The OVCIU rooms are under a negative pressure gradient, and are equipped with airlock and anterooms. Foot baths are

used and protective clothing is worn by the attending personnel. Handling of animals between and within rooms progressed from the uninfected to the infected, if there were any. In the present experiment, there was no evidence of accidental infection (seroconversion) in unvaccinated animals.

During their stay at the MRW, usually soon after their arrival, pups were examined, sexed, weighed, ear-tagged, and blood was collected under isoflurane anesthesia (AErrane®, Ohmeda Pharmaceutical Products, Division of BOC Canada Ltd., Mississauga, Ontario, Canada) to harvest serum for determination of their maternal antibody status using virus neutralization (see below).

Fecal flotation in sodium nitrate allowed detection of *Capillaria* sp. ova in the feces of two litters, *Physaloptera* sp. ova in one litter, and coccidia in another litter. All raccoons were given ivermectin (Ivomec®, Merck Agvet, Merck and Co., Inc., Whitehouse Station, New Jersey, USA) 200 µg/kg subcutaneously. Raccoons from the litter in which coccidia were identified were administered a trimethoprim-sulfamethoxazole combination (Apo-Sulfatrim®, Apotex Inc., Toronto, Ontario, Canada) 30 mg/kg/day orally for 5 days. Subsequent fecal samples were consistently negative for ova or oocysts. Each pup was prophylactically given 1 ml of

killed feline panleukopenia vaccine subcutaneously (Fel-O-Vax PCT®, Ayerst Veterinary Laboratories, Division of Wyeth-Ayerst Canada Inc., Guelph, Ontario, Canada) using aseptic methods. Feline panleukopenia epidemics may be devastating and the disease has been identified in sick or dead wild raccoons found on the MTZ site.

1.3.4 Group allotment

Upon arrival at the OVCIU, raccoons with detectable antibodies (seropositive) were randomly allotted to Groups E and F until these groups were filled. Raccoons without detectable antibodies (seronegative) were allotted randomly to Groups A, B, and C. Unexpectedly, however, only 23 out of 65 raccoon pups were found to be seronegative and consequently, Group A (seronegative unvaccinated controls) numbered seven, and not eight, raccoons.

Unvaccinated control raccoons (Groups A and E) and raccoons to be vaccinated (Groups B, C, and F) were kept in different rooms (Rooms 1 and 2, respectively) in order to avoid exposure of controls to the vaccine virus.

Due to the shortage of antibody negative raccoon pups, animals in Group D were not chosen randomly. They were raccoons with initially low maternal antibody titers that had waned to undetectable levels by 14 weeks of age (2 weeks prior to their single experimental CD vaccination). Group D

pups were kept in Room 1 with Group A and E until the day they were 16 weeks of age, at which time they were transferred to Room 2 with the other vaccinates.

Four additional pairs of seropositive raccoons were kept in a third isolation ward (Room 3), to be used later in an *in vivo* virus propagation procedure and a pilot study of challenge virus dose (see challenge study below). Raccoons which were not used experimentally were donated to the Ontario Ministry of Natural Resources.

1.3.5 Restraint and routine procedures

All raccoons were handled weekly, from the day of arrival in the OVCIU at 8 weeks of age (study week 0) to the end of the follow-up period (20 or 24 weeks of age). Each week, they were anesthetized, visually examined, weighed, bled, and when indicated, given the MLV CD vaccine. They were given ivermectin and feline panleukopenia vaccine on the day of arrival and biweekly thereafter for 6 and 8 weeks, respectively.

At the OVCIU, anesthesia for all procedures was induced with a combination of ketamine hydrochloride (Ketaset®, Ayerst Laboratories, Division of Wyeth-Ayerst Canada Inc., Montreal, Quebec, Canada) and xylazine (Rompun®, Bayer Inc., Agriculture Division, Animal Health, Etobicoke, Ontario,

Canada), at the dose of 10 mg/kg and 2 mg/kg respectively, administered IM.

Blood was collected from the base of the jugular vein, as it enters the thoracic inlet, using a blind technique developed for this study. The angle created by the manubrium and the sternal attachment of the first rib was located by digital palpation. A 25 gauge needle attached to a 3 ml syringe was inserted 0.5 cm lateral to the manubrium and directed perpendicular to the skin surface. The vein was always found, at a depth that increased with age. When raccoons were older, a 23 gauge needle and a 6 ml syringe were used. This venipuncture site proved reliable for withdrawing up to 2 to 3 ml of blood, even on very small pups (the smallest pup admitted in the study weighed 420 g).

The blood samples were refrigerated, centrifuged, and the sera separated within 24 hours of collection. Each serum sample was divided in two equal volumes and frozen at -70^{0} C until submission to the laboratory.

1.3.6 Serology

Virus neutralization assays for the detection of CDV antibody were performed by the Animal Health Laboratory, University of Guelph in microtiter format using standard laboratory techniques (Mahy and Kangro, 1996), 100 $CCID_{50}$ Onderstepoort strain CDV, Vero cells and known positive and

negative control sera. Vero cells were grown in Earle's minimum essential media (EMEM) (Flow Laboratories, Mississauga, Ontario, Canada), supplemented with 1% non-essential amino acids (NEEA)(Grand Island Biological Company, Grand Island, New York, USA) and irradiated fetal bovine sera (Can Sera, Rexdale, Ontario, Canada). Sera were serially diluted two-fold in duplicate, with antibody titers determined as the 50% endpoint for cytopathic effect (CPE) after 5 days incubation at 37°C in 5% CO_2.

Seroconversion was defined as a change from negative to a positive antibody titer over the course of the experiment, with titers < 1/2 being considered as negative. Except for week 12 samples (week 8 for Group D), samples from the vaccination trial were all submitted at once, and run simultaneously to eliminate inherent between-batch test variation. Titers are reported as reciprocals of the end point dilution.

1.3.7 Disposition of raccoons

All but four raccoons were euthanatized by lethal intracardiac injection of T-61® (Hoeschst Canada Inc., Regina, Saskatchewan, Canada) 0.3 ml/kg while under ketamine-xylazine anesthesia. Four vaccinated raccoons were returned to the MTZ and introduced to the raccoon exhibit.

1.4 Vaccination

Galaxy D® (Solvay Animal Health, Inc., Kitchener, Ontario, Canada), a readily available monovalent commercial dog CD vaccine was used in this trial. It is an Onderstepoort strain CD MLV propagated in a Vero (green monkey) cell culture line. This choice was dictated mostly by Galaxy D®'s popularity among people working with exotic carnivores. This popularity, in turn, stems from the fact that Galaxy D® was used as a successor to Fromm D®, a widely used avianized Onderstepoort strain CD MLV vaccine that was withdrawn from the market.

Raccoons were aseptically inoculated subcutaneously (SC), between the shoulder blades, with 1 ml of reconstituted vaccine, as per the manufacturer's recommendations. Vaccine controls in Groups A and E were injected with 1 ml of sterile saline SC. Each week, the inoculation site was evaluated for the presence of local reaction.

2. Challenge study

2.1 Objectives

This second phase of the experiment was designed as a challenge study to ascertain whether the immune response to vaccination, encompassing the measured antibody response,

was associated with actual protection from clinical CD.

2.2 **Experimental design**

Sixteen raccoons with various titers of CDV neutralizing antibodies, and four seronegative controls were used. Raccoons were inoculated via the oculonasal route with a virulent raccoon CDV isolate and followed for a period of 42 days. Humoral response to challenge was assessed by measuring serum virus-neutralizing antibody titers at days 0, 3, 7, 10, 14, 21, 28, 35, and 42, or, the case being, when euthanatized earlier. The outcomes of interest were protection from clinical disease (as defined below), and survival to 42 days.

2.3 **Challenge virus**

2.3.1 Source

The virus received on September 16th, 1996 (Agriculture Canada permit # AH.1996.687) was a raccoon CDV isolate labeled as California Raccoon Isolate A92-27/14, and generously provided by Dr Max J.G. Appel of Cornell University in Ithaca, New York, as triturated tissue suspensions of raccoon lymph node, lung, and brain.

2.3.2 Virus amplification and pooling procedures

A pair of 21-week-old seronegative raccoons, housed in Room 3 of the OVCIU, were each administered 0.75 ml of California Raccoon CD virus tissue suspension (pool of lymph node, lung and brain) intravenously. Six days later, they were euthanatized. The submandibular, retropharyngeal, prescapular, axillary, inguinal, popliteal, mediastinal, tracheobronchial, mesenteric and sublumbar lymph nodes, the tonsils, the thymus and the spleen were aseptically collected immediately following euthanasia of each raccoon. These tissues were trimmed, placed in media, homogenized and centrifuged, pooled, further clarified by centrifugation, and aliquotted. For a more detailed description of tissue pooling and processing of sample homogenates, refer to **Appendix II**. Aliquots of neat (undiluted), 1:10, and 1:100 dilution of virus inocula were stored at -70^0 C until use.

2.3.3 Pilot study: challenge dose

The six remaining raccoons in Room 3 were seronegative when placed in individual cages at 24 weeks of age. Two raccoons were each administered 1 ml of neat CDV inoculate, that had been thawed on ice immediately before. The inoculum was administered as follows: five drops were placed in the conjunctival sac of each eye, five drops were instilled in

each vertically held nostril, and the remainder of the dose was sprayed onto the oropharyngeal mucosa. The procedure was repeated with the 1:10 and the 1:100 dilutions of inoculum, using two raccoons each. The raccoons that had received the neat inoculum were euthanatized 28 days post challenge (PC), showing classical signs of disease (cf. criteria for disease and euthanasia, below). Those that had received the 1:10 dilution of inoculum were also euthanatized, at 26 and 33 days PC respectively, with clinical CD. Both raccoons that were administered the 1:100 dilution of inoculum were euthanatized at 33 days PC, with one animal showing clinical CD. The carcasses were not necropsied.

2.4 **Experimental animals and procedures**

2.4.1 Choice of raccoons

The antibody titer of each raccoon was determined at the end of the vaccination clinical trial (20 weeks of age for Groups A, B, C, E, F, and 24 weeks of age for group D). Groups A and E (unvaccinated controls) were pooled and four raccoons were randomly selected to act as susceptible controls; they had no detectable antibody titer. All raccoons from vaccinated groups (B, C, D, and F) had measurable antibody titers. Four raccoons were randomly selected from each group, for a total of 16 vaccinated

raccoons, with various serum neutralizing antibody titers (1:12 to 1:384), to be used in the challenge. Raccoons were housed individually in stainless steel dog or primate cages, in the same isolation room in the OVCIU. All 20 raccoons, ranging from 7 to 8 months of age, entered the challenge study on November 11th, 1996.

2.4.2 Procedures

On day 0, all 20 raccoons were challenged with the neat (undiluted) virulent CDV inoculum administered as described in the pilot study. Blood was collected and processed on days 0, 3, 7, 10, 14, 21, 28, 35, and on day 42, at which time all surviving animals were euthanatized. Blood samples were submitted for serology. If it was necessary to euthanatize raccoons earlier due to their clinical condition, samples were collected immediately prior to euthanasia.

2.4.3 Observation methods

All raccoons were visually inspected on a daily basis by one of two observers who were both blinded to the vaccination status of the animals. Alertness, responsiveness and general demeanor were evaluated. Food consumption, as well as fecal output and consistency were also assessed. All animals were monitored for cutaneous erythema, pustules,

foot pad and muzzle skin thickening and depigmentation, ocular and nasal discharge, sneezing, coughing, and any behavioral abnormality or neurologic disturbance. Furthermore, animal caretakers were instructed to relay any observation that they deemed abnormal. Observations were entered daily in each raccoon's individual file. On days when blood was collected, anesthesia allowed the observer to conduct a closer physical inspection.

2.4.4 Criteria for disease and/or euthanasia

Criteria for disease and euthanasia were established prior to the challenge study. Anorexia, severe lethargy, ocular and/or nasal mucoid discharge, vomiting, diarrhea, pustules or blisters, and neurologic signs of any kind were deemed indicative of disease. Raccoons convincingly demonstrating any of the above clinical signs for more than 3 consecutive days, or sooner if deemed appropriate on humane grounds, were euthanatized. An animal observed seizuring was euthanatized that day.

2.4.5 Disposition of animals

A thorough necropsy was conducted on all animals immediately after death. Tissue samples (see below) were collected and fixed in 10% buffered formalin.

2.4.6 Histopathologic assessment

Tissues were routinely processed into wax and sectioned for histopathology. Sections of the cerebrum (one through the basal ganglia, one through the hypothalamus), cerebellum, medulla oblongata, tongue, esophagus, stomach, duodenum, jejunum, ileum, colon, liver, pancreas, tonsils, thymus, spleen, mesenteric lymph node, thyroid glands, adrenal gland, trachea, lung, kidney, urinary bladder, testis/ovary, uterus, footpad, eyelids, and planum nasale were stained with hematoxylin and eosin (modified from Armed Forces Institute of Pathology, 1968) and examined microscopically, with knowledge of the experimental group. The presence of characteristic intracytoplasmic and intranuclear acidophilic inclusion bodies in epithelial, lymphoid, or glial cells was considered diagnostic for CD, whether or not other compatible lesions were present.

2.5 **Statistical methods**

Serologic results were recorded as the reciprocal of the end point dilution (50% endpoint for CPE), and transformed to the $\log_2$. Individual raw and transformed data for the vaccination trial are tabulated in **Appendix III** and **Appendix IV**, respectively. Group means and standard errors of the means of transformed data are found in **Appendix V**.

Individual raw and transformed data for the challenge study are tabulated in **Appendix VI** and **Appendix VII**, respectively. Group means and standard errors of the means of transformed data are found in **Appendix VIII**. In the Results, data are presented in the form of graphs, depicting each group's geometric mean antibody titer over the period of the trial. These were constructed to facilitate visual assessment of the experimental data. In all graphs, titers are expressed as the $\log_2$ of the reciprocal of the dilution. Statistical analysis was performed with PC-SAS 6.12 for Windows (SAS Institute Inc., Cary, North Carolina, USA) using repeated measures ANOVA on experimental groups. For the survival data, Cox proportional hazards regression accounted for censoring (Collett, 1994).

