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VACCINATION OF BLACK-FOOTED FERRET (*MUSTELA NIGRIPES*) × SIBERIAN POLECAT (*M. EVERSMANNI*) HYBRIDS AND DOMESTIC FERRETS (*M. PUTORIUS FURO*) AGAINST CANINE DISTEMPER

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ABSTRACT: An inactivated canine distemper vaccine with adjuvant and a modified-live virus (MLV) vaccine were evaluated using black-footed ferret (*Mustela nigripes*) × Siberian polecat (*Mustela eversmanni*) hybrids as surrogates for endangered black-footed ferrets. For comparative purposes, we also vaccinated domestic ferrets (*Mustela putorius furo*) with the MLV vaccine. Response to vaccination was measured by clinical observation, hematology, dynamics of serum virus neutralizing antibodies, and challenge with virulent canine distemper virus. No clinical signs attributable to the vaccines were observed. Transient leukopenia occurred in hybrid ferrets that received MLV vaccine and there was marked lymphopenia for approximately 52 days post-vaccination. Lymphopenia was present for approximately 21 days in domestic ferrets vaccinated with MLV vaccine. Neutralizing antibodies against canine distemper virus were detected 14 days post-vaccination in hybrids receiving MLV vaccine and most titers were >1:1024 for the 791 days of the study. Antibody titers in hybrids vaccinated with the inactivated vaccine were significantly lower. All eight hybrid ferrets that received MLV vaccine survived challenge with virulent canine distemper virus without clinical disease. However, one of seven hybrids vaccinated with the inactivated vaccine developed canine distemper and was euthanized; two other hybrids became clinically ill but survived. The MLV vaccine may be useful in prevention of canine distemper in black-footed ferrets, but until additional studies of efficacy and safety are completed, use of the inactivated vaccine is appropriate.

Key words: Black-footed ferret, *Mustela nigripes*, canine distemper, vaccination, Siberian polecat, *Mustela eversmanni*, domestic ferret, *Mustela putorius furo*, endangered species.

INTRODUCTION

Canine distemper (CD) is the most significant infectious disease of the endangered black-footed ferret (*Mustela nigripes*) (Thorne and Williams, 1988) and reported mortality has been 100%. In western short grass prairies, this disease cycles among carnivores, especially coyotes (*Canis latrans*) (Gese et al., 1991). Historic black-footed ferret habitat encompassed the range of prairie dogs (*Cynomys* spp.) on these prairies (Nowak, 1991). Consequently, management of CD in populations of black-footed ferrets is necessary for recovery of this species (U.S. Fish and Wildlife Service, 1988).

Many inactivated and modified-live virus (MLV) CD vaccines have been developed because of the importance of CD in domestic dogs and mustelids in the pet and fur industries. But there have been few controlled studies of CD vaccination in wild carnivores (Halbrooks et al., 1981; Montali et al., 1983; Hoover et al., 1985; Goodrich et al., 1994). In 1971, MLV CD vaccine of avian origin (American Scientific Laboratories, Madison, Wisconsin, USA), previously shown to be safe in domestic ferrets (*Mustela putorius furo*), caused fatal CD in four of six recently captured black-footed ferrets (Carpenter et al., 1976). Modified-live virus vaccines induced CD or significant immunosuppression in other highly susceptible species, including red panda (*Ailurus fulgens*) (Bush et al., 1976), kinkajou (*Potos flavus*) (Ka-

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zacos et al., 1981) and gray fox (*Urocyon cinereoargenteus*) (Halbrooks et al., 1981) and possibly African hunting dogs (*Lycaon pictus*) (McCormick, 1983) and South American bush dogs (*Speothos venaticus*) (McInnes et al., 1992).

Because of the endangered status of black-footed ferrets (International Union for the Conservation of Nature, 1988) and their susceptibility to MLV-induced CD, black-footed ferret × Siberian polecats (*Mustela eversmanni*) were used as surrogates for black-footed ferrets in our initial vaccination studies. Siberian polecats are the closest living relatives of black-footed ferrets (Anderson, 1989; O'Brien et al., 1989). These animals and black-footed ferrets × Siberian polecat hybrids have provided useful biological information when it was not safe or desirable to study black-footed ferrets directly. We tested both an inactivated and MLV CD vaccine in hybrid ferrets. For comparative purposes we also vaccinated domestic ferrets with the same MLV vaccine because they are commonly vaccinated with MLV vaccines without apparent adverse effects (Ryland et al., 1983).