The decay curve was established by standard least squares linear regression analysis of the data using the Corel Quattro Pro 7 analytical statistics package (Corel Corporation, Ottawa, Ontario, Canada). The slope was calculated using the equation: $y = \beta_0 + \beta_1 x$, where y is the antibody titer at time x, β_0 is the Y intercept and β_1 is the slope of the regression line.

Since sera collected from raccoons at 20 weeks of age (Groups A, B, C, E, and F) and 24 weeks of age (Group D) were not batch-tested with sera from earlier weeks of the

trial, they were not used in any longitudinal quantitative analysis of titers, but were used in comparative analysis between groups for the last week of the trial (cf. Discussion). The data of that final week are not displayed on the graphs for the same reasons.

RESULTS

Vaccination trial

1. Vaccine safety:

There were no discernible local nor systemic adverse reactions attributable to vaccination with Galaxy D. A total of 64 doses were administered in the trial.

2. Vaccine efficacy (Groups B, C, and D vs Group A) :

None of the initially seronegative unvaccinated control raccoons (Group A) developed a detectable serum CDV neutralizing antibody titer over the course of the trial, while all initially seronegative vaccinated raccoons (Groups B, C, and D) had measurable serum CDV neutralizing antibody titers by week 2 post vaccination (PV) ($P = 0.0001$) (**Figure 1**). Five of these 24 raccoons had very low titers by one week PV. In all raccoons, titers climbed abruptly between weeks 1 and 3 PV, and remained high (group means between 256 and 4,096) throughout the follow-up period.

3. Single vs multiple vaccination (Group B vs Group C):

There was no significant difference ($P > 0.05$) over the period of observation between the PV mean titer curves of initially seronegative eight-week-old raccoons vaccinated sequentially at 8, 12, and 16 weeks of age (Group B) and

those which received a single dose at 8 weeks of age (Group C) (**Figure 2**).

4. Immune response vs age of vaccination (Group C vs Group D):

There was no significant difference ($P > 0.05$) between the mean antibody titers following a single dose of vaccine in initially seronegative eight-week-old raccoons (Group C) and initially seronegative 16-week-old raccoons (Group D) over the period of observation (**Figure 3**).

5. Maternal antibody decay curve (Group E):

Half of the initially seropositive control eight-week-old raccoons (Group E) had no detectable antibody by 16 weeks of age, suggesting strongly that these were passively acquired maternal antibodies, rather than a product of active immunity. Standard least squares linear regression gave a slope of -0.663 with an intercept of 13.35 on the Y axis when the X axis is projected back to birth **(Figure 4)**. The half-life of maternal antibodies (the time it takes for the antibody level to be reduced by half) was estimated at 10.55 days.

6. Maternal antibodies and immune response (Group E vs Group F):

There was no significant difference ($P > 0.05$) between the mean titers of eight-week-old unvaccinated control raccoons with maternal antibodies (Group E) when compared to mean titers of eight-week-old raccoons with maternal antibodies vaccinated at 8, 12 and 16 weeks of age (Group F), until week 10 PV, or 2 weeks after the third vaccination at 16 weeks of age. From that point, vaccinates (Group F) developed significantly ($P = 0.0229$) higher titers than the controls over the remainder of the observation period (**Figure 5**).

7. Maternal antibody vs vaccine efficacy (B vs F):

Initially seronegative eight-week-old raccoons vaccinated at 8, 12, and 16 weeks of age (Group B) had higher mean PV titers to CD when compared to raccoons with maternal antibodies vaccinated with the same regime (Group F), from the third week PV onwards. The titers at 9, 10, 11, and 12 weeks PV (17, 18, 19 and 20 weeks of age) were significantly ($P = 0.0001$) higher in the former group (**Figure 6**).

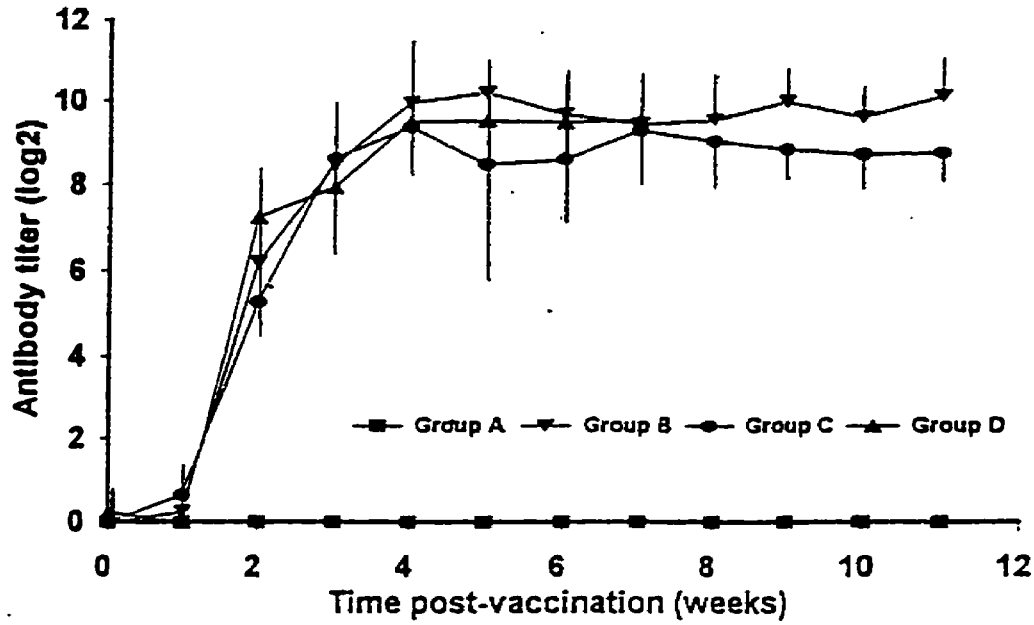


Figure 1. Antibody levels of initially seronegative raccoons following Galaxy D® vaccination using various protocols. Curves represent the geometric mean of $\log_2$ of the reciprocal of serum CDV neutralizing antibody titers of an experimental group of raccoons vs time (± SD). **Group A)** Seronegative eight-week-old unvaccinated control raccoons (n=7). **Group B)** Seronegative eight-week-old raccoons inoculated at 0, 4, and 8 weeks (8, 12, and 16 weeks of age) (n=8). **Group C)** Seronegative eight-week-old raccoons inoculated once at time 0 (8 weeks of age) (n=8). **Group D)** Seronegative sixteen-week-old raccoons inoculated once at time 0 (16 weeks of age) (n=8). The difference between vaccinates and controls was highly significant (P = 0.0001).

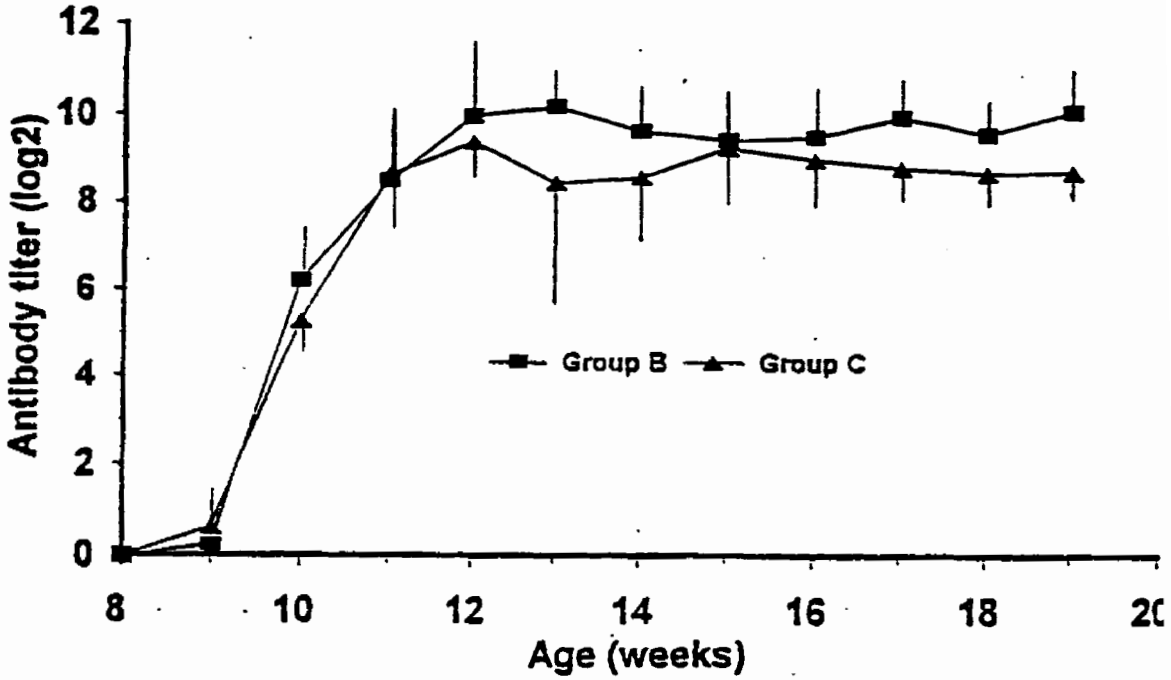


Figure 2. Antibody levels of raccoons following a single dose of Galaxy D® vaccine at 8 weeks of age (Group B) or three doses at 8, 12, and 16 weeks of age (Group C). Each curve represents the geometric mean of the $\log_2$ of the reciprocal of serum CDV neutralizing antibody titers of an experimental group of raccoons (n=8) vs age (± SD). No significant difference at any time between groups.

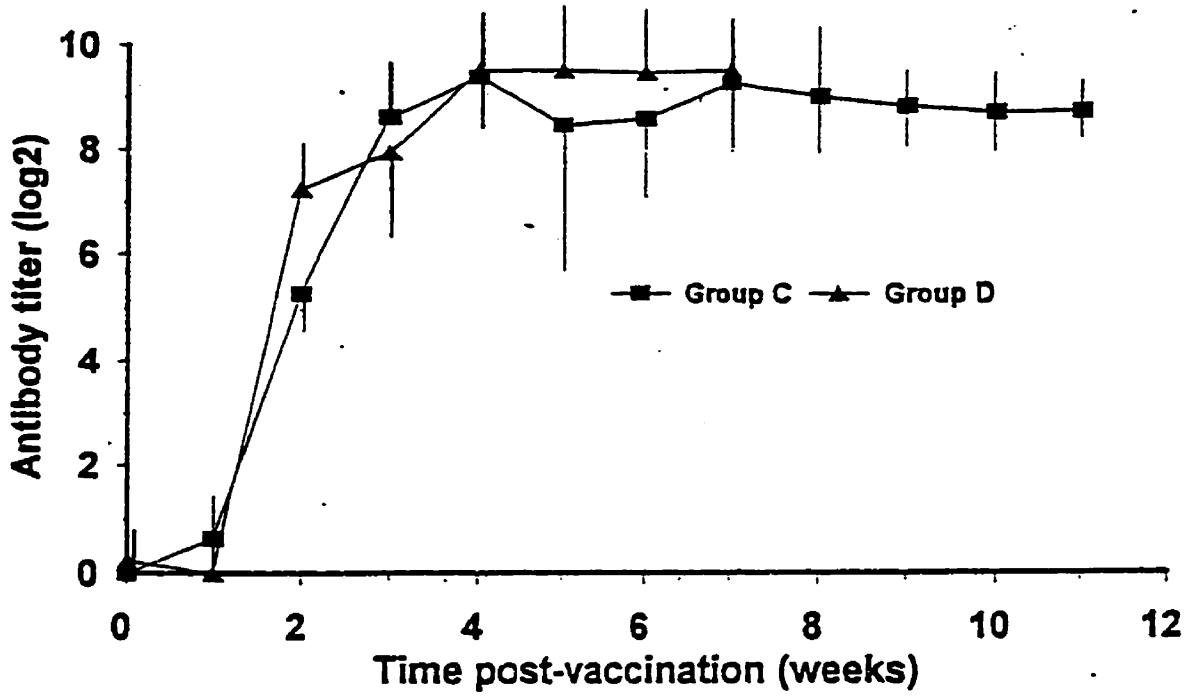


Figure 3. Effect of age at vaccination with Galaxy D® on antibody levels in initially seronegative raccoons vaccinated at 8 weeks of age (Group C) or 16 weeks of age (Group D). Each curve represents the geometric mean of the $\log_2$ of serum CDV neutralizing antibody titers of an experimental group of raccoons (n=8) plotted against time post vaccination (± SD). No significant difference at any time between groups.

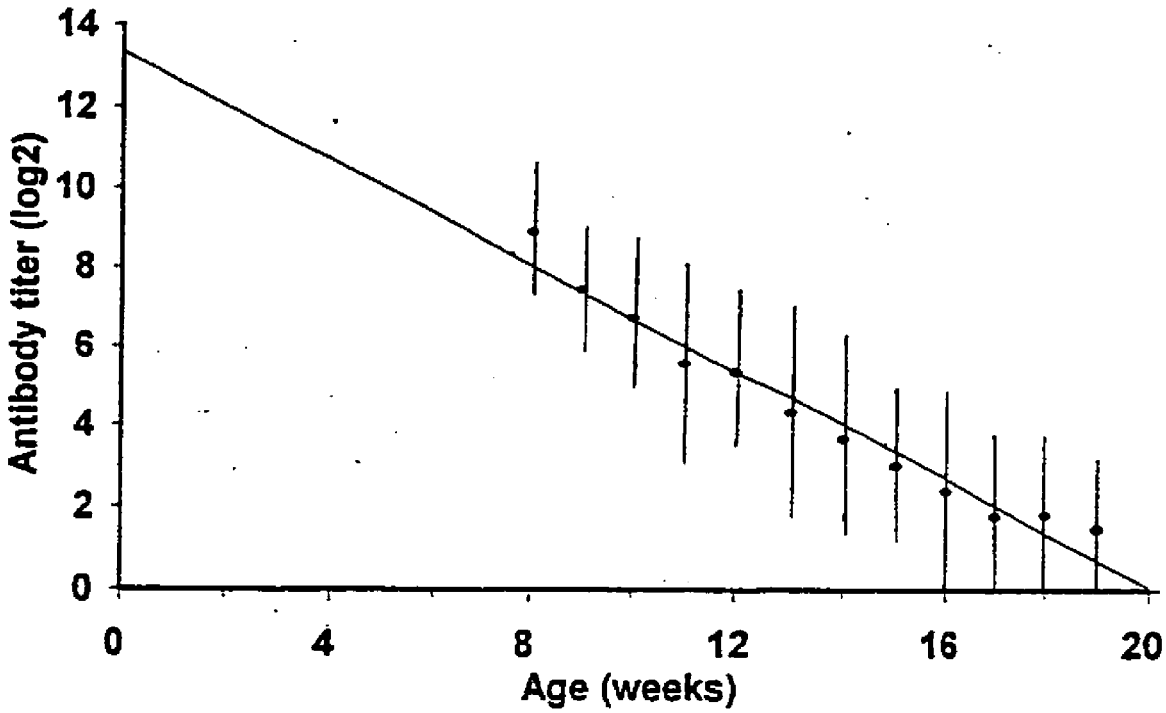


Figure 4. Maternal antibody decay curve in raccoons 8 to 20 weeks of age. The curve, with a slope of -0.663, represents a least squares linear regression line of best fit through the geometric means of the $\log_2$ of the reciprocal of serum CDV neutralizing antibody titers of Group E (n=8) plotted against age (± SD).

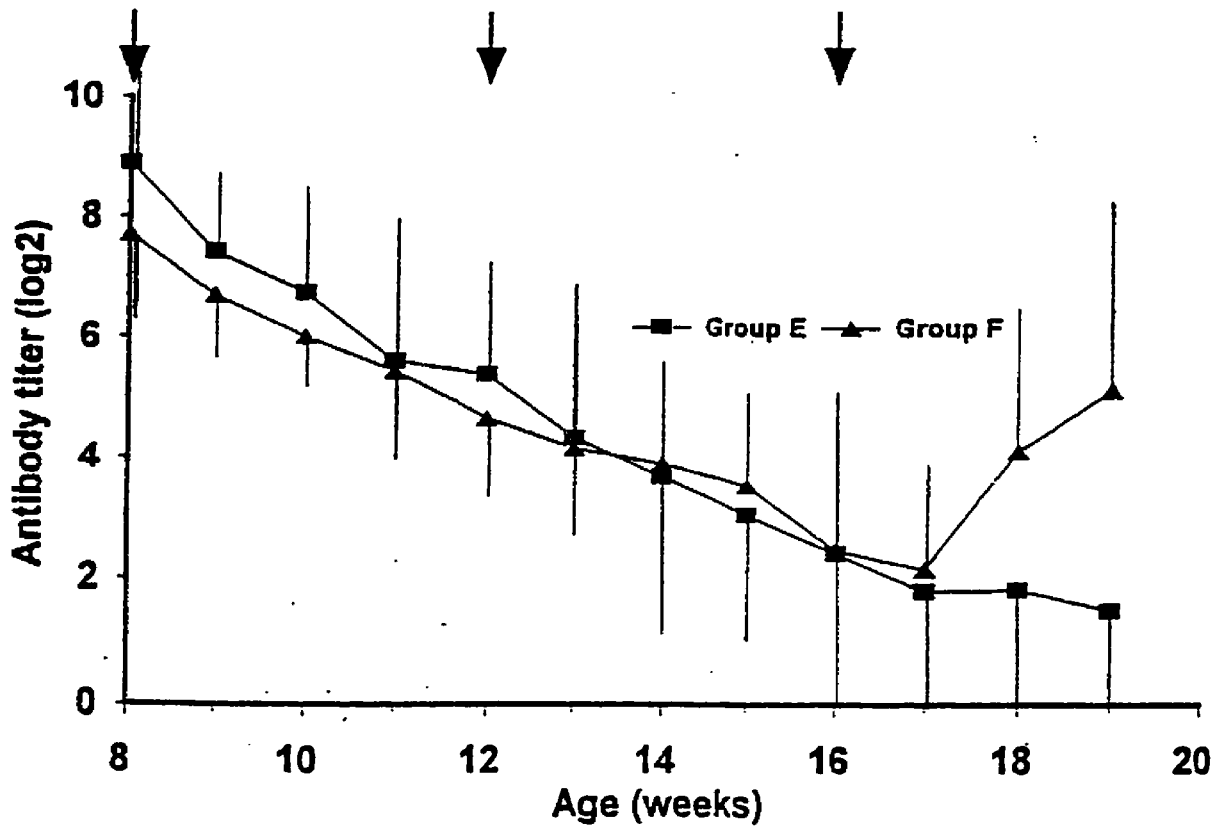


Figure 5. Antibody levels of raccoons with maternal antibodies following vaccination with Galaxy D® at 8, 12, and 16 weeks of age (Group F) compared with unvaccinated controls with maternal antibodies (Group E). Each curve represents the geometric mean of $\log_2$ of the reciprocal of serum CDV neutralizing antibody titers of an experimental group of raccoons (n=8) plotted against age (± SD) (↓=vaccination). Mean curves for weeks 17 to 20 differ significantly (P = 0.0229) (data for week 20 not shown).

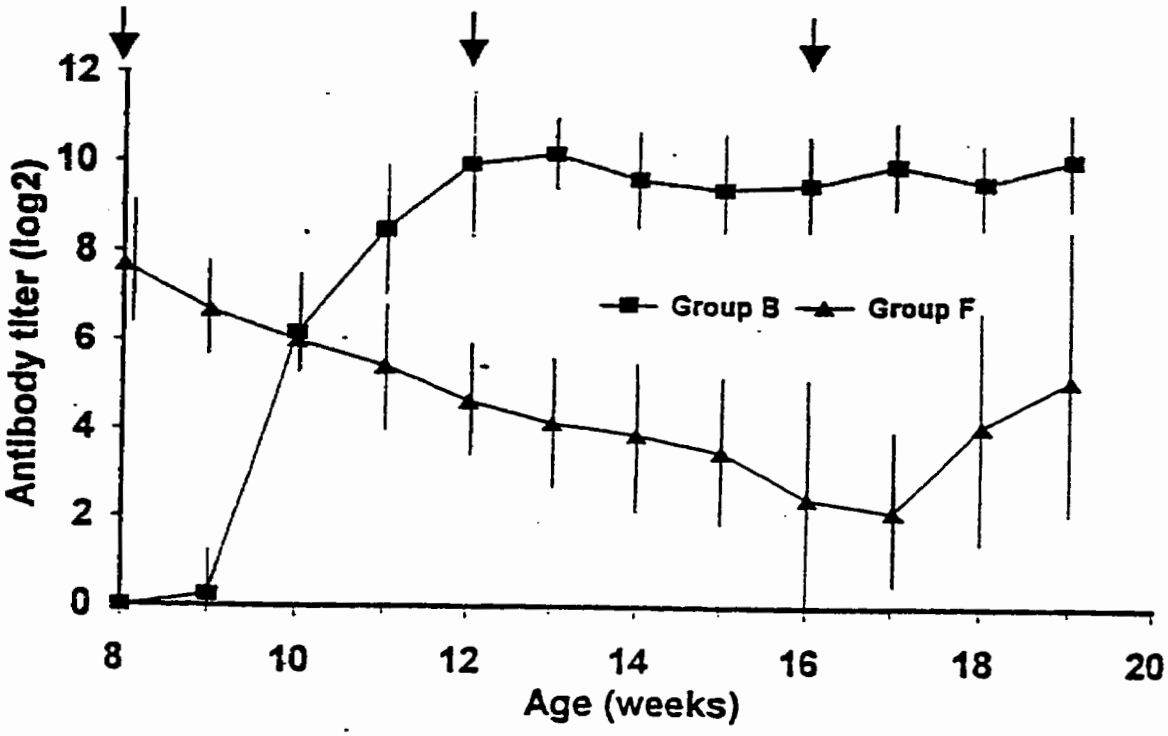


Figure 6. Antibody levels of initially seronegative raccoons vaccinated with Galaxy D® at 8, 12, and 16 weeks of age (Group B) compared with those in raccoons with maternal antibodies vaccinated at 8, 12, and 16 weeks of age (Group F). Each curve represents the geometric mean of the $\log_2$ of the reciprocal of serum CDV neutralizing antibody titers of an experimental group of raccoons (n=8) plotted against age (± SD) (↓=vaccination). Group means differ significantly (P = .0001) on weeks 17, 18, 19, and 20 (data for week 20 not shown).

Challenge study

1.Vaccine protection

All of the 16 vaccinated raccoons survived the challenge, and none met the criteria defined for clinical disease over the 42 day observation period. These raccoons experienced a significant rise ($P < 0.05$) in their antibody titer levels between challenge (day 0) and day 10 post challenge (PC) (**Figure 7**).

Three out of four seronegative controls developed disease on days 29, 30, and 30 post challenge (PC), and were euthanatized on days 33, 33, and 30 respectively. The first two animals were depressed, and had bilateral ocular and nasal mucopurulent discharge and crusting. The third was euthanatized because of sudden onset of seizures. These three raccoons never developed a detectable serum CDV neutralizing antibody titer.

The fourth seronegative control raccoon demonstrated vague signs of illness (inappetence, lethargy, cutaneous erythema) on days 21, 22, and 29 but survived the challenge and appeared clinically normal 42 days PC. In this individual, demonstrable serum antibody titers appeared at 21 days PC and climbed slowly over the remainder of the observation period (**Figure 7**).

There was a statistically significant ($P = 0.0008$)

protective effect of the vaccine against clinical disease and death from canine distemper. A survival curve, illustrating time to disease, summarizes the outcome of the challenge study (**Figure 8**).

2.Postmortem findings

Two of the three raccoons that were euthanatized due to disease had bilateral mucopurulent blepharoconjunctivitis and rhinitis. One developed depigmentation of the muzzle and footpads, while these were hyperkeratotic in the other. Histologically, in both animals, intracytoplasmic and intranuclear acidophilic inclusion bodies were identified in epithelial cells of the bladder, bronchioles, epidermis of the footpad, tongue, meibomian glands, and seminiferous tubules. Inclusions were also observed in splenic reticuloendothelial cells of one raccoon. Moderate interstitial pneumonia and suppurative bronchiolitis were observed in the lungs of both animals. Parakeratotic hyperkeratosis with multifocal necrosis in the stratum spinosum and a mild interface lymphoid infiltrate characterized the epidermis of the foot pads. Necrosis in the meibomian glands was associated with severe secondary bacterial invasion. Lymphoid depletion ranged from moderate in the spleen to severe in the mesenteric lymph node.

The third raccoon, euthanatized because of seizures, had a mild bilateral conjunctivitis. There were no abnormalities on internal examination at necropsy. Microscopically, intranuclear inclusion bodies were identified in the hippocampal neurons and in the epithelial cells of the bladder mucosa, with no associated inflammation.

The only unvaccinated raccoon that survived the challenge had no obvious external gross lesions upon necropsy at 42 days PC, but a chronic cystitis, with a thick, rugose, hyperemic bladder wall, and pyuria was noted internally. Histologically, a single but conspicuous focus of non-suppurative encephalitis was observed in the medulla. There was malacia and glial activation with moderate perivascular cuffing. Inclusion bodies were readily identifiable in surrounding neurons. Other histologic findings included mild to moderate interstitial pneumonia, chronic cystitis, mild focal interstitial nephritis, and mild diffuse lymphoplasmacytic infiltration of the nasal submucosa. Lymphoid follicles of the mesenteric lymph nodes were populated with blastic lymphoid cells.

There were very few gross external or internal lesions in the 16 vaccinated raccoons. These were limited to mild adhesions of the ventral left apical pulmonary lobe to the pericardium in one raccoon, and mild patchy atelectasis in

two others. Histologically, 10 of 16 raccoons had mild to moderate, patchy or diffuse interstitial pneumonia, but inclusion bodies were not detected.

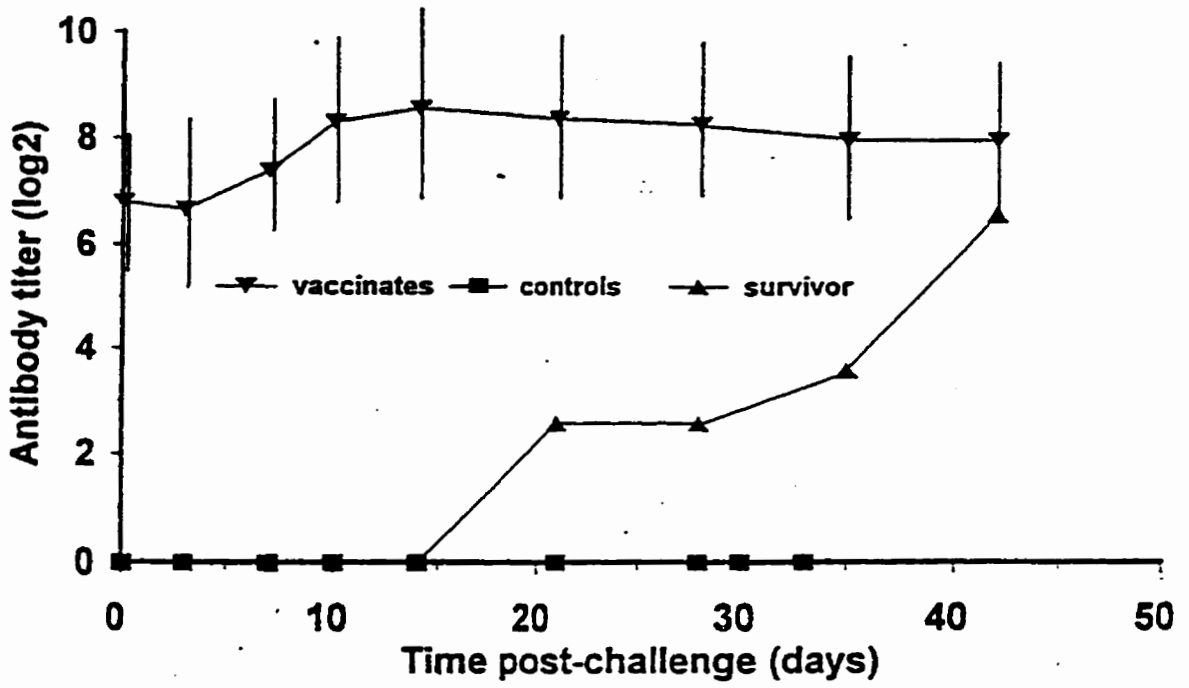


Figure 7. Antibody levels in vaccinated (n=16), euthanatized unvaccinated control (n=3), and surviving control (n=1) raccoons following challenge with CDV. Each curve represents the geometric mean of the $\log_2$ of the reciprocal of serum CDV neutralizing antibody titers of an experimental group of raccoons plotted against time post challenge (± SD).

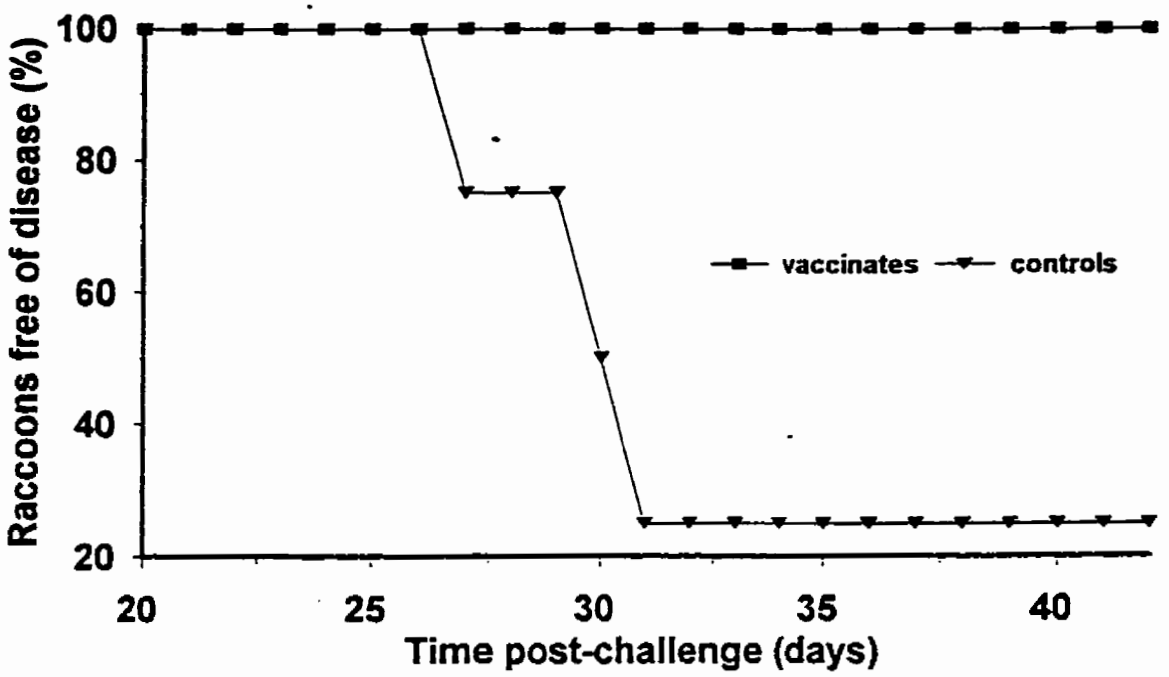


Figure 8. Survival of vaccinated (n=16) and unvaccinated control (n=4) raccoons following challenge with virulent CD virus. Time-to-event survival curve where the event is presence of CD clinical signs. The curve represents the proportion of raccoons in each group that remained free of clinical signs over the observation period of 42 days.

DISCUSSION

In this study, there were no local or systemic adverse reactions observed in 32 vaccinated raccoons (for a total of 64 doses) that could be attributed to the use of Galaxy D®. This relates to the issue of safety. It is in agreement with the absence of detectable adverse reaction through years of clinical use of this vaccine in raccoons at the MTZ and elsewhere, and suggests, but does not establish, sufficient attenuation of this vaccinal strain for use in raccoons.

The vaccine proved effective in promoting a humoral response, in that all initially seronegative raccoons developed measurable serum CDV neutralizing antibody titers by the second week PV. Titers then climbed abruptly, reaching a plateau at 4 weeks PV, after which time group mean titers were sustained in the range of 256 to 2,048 throughout the period of observation. Antibody response in this trial is consistent with investigations of humoral response to MLV vaccination in other species (Rockborn et al., 1965; Halbrooks et al., 1981; Montali et al., 1983; Hoover et al., 1989; Cooper et al., 1991; Goodrich et al., 1994; Williams et al., 1996).

While stress of repeated anesthesia, unavoidable for the purpose of bleeding the animals, conceivably could have affected the time of onset and/or the magnitude of the

humoral response, such an effect would not preclude comparisons between groups, since they were all treated in the same manner. A similar assumption can be made regarding the concurrent use of inactivated feline panleukopenia vaccine given to all raccoons in the trial. Concurrent use of vaccines reflects the field situation, in which such practice is common, as is worming with ivermectin.

A "protective" antibody titer cannot be determined in raccoons due to inter-laboratory variations in methodology and variations in dose and virulence of field virus, nor can it be extrapolated from studies in other species for the same reasons. Therefore it was not known, prior to the challenge study, if or how antibody titers observed in this vaccination trial correlated with true protection from disease.

Antibody titers in a single serum sample may vary considerably with the test, operator and time of testing, despite standardization of protocols within a laboratory. This phenomenon is exemplified in the titers from the final week of the vaccination trial (week 12 for Groups A, B, C, E, and F, and week 8 for Group D) in Appendix IV. These were run at a different time and yielded uniformly lower titers than the remainder of the sera. They were excluded from the graphs. This serves to emphasize the importance of running samples from any experiment simultaneously to avoid such

confounding variations and underlines the inappropriateness of extrapolating antibody titer levels deemed protective in any other study, even within the same species. However, in trying to compare different vaccination schedules for the purpose of determining which is the most effective, it is reasonable to assume that the higher the titer, the better.

There was no significant difference, in terms of titer levels, between single and multiple vaccinations in seronegative pups. Eight-week-old pups appeared just as capable of responding vaccination as 16-week-old pups. Raccoons are probably capable of mounting an immune response to CDV very early in life, as is the case in the dog (one day of age) and ferret (8 days of age) (Appel and Gillespie, 1972; Ott and Gorham, 1955; Ott et al., 1965).

In this experiment, maternal antibodies in all seropositive control raccoon pups declined gradually to negligible levels by the time they had reached 20 weeks of age. Four of eight pups had undetectable levels by 17 weeks of age. The half-life of maternal antibodies was estimated to be about 10.55 days. Similar but slightly shorter half-lives for decay of maternal antibodies against CDV have been documented in dogs (8.5 days) and in ferrets (9.4 days) (Gillespie et al., 1958; Appel and Harris, 1988). This decay reflects normal protein catabolism and is comparable for various antibodies against other pathogens (e.g. 9.7 days

for parvovirus antibodies in dogs (Pollock and Carmichael, 1982).

The present trial demonstrates that maternal antibodies will nullify or seriously interfere with active immunization in eight-week-old raccoon pups until they reach 14 to 16 weeks of age. Examination of individual data from raccoons of Group F (initially seropositive raccoons, vaccinated at 8, 12, and 16 weeks of age) reveals differences in the ability of pups to respond to vaccination in the presence of maternal antibodies. Vaccination failed to elicit a humoral response before the third vaccination (16 weeks of age) in all but one of the eight raccoons that possessed maternal antibodies (Group F). The exception (# 50), one of the three with the lowest titer at 12 weeks of age, experienced a rise in antibody titer two weeks after the second vaccination, at 12 weeks of age (Appendix IV). Two raccoons (#3 and #51), one of them (#51) a litter mate of # 50, with a similar titer at the time of the second vaccination, failed to respond until 16 weeks of age. For undetermined reasons, one raccoon from the same group (#18) did not respond to the third vaccine dose, in spite of a relatively low titer at 16 weeks of age. This raccoon could have benefitted from a fourth dose at 18 to 20 weeks of age.

Therefore, even within this experiment, it is difficult to determine a threshold antibody titer below which an

animal's maternal antibodies will not interfere with vaccination. From Figure 5, it appears that a titer of 32, but not 8, will nullify any vaccination attempt. However, in the field the immune status of a raccoon pup is rarely, if ever, known. It is of note that all pups collected from Scarborough, in which CD is endemic (Cranfield et al., 1984; Rosatte et al., 1991; Schubert-Kuehner, 1995), had maternal antibodies. Results from this study suggest that a vaccination protocol extending to 16 or 20 weeks of age for any raccoon under 16 weeks of age is prudent, particularly in a CD-endemic area.

Modified-live virus vaccines stimulate CMI, the second arm of the immune response, which acts in concert with the components of the humoral immune system to protect against CDV infection and disease (Krakowka and Wallace, 1979; Appel et al., 1982; Appel, 1987; Greene, 1990). Cell-mediated immunity was not measured in the present study and represents an unknown variable. Discussion of the challenge study will therefore address protection in relation to the humoral response, while acknowledging that part or possibly most of the protection imparted by vaccination may have been associated with CMI.

Ultimately, the true efficacy of a vaccine is best assessed by means of a challenge study. Although ideal, a field trial with field exposure would be logistically

complicated to conduct, so that challenge in a controlled environment was elected. In this experiment, all 16 vaccinated raccoons, with titers at challenge ranging from 12 to 384, were protected in that they remained free of clinical signs of disease over a 42 day follow-up period, and none had gross or microscopic lesions of CD when euthanatized at that time.

Fourteen of these sixteen raccoons experienced a rise in antibody titer in the 10 days following challenge. While in many animals this increase was slight, in others it was substantial. In raccoon #16, the titer climbed from 12 on the day of challenge to 1,536 on day 10 PC, demonstrating a strong anamnestic response. This particular animal had developed a relatively weak immune response by the end of the vaccination trial (Appendices IV and V).

Conversely, three of four unvaccinated seronegative animals developed clinical signs characteristic of CD that were severe enough to warrant euthanasia. These three animals never developed serum neutralizing antibody titers and typical CD inclusion bodies were observed histologically in their tissues. This is in accordance with the accepted concept of CD pathogenesis, in which an early vigorous humoral response is mandatory for recovery from distemper infection (Appel, 1987).

The fourth control raccoon developed equivocal, non-

specific signs of illness during the observation period, but appeared clinically normal when euthanatized at the end of the study. This raccoon had a sluggish humoral response to challenge. Measurable but low titers (1:6) were first detected at 21 days PC and climbed very slowly (see Figure 7). At necropsy, this raccoon had cystitis with pyuria, common in raccoons with CD (Monson and Stone, 1976). More convincingly, focal non-suppurative encephalitis, with typical CD inclusion bodies in glial cells, was identified histologically. Low-grade or sluggish humoral responses are often associated with chronic progressive encephalitis in CD-infected animals (Appel, 1987). It is likely that this raccoon would have developed neurological signs over a longer period, although recovery of animals such as this one is not impossible (Appel, 1987).

While only three of four control raccoons developed clinical signs over the 42 day follow-up period, all four had disease due to CD. Statistically significant protection from clinical or subclinical infection was therefore achieved with Galaxy D® (0/16 vs 4/4).

The only inferences pertaining to duration of protection that can be made from this study relate to the date at which the challenge study was conducted. In challenged raccoons, the time elapsed between the last CD vaccination in the vaccination trial and the day of

challenge ranged from 13 to 23 weeks. Raccoons #5, #8, and #11, vaccinated only once at eight weeks of age, did not develop CD when challenged 23, 22 and 22 weeks later respectively, suggesting that a single vaccination in a seronegative pup confers protection for a minimum of five and a half months. This is consistent with the notion that immunity conferred by MLV CD vaccines is long-lasting (Appel, 1987).

In retrospect a higher number of unvaccinated controls would have been desirable in the challenge study. On humane grounds, the minimum of animals needed to achieve reasonable statistical power should be used. Since two animals inoculated with a 1:10 dilution of stock challenge virus, as well as both animals receiving the undiluted inoculum, developed clinical CD during the pilot trial to determine dose and confirm virulence of the challenge inoculum, it was assumed that all four challenge controls inoculated with undiluted tissue suspension would also develop clinical signs, and therefore the size of the control group was deemed adequate.

Interstitial pneumonia of mild to moderate intensity was identified histologically in many challenged vaccinated raccoons. However, bronchiolitis characteristic of CD in raccoons was not seen and never were any inclusion bodies found. An etiology was not ascertained. It is conceivable

that these lesions are the result of replication of the challenge virus in lung lymphoid tissue prior to its elimination. Interstitial pneumonia of the type seen in this trial is common in wild raccoons that have died from a variety of causes other than CD (I.K. Barker, personal communication).

Based on the results of this study, a CD vaccination schedule for raccoons consisting of sequential monthly inoculations with a MLV CD vaccine, and starting at 6 or 8 weeks of age, can be recommended. It would appear unsafe to terminate such a schedule before 16 weeks of age since, in some raccoon pups, maternal antibodies may still be present at high enough concentration to neutralize the vaccine.

Although all seropositive pups were from wild unvaccinated mothers, some had very high titers (1:1536) when initially tested at 4 to 6 weeks of age, suggesting that the antibody titer in their dam was even higher (Gillespie et al., 1958). This, in turn, corroborates the observation that CDV is circulating in the Scarborough and Barrie populations of free-ranging raccoons. By inference, it also reflects a relatively high rate of survival following CD infection in raccoons, with a theoretically lifelong protection from reinfection.

In addition to naive adults, the susceptible subsets of raccoons in an endemic population would consist of animals

born to naive dams, plus juvenile raccoons 16 weeks or older in which maternal immunity has waned, leaving them vulnerable to infection. A TVR program would ideally target these population subsets, but realistically such an objective is difficult to achieve in a field situation. Multiple vaccinations are also impractical in TVR programs for free-ranging raccoons, but are essential in raccoon orphanages or rehabilitation centers when dealing with young animals of unknown antibody status, since maternal immunity is likely to interfere with successful vaccination and the risk of CDV transmission is high due to population density and conditions of rearing. Provided animals involved in a TVR program are old enough to be free of maternal antibodies, a single vaccination with Galaxy D® appears to be adequate to protect against clinical disease.

The results obtained in this work may shed some light on possible explanations for the occurrence of CD in vaccinated raccoons (see Appendix I). The exact duration of vaccinal immunity remains speculative in the dog in spite of the volume of work that has been done on CD in this species. Duration of vaccinal protection is unknown in raccoons but data from this study suggests that a single vaccination protects for at least 5 months, so that poor persistence of active resistance can hardly explain the apparent failure of vaccination in Cases 1 and 3. Stress and immunosuppression,

undetected concurrent predisposing infections, or overwhelming infective doses may have been contributing factors. In Case 3, vaccine-induced CD cannot be ruled out but is less likely. The raccoon in Case 1 may have been incubating the disease when vaccinated. Another likely scenario, in Case 3, is interference with vaccine response due to maternal antibodies in these two raccoons from known endemic areas. Even three sequential vaccinations may be ineffective if the last one is given before 16 weeks of age.

There is considerable variation in the size of 12-week-old raccoons (1.45 kg to 3.6 kg in this study), and some may appear older than they really are. Pattern of tooth eruption may vary, and the exact day of eruption may not be identifiable when pups are examined weekly, so that age estimation using this criterion carries a margin of error (Montgomery, 1964). Because litters in this study were collected from the wild and the exact date of birth was not known, it was not possible to compare the pattern of dental eruption between litters, although it certainly was consistent among pups within each litter. This contrasts with weight, the second criterion, which varied considerably within litters and appeared to be inversely related to the size of the litter, making it a much less reliable means of estimating age. All raccoons in this study had their permanent canines by 16 weeks of age, so this may be used as

a rough guide in assessing a juvenile raccoon's minimum age and, hence, its possible need for a second vaccination at a later date.

CONCLUSION

Vaccination of raccoons with Galaxy D® did not cause any adverse reaction and, within the limits of the number of animals used, appears safe. It also proved efficacious in providing full protection against challenge. Multiple vaccinations did not result in higher serum CD neutralizing antibody titers than a single vaccination, but since the immune status of raccoon pups is rarely known, a protocol consisting of serial monthly vaccinations is recommended in raccoons 16 weeks of age and younger to circumvent possible interference by maternal antibodies. Eight-week-old CD antibody-negative raccoon pups were fully capable of responding to vaccination with Galaxy D® and their humoral response did not differ from that of 16-week-old raccoons. Maternal passive protection had waned to negligible levels by 20 weeks of age. The half-life of maternal antibodies was estimated at 10.55 days.

It is a fundamental responsibility of managers to reduce any risk of transmission of infectious disease to a collection of captive animals. It is incumbent upon zoos to direct every possible effort towards that goal, more so since the future of some endangered species is increasingly linked to zoological institutions. In recent decades, the

ubiquitous raccoon has emerged as an important wildlife reservoir for canine distemper. The work reported here is a significant step towards a better understanding of CD prophylaxis in this species. It offers some guidelines as to when to vaccinate and documents protection conferred by vaccination with Galaxy *D*®. Such knowledge should be applied to sound management of free-ranging raccoons on zoo sites. Data from this thesis will also be helpful to researchers working with raccoons as experimental animals, as well as rehabilitators striving to impart solid vaccinal protection to the animals in their care.

LITERATURE CITED

Alexander, K.A., P.W. Kat, L.G. Frank, K.E. Holekamp, L. Smale, C. House, and M.J.G. Appel. 1995. Evidence of canine distemper virus infection among free-ranging spotted hyenas (*Crocuta crocuta*) in the Masai Mara, Kenya. Journal of Zoo and Wildlife Medicine 26: 201-206.

Appel, M.J.G. 1969. Pathogenesis of canine distemper. American Journal of Veterinary Research 30: 1167-1182.

Appel, M.J.G. 1987. Canine distemper virus. *In* Virus Infections of Carnivores, M.J. Appel (editor). Elsevier Science Publishers B.V., New York, New York, pp. 133-159.

Appel, M.J.G., and J.H. Gillespie. 1972. Canine distemper virus. *In* Virology Monographs, S. Gard, C. Hallauer, and K.F. Meyer (editors). Springer-Verlag, New York-Wien, pp. 1-96.

Appel, M.J.G., B.E. Sheffy, D.H. Percy, and J.M. Gaskin. 1974. Canine distemper virus in domesticated cats and pigs. American Journal of Veterinary Research 35: 803-806.

Appel, M.J.G., E.P.J. Gibbs, S.J. Martin, V. ter Meulen, B.K. Rima, J.R. Stephenson, and W.P. Taylor. 1981. Morbillivirus diseases of animal and man. *In*

Comparative Diagnosis of Viral Diseases IV. E. Kurstack, and C. Kurstack (editors). Academic Press, New York, New York, pp. 235-297.

Appel, M.J.G., W.R. Shek, and B.A. Summers. 1982. Lymphocyte-mediated immune cytotoxicity in dogs infected with virulent canine distemper virus. Infection and Immunity 37: 592-600.

Appel, M.J.G., W.R. Shek, H. Shesberadaran, and E. Norrby. 1984. Measles virus and inactivated canine distemper virus induce incomplete immunity to canine distemper. Archives of Virology 82: 73-82.

Appel, M.J.G., and W.V. Harris. 1988. Antibody titers in domestic ferret jills and their kits to canine distemper virus vaccine. Journal of the American Veterinary Medical Association 193: 332-333.

Appel, M.J.G., C. Reggiardo, B.A. Summers, S. Pearce-Kelling, C.J. Maré, T.H. Noon, R.E. Reed, J.N. Shively, and C. Örvell. 1991. Canine distemper virus infection and encephalitis in javelinas (collared peccaries). Archives of Virology 119: 147-152.

Appel, M.J.G., and R.J. Montali. 1994. Canine distemper and emerging morbillivirus diseases in exotic species. Proceedings of the American Association of Zoo Veterinarians, pp. 336-339.

Appel, M.J.G., R.A. Yates, G.L. Foley, J.J. Bernstein, S.

Santinelli, L.H. Spelman, L.D. Miller, L.H. Arp, M. Anderson, M. Barr, S. Pearce-Kelling, and B.A. Summers. 1994. Canine distemper epizootic in lions, tigers, and leopards in North America. Journal of Veterinary Diagnostic Investigation 6: 277-288.

Armed Forces Institute of Pathology. 1968. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, Third Edition, L.G. Luna (editor). The Blackiston Division, McGraw-Hill Book Company, New York, New York, USA, 258 pp.

Armstrong, W.H., and C.H. Anthony. 1942. An epizootic of canine distemper in a zoological park. Cornell Veterinarian 32: 286-288.

Baker, J.A., D.S. Robson, and J.H. Gillespie. 1959. A nomograph that predicts the age to vaccinate puppies against distemper. Cornell Veterinarian 49: 158-167.

Baumgärtner, W., R.W. Boyce, S.E. Weisbrode, S. Alldinger, M.K. Axthelm, and S. Krakowka. 1995. Histologic and immunocytochemical characterization of canine distemper-associated metaphyseal bone lesions in young dogs following experimental infection. Veterinary Pathology 32: 702-709.

Bittle, J.L. 1993. Use of vaccines in exotic animals. Journal of Zoo and Wildlife Medicine 24: 352-356.

Budd, J. Distemper. 1981. *In* Infectious Diseases of Wild

Mammals, Davis, J.W., L.H. Karstad, and D.O. Trainer (editors). The Iowa State University Press, Ames, Iowa, pp. 31-44.

Bush, M., R.J. Montali, D. Brownstein, A.E. James Jr, and M.J.G. Appel. 1976. Vaccine-induced canine distemper in a lesser panda. Journal of the American Veterinary Medical Association 169: 959-960.

Bush, M., and M. Roberts. 1977. Distemper in captive red pandas. International Zoo Yearbook 17: 194-196.

Carpenter, J.W., M.J.G. Appel, R.C. Erickson, and M.N. Novilla. 1976. Fatal vaccine-induced canine distemper virus infection in black-footed ferrets. Journal of the American Veterinary Medical Association 169: 961-964.

Carré, H. 1905. Sur la maladie des jeunes chiens. Compte Rendu de l'Académie des Sciences 140: 689-690, 1489-1491.

Chappuis, G. 1994. La maladie de Carré. Recueil de Médecine Vétérinaire 170: 645-652.

Chappuis, G. 1995. Control of canine distemper. Veterinary Microbiology 44: 351-358.

Collett, D. 1994. Modelling Survival Data in Medical Research. Chapman & Hall, New York, New York, USA, 347 pp.

Cooper, P.E., G. Chappuis, A.L. Saint-Gerand, and C .Duret. Comparaison de l'efficacité de différents vaccins du

chien, utilisés sous forme monovalente ou associée, par évaluation des réponses sérologiques et après épreuves virulentes 12, 22, et 26 mois après vaccination. Bulletin de la Société des Vétérinaires Praticiens de France 75: 131-152.

Cranfield M.R., I.K. Barker, K.G. Mehren, and W.A. Rapley. 1984. Canine distemper in wild raccoons (*Procyon lotor*) at the Metropolitan Toronto Zoo. Canadian Veterinary Journal 25: 63-66.

Crook, E., J.R. Gorham, and S.H. McNutt. 1958. Experimental distemper in mink and ferrets. I. Pathogenesis. American Journal of Veterinary Research 19: 955-957.

Davidson, M. 1986. Canine distemper virus infection in the domestic ferret. Compendium on Continuing Education for the Practicing Veterinarian 8: 448-453.

Davis, H.B. 1907. The raccoon: a study in animal intelligence. American Journal of Psychology 18: 447-489.

de la Torre, S. 1989. Closantel: effectivo en tratamiento del moquillo canino. Veterinaria Argentina 6: 470-474.

De Vries, P., F.G.C.M. Uytdehaag, and A.D.M.E. Osterhaus. 1988. Canine distemper virus (CDV) immune-stimulating complexes (Iscoms), but not measles iscoms, protect dogs against CDV infection. Journal of General Virology

69: 2071-2083.

Diters, R.W., and S. W. Nielsen. 1978. Toxoplasmosis, distemper, and herpesvirus infection in a skunk *(Mephitis mephitis)*. Journal of Wildlife Diseases 14: 132-136.

Dungworth, D.L. 1993. The respiratory system. *In* Pathology of Domestic Animals, Fourth Edition, Volume 2, K.V.F. Jubb, P.C. Kennedy, and N. Palmer (editors). Academic Press, Inc., Harcourt Brace Jovanovich, Publishers, Toronto, Canada, pp. 539-699.

Erken, A.H.M., and E.F. Jacobi. 1972. Successful breeding of lesser panda and loss through inoculation. Bijdragen tot de Dierkunde 42: 92-95.

Eulenberger, Von K., P. Müller, K-F. Schüppel, and V. Pohle. Hundestaupe bei Kleinen Pandas *(Ailurus fulgens)* im zoologischen garten Leipzig. 1993. Verhandlungsbericht des Internationalen Symposiums uber die Erkrankungen der Zootiere 35: 79-82.

Evans, R.H. 1984. Studies of a virus in a biological system: naturally occurring and experimental canine distemper in the raccoon (*Procyon lotor*). MSc thesis, Southern Illinois University, 135 pp.

Fenner, F. 1976. Paramyxoviridae. Intervirology 7: 59-60.

Fix, A.S., D.P. Riordan, H.T. Hill, M.A. Gill, and M.B.

Evans. 1989. Feline panleukopenia virus and subsequent canine distemper infection in two snow leopards. Journal of Zoo and Wildlife Medicine 20: 273-281.

Fowler, M.E. 1986. Carnivora. *In* Zoo and Wild Animal Medicine, M.E. Fowler (editor). W. B. Saunders Company, Philadelphia, Pennsylvania, pp. 799-882.

Fox, H. 1923. Diseases in Captive Wild Mammals and Birds. J.B. Lipincott Company, Philadelphia, Pennsylvania, pp. 599-600.

Gillespie, J.H. 1965. A study of inactivated distemper virus in the dog. Cornell Veterinarian 55: 3-8.

Gillespie, J.H., J.A. Baker, J. Burgher, D. Robson, and B. Gilman. 1958. The immune response of dogs to distemper virus. Cornell Veterinarian 48: 103-126.

Glasgow, L.A., and G.J. Galasso. 1972. Isoprinosine: Lack of antiviral activity in experimental model infections. Journal of Infectious Diseases 126: 162-163.

Goodrich, J.M., E.S. Williams, and S.W. Buskirk. 1994. Effects of a modified-live virus canine distemper vaccine on captive badgers (*Taxidea taxus*). Journal of Wildlife Diseases 30: 492-496.

Gorham, J.R. 1966. The epizootiology of distemper. Journal of the American Veterinary Medical Association 149: 610-622.

Gorham, J.R., and C.A. Brandly. 1953. The transmission of distemper among ferrets and mink. Proceedings of the 90th American Veterinary Medical Association meeting, Toronto, Ontario, pp. 129-141.

Gorham, J.R., R. Farrell, and L. Lauerman. 1962. Immunologic responses of young mink. Veterinary Medicine 57: 896-900.

Gorska, C., and J. Gorski. 1983a. Effects of aerosol and parenteral vaccinations against distemper in polecat and mink farms. Bulletin of the Veterinary Institute in Pulawy 26: 27-35.

Gorska, C. and J. Gorski. 1983b. Attempts of vaccination of foxes against distemper and Rubarth's disease by an aerosol route. Bulletin of the Veterinary Institute in Pulawy 26: 35-39.

Goss, L.J. 1948. Species susceptibility to the viruses of Carré and feline enteritis. American Journal of Veterinary Research 9: 65-68.

Gould, D.H. 1983. Paramyxovirus-like nucleocapsids associated with encephalitis in a captive Siberian tiger. Journal of the American Veterinary Medical Association 183: 1319-1322.

Grachev, M.A., V.P. Kumarev, L.V. Mamev, V.L. Zorin, L.V. Baranova, N.N. Denikina, S.I. Belikova, E.A. Petrov, V.S. Kolesnik, V.N. Dorofeev, A.M. Beim, V.N. Kudelin,

F.G. Nagieva, and V.N. Sidorov. 1989. Distemper virus in Baikal seals. Nature 338: 209.

Greene, C.E. Immunoprophylaxis and immunotherapy. 1990. *In* Infectious Diseases of the Dog and Cat, C.E. Greene (editor). W.B. Saunders Company, Harcourt Brace Jovanovich, Inc., Toronto, Canada, pp. 21-54.

Haas, L. H. Hofer, M. East, P. Wohlsein, B. Liess, and T. Barrett. 1996. Canine distemper virus infection in Serengeti spotted hyaenas. Veterinary Microbiology 49: 147-152.

Habermann, R.T., C.M. Herman, and F.P. Williams. 1958. Distemper in raccoons and foxes suspected of having rabies. Journal of the American Veterinary Medical Association 132: 31-35.

Hagen, K.W., and J.R. Gorham. 1970. Distemper control by aerosol vaccination. U.S. Fur Rancher 49(10): 16-17.

Haig, D.A. 1956. Canine distemper - immunization with avianized virus. Onderstepoort Journal of Veterinary Research 27: 19-53.

Halbrooks, R.D., L.J. Swango, P.R. Schnurrenberger, F.E. Mitchell, and E.P. Hill. 1981. Response of gray foxes to modified live-virus canine distemper vaccines. Journal of the American Veterinary Medical Association 179: 1170-1174.

Hamilton, H.W. Jr. 1936. The food and breeding habits of the raccoon. Ohio Journal of Science 36: 131-140.

Hamir, A.N., and C.E. Rupprecht. 1990. Absence of rabies encephalitis in a raccoon with concurrent rabies and canine distemper infections. Cornell Veterinarian 80: 197-201.

Hamir, A.N., N. Raju, C. Hable, and C.E. Rupprecht. 1992. Retrospective study of testicular degeneration in raccoons with canine distemper infection. Journal of Veterinary Diagnostic Investigation 4: 159-163.

Harder, T.C., M. Kenter, M.J.G. Appel, M.E. Roelke-Parker, T. Barrett, and A.D.M.E. Osterhaus. 1995. Phylogenetic evidence of canine distemper virus in Serengeti's lions. Vaccine 13: 512-523.

Harder, T.C., M. Kenter, H. Vos, K. Siebelink, W. Huisman, G. van Amerongen, C. Örvell, T. Barrett, M.J.G. Appel, and A.D.M.E. Osterhaus. 1996. Canine distemper virus from diseased large felids: Biological properties and phylogenetic relationships. Journal of General Virology 77: 397-405.

Helmboldt, C.F., and E.L. Jungherr. 1955. Distemper complex in wild carnivores simulating rabies. American Journal of Veterinary Research 16: 463-469.

Hoff, G.L., W.J. Bigler, S.J. Proctor, and L.P. Stallings. 1974. Epizootic of canine distemper virus infection

among urban raccoons and gray foxes. Journal of Wildlife Diseases 10: 423-428.

Hoover, J.P., C.A. Baldwin, and C.E. Rupprecht. 1989. Serologic response of domestic ferrets (*Mustela putorius furo*) to canine distemper and rabies virus vaccine. Journal of the American Veterinary Medical Association 194: 234-238.

Itakura, C., K. Nakamura, J. Nakatsuka, and M. Goto. 1979. Distemper infection in lesser pandas due to adminstration of a canine distemper live vaccine. Japanese Journal of Veterinary Science 41: 561-566.

Jacobson, E.R., G.V. Kollias Jr, and D. Heard. 1988. Viral diseases and vaccination considerations for nondomestic carnivores. In Exotic Animals, E.R. Jacobson, and G.V. Kollias (editors). Churchill Livingstone, New York, New York, pp. 231-247.

Jakowski, R.M., and D.S. Wyand. 1971. Listeriosis associated with canine distemper in a gray fox. Journal of the American Veterinary Medical Association 159: 626-628.

Jamison, R.K., E.C. Lazar, L.N. Binn, and A.D. Alexander. 1973. Survey for antibodies to canine viruses in selected wild mammals. Journal of Wildlife Diseases 9: 2-3.

Karstad, L., and J. Budd. 1964. Distemper in raccoons

characterized by giant-cell pneumonitis. Canadian Veterinary Journal 5: 326-330.

Kauffman, C.A., A.G. Bergman, and R.P. O'Connor. 1982. Distemper virus infection in ferrets: an animal model of measles-induced immunosuppression. Clinical and Experimental Immunology 47: 617-625.

Kaufmann, J.H. 1982. Raccoons and allies. *In* Wild Mammals of North America, J.A. Chapman and G.A. Feldhamer (editors). The John Hopkins University Press, Baltimore, Maryland, pp. 567-585.

Kazacos, K.R., H.L. Thacker, H.L. Shivaprasad, and P.P. Burger. 1981. Vaccination-induced distemper in kinkajous. Journal of the American Veterinary Medical Association 179: 1166-1169.

Kilham, L., and C.M. Herman. 1954. Isolation of an agent causing hyperbilirubinemia and jaundice in raccoons. Proceedings of the Society for Experimental Biology and Medicine 85: 272-275.

Kilham, L., R.T. Habermann, and C.M. Herman. 1956. Jaundice and bilirubinemia as manifestations of canine distemper in raccoons and ferrets. American Journal of Veterinary Research 17: 144-148.

Kingsbury, D.W. 1991. The Paramyxoviruses. Plenum Press, New York, New York, 596 pages.

Kotani, T., M. Jyo, Y. Odagiri, Y. Sakakibara, and T. Horiuchi. 1989. Canine distemper virus infection in lesser pandas (*Ailurus fulgens*). Japanese Journal of Veterinary Science 51: 1263-1266.

Krakowka, S., G. Cockerell, and A. Koestner. 1975. Effects of canine distemper virus infection on lymphoid function *in vitro* and *in vivo*. Infection and Immunity 11: 1069-1078.

Krakowka, S., and A.L. Wallace. 1979. Lymphocyte-associated immune responses to canine distemper and measles viruses in distemper-infected gnotobiotic dogs. American Journal of Veterinary Research 40: 669-672.

Laidlaw, P.P., and G.W. Dunkin. 1926. Studies in dog distemper.II. Experimental distemper in the dog. Journal of Comparative Pathology 39: 213-221.

Laperle, A. 1993. Distemper chez les ratons laveurs. Médecin Vétérinaire du Québec 23: 83-85.

Machida, N., N. Izumisawa, T. Nakamura, and K. Kiryu. 1992. Canine distemper virus infection in a masked palm civet (*Paguma larvata*). Journal of Comparative Pathology 107: 439-443.

Mahy, B.W.J., and H.O. Kangro. 1996. Virology Methods Manual, B.W.J. Mahy, and H.O. Kangro (editors). Academic Press, Harcourt Brace & Company, Publishers,

Toronto, Ontario, Canada, 374pp.

Mainka, S.A., Q. Xianmeng, H. Tingmei, and M.J.G. Appel. 1994. Serologic survey of giant pandas (*Ailuropoda melanoleuca*), and domestic dogs and cats in the Wolong reserve, China. Journal of Wildlife diseases 30: 86-89.

Malik, R., M. Dowden, P.E. Davis, G.S. Allan, V.R. Barrs, P.J. Canfield, and D.N. Love. 1995. Concurrent juvenile cellulitis and metaphyseal osteopathy: An atypical canine distemper virus syndrome? Australian Veterinary Practitioner 25: 62-67.

Mamaev, L.V., I.K.G. Visser, S.I. Belikov, N.N. Denikina, T. Harder, L. Goatley, B. Rima, B. Edginton, A.D.M.E. Osterhaus, and T. Barrett. 1996. Canine distemper virus in Lake Baikal seals (*Phoca sibirica*). Veterinary Record 138: 437-439.

Mangi, R.J., T.P. Munyer, S. Krakowka, R.O. Jacoby, and F.S. Kantor. 1976. A canine distemper model of virus-induced anergy. Journal of Infectious Diseases 133: 556-563.

Marsilio, F., P-G. Tiscar, L. Gentile, H.U. Roth, G. Boscagli, M. Tempesta, and A. Gatti. 1997. Serologic survey for selected viral pathogens in brown bears from Italy. Journal of Wildlife Diseases 33: 304-307.

Maurer, K.E., and S.W. Nielsen. 1981. Neurologic disorders in the raccoon in Northeastern United States. Journal

of the American Veterinary Medical Association 179: 1095-1098.

Mickwitz, C.U. 1968. Zur Staupe der Kleinbären (Procyoniden). Kleintier-Praxis 13: 80-84,86,88-90.

Miller, R.M. 1971. Raccoons, coatis and kinkajous. *In* Current Veterinary Therapy IV, Small Animal Practice. W.B. Saunders Company, Philadelphia, Pennsylvania, pp.457-459.

Miller, R.E. 1995. Quarantine procedures for AZA-accredited zoological parks. Proceedings of the Joint Conference of the American Association of Zoo Veterinarians, the Wildlife Disease Association, and American Association of Wildlife Veterinarians, pp. 165-172.

Monson, R.A., and W.B. Stone. 1976. Canine distemper in wild carnivores in New York. New York Fish and Game Journal 23: 149-154.

Montali, R.J., C.R. Bartz, J.A. Teare, J.T. Allen, M.J.G. Appel, and M. Bush. 1983. Clinical trials with canine distemper vaccines in exotic carnivores. Journal of the American Veterinary Medical Association 183: 1163-1167.

Montali, R.J., C.R. Bartz, and M. Bush. 1987. Canine distemper virus. *In* Virus Infections of Carnivores, M.J. Appel (editor). Elsevier Science Publishers B.V., New York, New York, pp. 437-443.

Montali, R.J., L. Tell, M. Bush, R.C. Cambre, D. Kenny, M. Sutherland-Smith, and M.J.G. Appel. 1994. Vaccination against distemper in exotic carnivores: successes and failures. Proceedings of the American Association of Zoo Veterinarians, pp. 340-344.

Montgomery, G.G. 1964. Tooth eruption in preweaned raccoons. Journal of Wildlife Management 28: 582-584.

Montgomery, G.G. 1968. Pelage development of young raccoons. Journal of Mammalogy 49: 142-145.

Montgomery, G.G. 1969. Weaning of captive raccoons. Journal of Wildlife Management 33: 154-159.

Morris, J.A., D.R. Coburn, and J.R. O'Connor. 1954. Rapid protection of ferrets against fully virulent distemper virus with nebulized attenuated distemper virus. Cornell Veterinarian 44: 198-207.

Muneer, M.A., I.O. Farrah, J.A. Newman, and S.M. Goyal. 1988. Immunosuppression in animals. British Veterinary Journal 144: 288-301.

Nicolle, C. 1931. La maladie du jeune âge des chiens est transmissible expérimentalement à l'homme sous forme inapparente. Archives de l'Institut Pasteur, Tunis 20: 312-323.

Nowak, R.M. 1991. Walker's Mammals of the World, Fifth Edition, Volume II. The John Hopkins University Press, Baltimore, Maryland, 1362pp.

Oervell, C., H. Sheshberadaran, and E. Norrby. 1985. Preparation and characterization of monoclonal antibodies directed against four structural components of canine distemper virus. Journal of General Virology 66: 443-456.

Ott, R.L., and J.R. Gorham. 1955. The response of newborn and young ferrets to intranasal administration with egg-adapted distemper virus. American journal of Veterinary Research 16: 571-572.

Ott, R.L., J.R. Gorham, and R.K. Farrell. 1965. A note on the use of adjuvated vaccines in ferrets. Canadian Journal of Comparative Medicine and Veterinary Science 29: 214-215.

Parihar, N.S., and I.B. Chakravarty. 1980. Canine distemper-like disease in a lesser panda (*Ailurus fulgens*). Indian Veterinary Journal 57: 198-199.

Parker, R.L., V.J. Cabasso, D.J. Dean, and E.L. Cheatum. 1961. Serologic evidence of certain virus infections in wild animals. Journal of the American Veterinary Medical Association 138: 437-440.

Pollock, R.V.H., and L.E. Carmichael. 1982. Maternally derived immunity to canine parvovirus infection: transfer, decline and interference with vaccination. Journal of the American Veterinary Medical Association

180: 37-42.

Potgieter, L.N.D., and C.S. Patton. 1984. Multifocal cerebellar cortical necrosis caused by canine distemper virus infection in a raccoon. Journal of the American Veterinary Medical Association 185: 1397-1399.

Pringle, C.R. 1992. *Paramyxoviridae*. Classification and Nomenclature of Viruses. Fifth Report of the International Committee on Nomenclature of Viruses, R.I.B. Francki, C.M. Fauquet, D.L. Knudson, and F. Brown (editors). Springer-Verlag, New York, New York, 459 pp.

Qiu, X., and S. A. Mainka. 1993. Review of mortality of the giant panda (*Ailuropoda melanoleuca*). Journal of Zoo and Wildlife Medicine 24: 425-429.

Robinson, V.B., J.W. Newberne, and D.M. Brooks. 1957. Distemper in the American raccoon (*Procyon lotor*). Journal of the American Veterinary Medical Association 131: 276-278.

Rockborn, G. 1960. A preliminary report on efforts to produce a living distemper vaccine in tissue culture. Journal of Small Animal Practice 1: 53.

Rockborn, G., E. Norrby, and N. Lannek. 1965. Comparison between the immunizing effect in dogs and ferrets of living distemper vaccines, attenuated in dog tissue

cultures and embryonated eggs. Research in Veterinary Science 6: 423-427.

Roelke-Parker, M.E., L. Munson, C. Packer, R. Kock, S. Cleaveland, M. Carpenter, S.J. O'Brien, A. Pospischil, R. Hofmann-Lehmann, H. Lutz, G.L.M. Mwamengele, M.N. Mgasa, G.A. Machange, B.A. Summers, and M.J.G. Appel. 1996. A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). Nature 379: 441-445.

Rosatte, R.C., M.J. Power, and C.D. MacInnes. 1991. Ecology of urban skunks, raccoons, and foxes in Metropolitan Toronto. *In* Wildlife Conservation in Metropolitan Environments, L.W. Adams, and D.L. Leedy (editors). Published by the National Institute for Urban Wildlife, Columbia, Maryland, pp. 31-38.

Roscoe, D.E. 1993. Epizootiology of canine distemper in New Jersey raccoons. Journal of Wildlife Diseases 29: 390-395.

Sanderson, G.C. 1987. Raccoon. *In* Wild Furbearer Management and Conservation in North America, M. Novak, J.A. Baker, M. E. Obbard, and B. Malloch (editors). The Ontario Trappers Association, North Bay, Ontario, Canada, pp.487-499.

Schönbauer, von M., S. Kölbl, and A. Shönbauer-Längle. 1984. Perinatale staupeinfektion bei drei eisbären (*Ursus*

maritimus) und bei einem brillenbären (*Tremarctos ornatus*). Verhandlungsbericht des Internationalen Symposiums über die Erkrankungen der Zootiere 26: 131-136.

Schubert-Kuehner, C.A. 1995. The effects of disease management on urban wildlife populations: an experimental approach. PhD thesis, University of Guelph, 100 pp.

Sciesinski, K. 1990. Cellular immunity after acupuncture stimulation at the points Dazhui, Hegu and Zusanli together with prophylactic vaccinations in young polar foxes. Scientifur 14: 274-278.

Sedgwick, C.J., and W.A. Young. 1968. Distemper outbreak in a zoo. Modern Veterinary Practice 49: 39-44.

Shapshak, P., M.C. Graves, and D.T. Imagawa. 1982. Polypeptides of canine distemper virus strains derived from dogs with chronic neurological diseases. Virology 122: 158-170.

Shek, W.R., R.D. Schultz, and M.J.G. Appel. 1980. Natural and immune cytolysis of canine distemper virus-infected target cells. Infection and Immunity 28: 724-734.

Shell, L.G. 1990. Canine distemper. Compendium on Continuing Education for the Practicing Veterinarian 12: 173-179.

Shin, Y-S., T. Mori, M. Okita, T. Gemma, C. Kai, and T.

Mikami. Detection of canine distemper virus nucleocapsid protein gene in canine peripheral blood mononuclear cells by RT-PCR. 1995. Journal of Veterinary Medical Science 57: 439-445.

Sikarskie, J.G., C.T. Lowrie, F.A. Kennedy, and G.L. Brady. 1991. Canine distemper in a vaccinated red panda (*Ailurus fulgens fulgens*). Proceedings of the American Association of Zoo Veterinarians, pp. 292-293.

Stephensen, C.B., J. Welter, S.R. Thaker, J. Taylor, J. Tartaglia, and E. Paoletti. 1997. Canine distemper virus (CDV) infection of ferrets as a model for testing *Morbillivirus* vaccine strategies: NYVAC- and ALVAC-based CDV recombinants protect against symptomatic infection. Journal of Virology 71: 1506-1513.

Stoffregen, D.A., and J.P. Dubey. 1991. A *Sarcocystis* sp.-like protozoan and concurrent canine distemper virus infection associated with encephalitis in a raccoon (*Procyon lotor*). Journal of Wildlife Diseases 27: 688-692.

Summers, B.A., H.A. Greisen, and M.J.G. Appel. 1984. Canine distemper encephalomyelitis: variation with virus strain. Journal of Comparative Pathology 94: 65-75.

Thulin, J.D., D.E. Granstrom, H.B. Gelberg, D.G. Morton, R.A. French, and R.C. Giles. Concurrent protozoal

encephalitis and canine distemper virus infection in a raccoon. Veterinary Record 130: 162-164.

Visser, I.K.G., M.W.G. v.d. Bildt, H.N. Brugge, P.J.H. Reijnders, E.J. Vedder, J. Kuiper, P. de Vries, J. Groen, H.C. Walvoort, F.G.C.M. UytdeHaag, and A.D.M.E. Osterhaus. 1989. Vaccination of harbour seals (*Phoca vitulina*) against phocid distemper with two different inactivated canine distemper virus (CDV) vaccines. Vaccine 7: 521-526.

Visser, I.K.G., E.J. Vedder, M.W.G. van de Bildt, C. Örvell, T. Barrett, and A.D.M.E. Osterhaus. 1992. Canine distemper virus ISCOMs induce protection in harbour seals (*Phoca vitulina*) against phocid distemper but still allow subsequent infection with phocid distemper virus-1. Vaccine 10: 435-438.

Visser, I.K.G., M.F. van Bressem, T. Barrett, and A.D.M.E. Osterhaus. 1993. Morbillivirus infections in aquatic mammals. Veterinary Research 24: 169-178.

Wild, F., A. Bernard, D. Spehner, D. Villeval, and R. Drillien. 1993. Vaccination of mice against canine distemper virus induced encephalitis with vaccinia virus recombinants encoding measles or canine distemper antigens. Vaccine 11: 438-444.

Wild, T.F., D. Naniche, C. Rabourdin-Combe, D. Gerlier, E.

Malvoisin, V. Lecouturier, and R. Buckland. 1995. Mode of entry of morbilliviruses. Veterinary Microbiology 44: 267-270.

Williams, E.S., E.T. Thorne, M.J.G. Appel, and D.W. Belitsky. 1988. Canine distemper in black-footed ferrets (*Mustela nigripes*) from Wyoming. Journal of Wildlife Diseases 24: 385-398.

Williams, E.S., S.L. Anderson, J. Cavender, C. Lynn, K. List, C. Hearn, and M.J.G. Appel. 1996. Vaccination of black-footed ferrets (*Mustela nigripes*) x siberian polecat (*M. eversmanni*) hybrids and domestic ferrets (*M. putorius furo*) against canine distemper. Journal of Wildlife Diseases 32: 417-423.

Wojcinski, Z.W., and I.K. Barker. 1986. Tyzzer`s disease as a complication of canine distemper in a raccoon. Journal of Wildlife Diseases 22: 55-59.

Womer, R. 1973. A study on the efficacy of diethyl ether inhalation in the treatment of canine distemper. Auburn Veterinarian 29: 52-54.

Wozencraft, W.C. 1993. Order Carnivora. *In* Mammal Species of the World, a Taxonomic and Geographic Reference, D.E. Wilson, and DA. M. Reeder (editors). Smithsonian Institute Press, Washington, D.C., pp. 279-344.

Yoshikawa, Y., F. Ochikubo, Y. Matsubara, H. Tsuruoka, M.

Ishii, K. Shirota, Y. Nomura, M. Sugiyama, and K. Yamanouchi. 1989. Natural infection with canine distemper virus in a Japanese monkey (*Macaca fuscata*). Veterinary Microbiology 20: 193-205.

APPENDIX I

Evidence of canine distemper in vaccinated raccoons

Case 1. An adult vasectomized male raccoon (MTZ PM# 13878) was observed sick on the zoo site. It was caught and euthanatized on December 22nd 1994. Canine distemper was diagnosed histologically. This raccoon of undetermined age had been vaccinated with Galaxy 6® on September 27th 1994.

Case 2. An adult hysterectomized female raccoon MTZ PM # 13911 was found dead on the zoo site on January 16th 1995. Again, canine distemper was diagnosed histologically. This raccoon, also of unknown age, had been vaccinated with Galaxy 6® on the 30th of June 1992.

Case 3. A juvenile male raccoon from a research station of the Ontario Ministry of Natural Resources in Midhurst, Ontario, was euthanatized when found convulsing on November 16th 1995 (Ontario Veterinary College PM# W355-95). Canine distemper infection was diagnosed as the cause of a severe acute necrotizing encephalitis. This raccoon had been vaccinated with a MLV vaccine in July, August, and September

of 1995, and had a serum neutralizing antibody titer of 1:256.

There were several additional anecdotal accounts from various individuals dealing in raccoon rehabilitation (Christine Mason, personal communication).

APPENDIX II

Canine distemper virus retrieval and preparation protocol:

Tissues from 2 raccoons, #41 and #45, were received. Sterile scalpel blades and petri dishes were used to isolate samples. Collected samples were placed immediately on ice for transport. In a laminar flow hood, 30 ml of sterile RPMI 1640 (Life Technologies Inc., Burlington, Ontario, Canada) media was dispensed into 50 ml conical tubes (Fisher Scientific, Unionville, Ontario, Canada) and stored in ice bath. Tissues were trimmed of excess fat and connective tissue and cut into approximately 1 cm^2 pieces, placed in media and homogenized with a tissue grinder (Tekmar Company, Cincinnati, Ohio, USA)). Attempts were made to have an entire sample placed in one aliquot of media resulting in one sample of homogenized tissue for each separate tissue received. After processing, each sample homogenate was returned to the ice bath.

Sample homogenates were labeled: Spleen Tissue Homogenate, Node and Tonsil Tissue Homogenate, or Node Tissue Homogenate. All samples were spun in a refrigerated centrifuge (Sorvall RT 6000, Dupont Canada Inc.,

Mississauga, Ontario, Canada) at 500 X G for 15 minutes, 4°C. Equal volumes of each tissue homogenate for both raccoons were pooled (6 ml of Spleen, Node and Tonsil, and Node each for Raccoon #41 combined with 6 ml of Spleen, Tonsil and Node, and Node each for Raccoon #45). This pooled sample was further clarified by centrifugation at 600 X G for 15 minutes, 4°C. One ml aliquots were pipetted into 30 storage vials (Sarsted Inc., Saint-Laurent, Quebec, Canada)), labeled as: Raccoon Distemper Pool 96.10.02 Neat. One hundred µl of the pooled sample was pipetted into 30 vials of previously aliquotted 900 µl volumes of RPMI 1640 labeled as: R.D.P. 96.10.02 1/10. One hundred µl of the above 1/10 dilution (after mixing by inversion) was dispensed into 30 previously aliquotted 900 µl volumes of RPMI 1640 and labeled as: R.D.P. 96.10.02 1/100. All pipetting and dispensing procedures were performed on ice. Samples were mixed several times by inversion, placed in storage boxes in a -70°C freezer. Residual tissue homogenates and tissue cell pellets were similarly stored in labeled boxes.

APPENDIX III

Vaccination trial

Serum CD neutralizing antibody titers

reciprocal of the end dilution

Raccoon	Group	wk 0	wk 1	wk 2	wk 3	wk 4	wk 5	wk 6	wk 7	wk 8	wk 9	wk 10	wk 11	wk 12
29	A	1	1	1	1	1	1	1	1	1	1	1	1	1
30	A	1	1	1	1	1	1	1	1	1	1	1	1	1
55	A	1	1	1	1	1	1	1	1	1	1	1	1	1
56	A	1	1	1	1	1	1	1	1	1	1	1	1	1
57	A	1	1	1	1	1	1	1	1	1	1	1	1	1
58	A	1	1	1	1	1	1	1	1	1	1	1	1	1
65	A	1	1	1	1	1	1	1	1	1	1	1	1	1
6	B	1	1	48	384	768	768	384	192	768	512	768	384	96
7	B	1	1	24	32	96	1536	256	256	384	384	384	512	64
9	B	1	1	32	192	512	384	1024	1024	512	512	384	768	64
10	B	1	4	48	1024	2048	1024	768	512	192	1536	384	768	96
33	B	1	1	96	1024	3072	2048	768	1024	1536	1024	1024	2048	128
35	B	1	1	192	384	3072	2048	1536	1024	2048	1536	1024	1536	128
36	B	1	1	256	768	768	1024	768	768	1536	1024	1024	1024	128
38	B	1	1	96	384	1536	1536	2048	2048	512	3072	2048	4096	64

Group A: Eight-week-old seronegative controls

Group B: Eight-week-old seronegatives, vaccinated at weeks 0, 4, and 8.

Appendix III (continued)

Vaccination trial

Serum CD neutralizing antibody titers

reciprocal of the end dilution

Raccoon	Group	wk 0	wk 1	wk 2	wk 3	wk 4	wk 5	wk 6	wk 7	wk 8	wk 9	wk 10	wk 11	wk 12
5	C	1	3	24	384	384	4	192	768	384	192	192	256	96
8	C	1	1	48	96	384	128	48	96	96	512	384	384	192
11	C	1	1	24	256	384	512	768	512	768	512	384	384	48
31	C	1	2	48	192	1024	1024	768	512	768	512	512	512	64
32	C	1	2	48	768	1024	1536	512	768	768	384	384	384	128
34	C	1	1	32	512	1536	1024	768	1536	512	768	512	384	48
37	C	1	3	96	1536	768	1024	1024	1536	1024	768	1024	768	128
64	C	1	1	24	512	512	512	192	512	512	256	256	384	48
1	D	1	1	192	48	384	512	384	768	384				
2	D	1	1	192	384	2048	1536	1536	1024	512				
4	D	1	1	256	128	384	768	512	384	192				
12	D	1	1	96	768	2048	1536	1536	2048	32				
13	D	1	1	48	256	1024	1536	1536	1536	256				
20	D	2	1	128	96	192	128	96	192	64				
21	D	2	1	384	1536	1536	1536	2048	1024	512				
22	D	1	1	128	192	384	256	384	384	128				

Group C: Eight-week-old seronegatives, vaccinated at week 0.

Group D: Sixteen-week-old seronegatives, vaccinated at week 0.

Appendix III (continued)
Vaccination trial
Serum CD neutralizing antibody titers
reciprocal of the end dilution

Raccoon	Group	wk 0	wk 1	wk 2	wk 3	wk 4	wk 5	wk 6	wk 7	wk 8	wk 9	wk 10	wk 11	wk 12
19	E	192	192	48	64	24	24	24	12	12	4	4	4	1
48	E	192	48	24	16	24	6	3	4	1	1	1	1	1
49	E	64	48	48	3	24	8	6	3	1	1	1	1	1
52	E	384	48	48	12	16	8	3	3	1	1	1	1	1
54	E	768	256	64	48	6	1	1	1	1	1	1	1	1
59	E	1536	512	768	512	192	256	128	32	48	24	24	12	1
60	E	1536	768	768	384	192	192	96	48	48	16	24	12	1
61	E	1536	384	192	96	192	64	48	32	24	16	12	8	1
3	F	40	64	24	8	8	6	3	3	1	1	192	512	512
15	F	256	64	64	96	48	32	24	16	3	4	4	12	96
16	F	128	192	128	96	128	96	64	48	1	3	4	8	1
17	F	512	96	128	64	24	12	4	4	24	12	4	4	96
18	F	512	256	96	96	32	32	24	16	3	3	4	3	1
50	F	96	64	64	24	8	6	48	64	248	64	128	64	12
51	F	128	48	32	12	12	8	4	3	1	1	256	1536	192
62	F	768	256	64	96	48	32	32	12	16	6	6	48	24

Group E: Eight-week-old seropositive controls.

Group F: Eight-week-old seropositives, vaccinated at weeks 0, 4, and 8.

APPENDIX IV

Vaccination trial

Serum CD neutralizing antibody titers

Log_2 of end point dilution

Raccoon	Group	wk 0	wk 1	wk 2	wk 3	wk 4	wk 5	wk 6	wk 7	wk 8	wk 9	wk 10	wk 11	wk 12
29	A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
55	A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
56	A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
57	A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
58	A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
65	A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	B	0.00	0.00	5.58	8.58	9.58	9.58	8.58	7.58	9.58	9.00	9.58	8.58	6.58
7	B	0.00	0.00	4.58	5.00	6.58	10.58	8.00	8.00	8.58	8.58	8.58	9.00	6.00
9	B	0.00	0.00	5.00	7.58	9.00	8.58	10.00	10.00	9.00	9.00	8.58	9.58	6.00
10	B	0.00	2.00	5.58	10.00	11.00	10.00	9.58	9.00	7.58	10.58	8.58	9.58	6.58
33	B	0.00	0.00	6.58	10.00	11.58	11.00	9.58	10.00	10.58	10.00	10.00	11.00	7.00
35	B	0.00	0.00	7.58	8.58	11.58	11.00	10.58	10.00	11.00	10.58	10.00	10.58	7.00
36	B	0.00	0.00	8.00	9.58	9.58	10.00	9.58	9.58	10.58	10.00	10.00	10.00	7.00
38	B	0.00	0.00	6.58	8.58	10.58	10.58	11.00	11.00	9.00	11.58	11.00	12.00	6.00

Group A: Eight-week-old seronegative controls

Group B: Eight-week-old seronegatives, vaccinated at weeks 0, 4, and 8.

Appendix IV (continued)

Vaccination trial

Serum CD neutralizing antibody titers

Log_2 of end point dilution

Raccoon	Group	wk 0	wk 1	wk 2	wk 3	wk 4	wk 5	wk 6	wk 7	wk 8	wk 9	wk 10	wk 11	wk 12
5	C	0.00	1.58	4.58	8.58	8.58	2.00	7.58	9.58	8.58	7.58	7.58	8.00	6.58
8	C	0.00	0.00	5.58	6.58	8.58	7.00	5.58	6.58	6.58	9.00	8.58	8.58	7.58
11	C	0.00	0.00	4.58	8.00	8.58	9.00	9.58	9.00	9.58	9.00	8.58	8.58	5.58
31	C	0.00	1.00	5.58	7.58	10.00	10.00	9.58	9.00	9.58	9.00	9.00	9.00	6.00
32	C	0.00	1.00	5.58	9.58	10.00	10.58	9.00	9.58	9.58	8.58	8.58	8.58	7.00
34	C	0.00	0.00	5.00	9.00	10.58	10.00	9.58	10.58	9.00	9.58	9.00	8.58	5.58
37	C	0.00	1.58	6.58	10.58	9.58	10.00	10.00	10.58	10.00	9.58	10.00	9.58	7.00
64	C	0.00	0.00	4.58	9.00	9.00	9.00	7.58	9.00	9.00	8.00	8.00	8.58	5.58
1	D	0.00	0.00	7.58	5.58	8.58	9.00	8.58	9.58	8.58				
2	D	0.00	0.00	7.58	8.58	11.00	10.58	10.58	10.00	9.00				
4	D	0.00	0.00	8.00	7.00	8.58	9.58	9.00	8.58	7.58				
12	D	0.00	0.00	6.58	9.58	11.00	10.58	10.58	11.00	5.00				
13	D	0.00	0.00	5.58	8.00	10.00	10.58	10.58	10.58	8.00				
20	D	1.00	0.00	7.00	6.58	7.58	7.00	6.58	7.58	6.00				
21	D	1.00	0.00	8.58	10.58	10.58	10.58	11.00	10.00	9.00				
22	D	0.00	0.00	7.00	7.58	8.58	8.00	8.58	8.58	7.00				

Group C: Eight-week-old seronegatives, vaccinated at week 0.

Group D: Sixteen-week-old seronegatives, vaccinated at week 0.

Appendix IV (continued)

Vaccination trial

Serum CD neutralizing antibody titers

Log_2 of end point dilution

Raccoon	Group	wk 0	wk 1	wk 2	wk 3	wk 4	wk 5	wk 6	wk 7	wk 8	wk 9	wk 10	wk 11	wk 12
19	E	7.58	7.58	5.58	6.00	4.58	4.58	4.58	3.58	3.58	2.00	2.00	2.00	0.00
48	E	7.58	5.58	4.58	4.00	4.58	2.58	1.58	2.00	0.00	0.00	0.00	0.00	0.00
49	E	6.00	5.58	5.58	1.58	4.58	3.00	2.58	1.58	0.00	0.00	0.00	0.00	0.00
52	E	8.58	5.58	5.58	3.58	4.00	3.00	1.58	1.58	0.00	0.00	0.00	0.00	0.00
54	E	9.58	8.00	6.00	5.58	2.58	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
59	E	10.58	9.00	9.58	9.00	7.58	8.00	7.00	5.00	5.58	4.58	4.58	3.58	0.00
60	E	10.58	9.58	9.58	8.58	7.58	7.58	6.58	5.58	5.58	4.00	4.58	3.58	0.00
61	E	10.58	8.58	7.58	6.58	7.58	6.00	5.58	5.00	4.58	4.00	3.58	3.00	0.00
3	F	5.58	6.00	4.58	3.00	3.00	2.58	1.58	1.58	0.00	0.00	7.58	9.00	9.00
15	F	8.00	6.00	6.00	6.58	5.58	5.00	4.58	4.00	1.58	2.00	2.00	3.58	6.58
16	F	7.00	7.58	7.00	6.58	7.00	6.58	6.00	5.58	0.00	1.58	2.00	3.00	0.00
17	F	9.00	6.58	7.00	6.00	4.58	3.58	2.00	2.00	4.58	3.58	2.00	2.00	6.58
18	F	9.00	8.00	6.58	6.58	5.00	5.00	4.58	4.00	1.58	1.58	2.00	1.58	0.00
50	F	6.58	6.00	6.00	4.58	3.00	2.58	5.58	6.00	7.95	6.00	7.00	6.00	3.58
51	F	7.00	5.58	5.00	3.58	3.58	3.00	2.00	1.58	0.00	0.00	8.00	10.58	7.58
62	F	9.58	8.00	6.00	6.58	5.58	5.00	5.00	3.58	4.00	2.58	2.58	5.58	4.58

Group E: Eight-week-old seropositive controls.

Group F: Eight-week-old seropositives, vaccinated at weeks 0, 4, and 8.

APPENDIX V

Vaccination trial

Groups means and standard deviations

	Group	wk 0	wk 1	wk 2	wk 3	wk 4	wk 5	wk 6	wk 7	wk 8	wk 9	wk 10	wk 11	wk 12
mean	A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SD	A	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
mean	B	0.00	0.25	6.19	8.49	9.94	10.17	9.62	9.40	9.49	9.92	9.54	10.04	6.52
SD	B	0.00	0.66	1.13	1.53	1.56	0.76	0.91	1.07	1.10	0.95	0.83	1.04	0.44
mean	C	0.00	0.65	5.26	8.62	9.37	8.45	8.56	9.24	8.99	8.79	8.67	8.69	6.37
SD	C	0.00	0.68	0.66	1.16	0.73	2.65	1.42	1.18	1.00	0.66	0.67	0.42	0.73
mean	D	0.25	0.00	7.24	7.94	9.49	9.49	9.44	9.49	7.52				
SD	D	0.43	0.00	0.86	1.52	1.23	1.30	1.42	1.08	1.36				
mean	E	8.89	7.44	6.76	5.62	5.39	4.34	3.69	3.04	2.42	1.82	1.84	1.52	0.00
SD	E	1.62	1.54	1.81	2.35	1.81	2.56	2.44	1.90	2.49	1.95	1.99	1.59	0.00
mean	F	7.72	6.72	6.02	5.44	4.67	4.17	3.92	3.54	2.46	2.17	4.15	5.17	4.74
SD	F	1.31	0.93	0.82	1.40	1.32	1.35	1.66	1.61	2.66	1.84	2.64	3.06	3.15

APPENDIX VI

Challenge study: serum CD neutralizing antibody titers

reciprocal of the end dilution

Raccoon	Group	day 0	day 3	day 7	day 10	day 14	day 21	day 28	day 35	day 42
9	B	48	24	64	96	64	64	48	96	48
10	B	48	64	96	192	192	128	96	48	96
33	B	256	512	384	256	384	384	384	256	512
36	B	128	192	256	256	192	192	192	256	256
5	C	96	96	192	512	768	384	384	512	256
8	C	128	192	96	128	192	192	192	192	192
11	C	96	96	64	128	96	128	192	128	128
34	C	128	256	384	412	768	768	768	768	768
1	D	96	96	128	192	96	96	96	64	96
4	D	256	96	128	128	192	256	192	192	192
20	D	64	64	96	96	256	192	192	96	96
21	D	384	384	1024	1536	3072	2048	1536	1536	1536
3	F	384	256	192	384	384	384	256	192	256
15	F	256	192	384	412	512	512	768	192	128
16	F	12	6	64	1536	1536	768	768	768	512
50	F	64	24	192	3072	4096	3072	2048	1536	1536

Raccoon	Group	day 0	day 3	day 7	day 10	day 14	day 21	day 28	day 30	day 33	day 35	day 42
19	E	1	1	1	1	1	1	1		1		
29	A	1	1	1	1	1	6	6			12	96
48	E	1	1	1	1	1	1	1		1		
61	E	1	1	1	1	1		1	1			

APPENDIX VII

Challenge study: serum CD neutralizing antibody titers

Log_2 of the end dilution

Raccoon	Group	day 0	day 3	day 7	day 10	day 14	day 21	day 28	day 35	day 42		
9	B	5.58	4.58	6	6.58	6	6	5.58	6.58	5.58		
10	B	5.58	6	6.58	7.58	7.58	7	6.58	5.58	6.58		
33	B	8	9	8.58	8	8.58	8.58	8.58	8	9		
36	B	7	7.58	8	8	7.58	7.58	7.58	8	8		
5	C	6.58	6.58	7.58	9	9.58	8.58	8.58	9	8		
8	C	7	7.58	6.58	7	7.58	7.58	7.58	7.58	7.58		
11	C	6.58	6.58	6	7	6.58	7	7.58	7	7		
34	C	7	8	8.58	8.69	9.58	9.58	9.58	9.58	9.58		
1	D	6.58	6.58	7	7.58	6.58	6.58	6.58	6	6.58		
4	D	8	6.58	7	7	7.58	8	7.58	7.58	7.58		
20	D	6	6	6.58	6.58	8	7.58	7.58	6.58	6.58		
21	D	8.58	8.58	10	10.58	11.58	11	10.58	10.58	10.58		
3	F	8.58	8	7.58	8.58	8.58	8.58	8	7.58	8		
15	F	8	7.58	8.58	8.69	9	9	9.58	7.58	7		
16	F	3.58	2.58	6	10.58	10.58	9.58	9.58	9.58	9		
50	F	6	4.58	7.58	11.58	12	11.58	11	10.58	10.58		
Raccoon	Group	day 0	day 3	day 7	day 10	day 14	day 21	day 28	day 30	day 33	day 35	day 42
19	E	1	1	1	1	1	1	1		1		
29	A	0	0	0	0	0	2.58	2.58			3.58	6.58
48	E	0	0	0	0	0	0	0		0		
61	E	0	0	0	0	0		0	0			

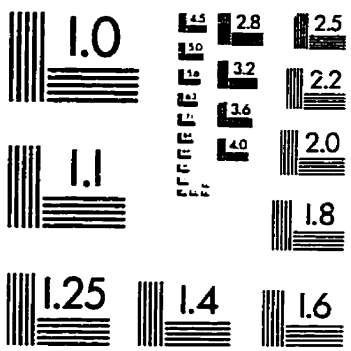
APPENDIX VIII

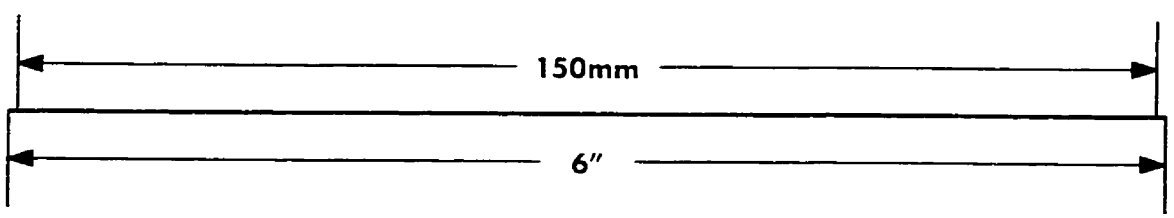
Group means and standard deviations

vaccinates, controls, and surviving control

	Group	day 0	day 3	day 7	day 10	day 14	day 21	day 28	day 35	day 42
mean	vacc	6.79	6.65	7.39	8.32	8.56	8.37	8.26	7.96	7.95
SD		1.26	1.61	1.11	1.46	1.69	1.48	1.45	1.48	1.41
mean	cont	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SD		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
mean	surv	0.00	0.00	0.00	0.00	0.00	2.58	2.58	3.58	6.58
SD		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

TEST TARGET (QA-3)





APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989