MATERIALS AND METHODS

Twenty-four 4 to 5-mo-old F_1 hybrids (three female, 21 male) were obtained from the black-footed ferret captive breeding facility at the Wyoming Game and Fish Department's Sybille Wildlife Research and Conservation Education Unit (Wheatland, Wyoming, USA). Animals were held groups of two or three in vinyl clad wire cages (60 × 80 × 20 cm) with wooden nest boxes within isolation facilities at the Department of Veterinary Sciences (University of Wyoming, Laramie, Wyoming), and they were fed commercial ferret feed (Purina Ferret Chow, St. Louis, Missouri, USA) or a mixture of dry cat food (Wayne KitKat Glo, Pet Products Plus, Inc., St. Charles, Missouri) and pelleted mink food (Gro-fur Dark Pellets, Milk Specialists, Co., New Holstein, Wisconsin, USA). Water was available ad libitum. Animals were observed daily during cleaning and feeding. One female and seven male hybrids were assigned to each of three experimental groups with essentially equal representation of each litter in each group. Hybrids were weighed and anesthetized weekly for the first 8 wk following vaccination and approximately monthly for 791 days until challenge. Hybrids were anesthetized with a mixture of 32 mg/kg ketamine hydrochloride (Vetalar, Avenco Co., Inc. Fort Dodge, Iowa, USA) and 0.16 mg/kg diazepam (Diazepam CIV, Elkins-Sinn, Inc., Cherry Hill, New Jersey, USA) by intramuscular injection. Three ml of blood were collected from the jugular vein each time the animals were anesthetized. Rectal body temperatures were determined using a digital thermometer. Males were castrated at approximately 1 yr of age to minimize fighting.

Eleven unvaccinated 3 to 4-mo-old castrated male domestic ferrets were obtained from Marshall Farms (North Rose, New York, USA). These animals were housed in 68 × 44 × 58 cm stainless steel cat cages and were fed and handled as the other ferrets. Blood samples were collected weekly for 6 wk post-vaccination.

Specific vaccines were chosen because of previous testing in highly susceptible species (Halbrooks et al., 1981; Montali et al., 1983) or because of preliminary use in black-footed ferrets (Williams et al., 1988). One group of eight hybrid ferrets was vaccinated subcutaneously between the shoulders with 1 ml inactivated CD vaccine (beta-propiolactone inactivated, Onderstepoort strain, $\geq 10^6$ 50% tissue culture infectious doses [$TCID_{50}$]/ml) and adjuvant (Stimulin, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) on days 0, 14, and 42; a booster was given on day 307. A second group of eight hybrids received 1 ml MLV CD vaccine (Fromm-D, Solvay Veterinary Supply, Princeton, New Jersey) subcutaneously between the shoulders on days 0 and 28. A third group of hybrids received only vaccine diluent on days 0 and 28 as above; these animals served as the control group. Seven domestic ferrets were vaccinated with the MLV vaccine and four ferrets received only diluent and served as controls.

Freshly collected blood was used immediately to fill heparinized capillary tubes and make smears; the remainder was allowed to clot for serology. White cell counts and packed cell volume determinations were generally made within 4 hr of collection using an automated cell counter (Coulter Electronics, Inc., Hialeah, Florida, USA). Differential cell counts were made on Wright's stained smears and absolute white blood cell counts were calculated.

Sera were separated from blood clots within 24 hr of collection and frozen at −70 C. Antibodies against CD virus were detected by serum neutralization tests using minor modifications of the technique of Appel and Robson (1973). After thawing, sera were heated at 56

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C for 30 min. Twelve duplicate serial two-fold dilutions of the sera were made in 96-well microtiter plates. A constant volume of CD virus (Onderstepoort strain) containing approximately 100 $TCID_{50}$ and medium (199 Earle's, Gibco, Grand Island, New York with 10% fetal calf serum) was added to all wells. Plates were incubated at 37 C in 5% CO_2 and 95% air for 1 hr. Vero maru cells (Middle America Research Unit, Ancon, Canal Zone, Panama) in Earle's medium were added at a concentration of 1×10^4 cells per well. Plates were incubated at 37 C in 5% CO_2 for 72 hr. Antibody titer against CD virus was determined to be the dilution of serum that resulted in complete protection of the cell monolayer.

At 798 days post-vaccination, hybrid ferrets that received MLV vaccine, inactivated vaccine, and three nonvaccinated hybrid ferrets from another study (List, 1994) were challenged by intraperitoneal injection of 0.5 ml tissue suspension containing virulent CD virus. The inoculum was a 20% Roswell Park Memorial Institute (RPMI)-1640 culture medium (Sigma Chemical Co., St. Louis, Missouri, USA) suspension of ground lung and lymph node from a hybrid ferret with experimental CD. The virulent virus originated from black-footed ferrets that died of CD in 1985 (Williams et al., 1988). This virus had only minor antigentic variation in comparison to other virulent and attenuated CD viruses (Blixenkrone-Møller et al., 1992). Hybrid ferrets were observed daily for clinical evidence of CD. They were weighed, anesthetized, and bled weekly for 4 wk and on day 55 post-inoculation. Fecal samples were collected weekly for negative stain electron microscopy to detect shedding of paramyxovirus (Williams et al., 1988). One vaccinated and three control animals were euthanized by intracardiac pentobarbital solution (Sleepaway, Fort Dodge Laboratories, Inc.) following anesthesia when they developed clinical CD from which we determined they could not recover.

At the end of the study, all animals were euthanized and necropsied. Sections of lung, lymph node, stomach, liver, spleen, small and large intestine, urinary bladder, kidney, heart, conjunctiva, and brain were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 to 6 μm, and stained with hematoxylin and eosin for light microscopy. Scrapings of small intestine mucosa were examined by negative stain electron microscopy for the presence of viruses.

Geometric mean neutralizing antibody titers were calculated for each group on each sampling day. Hematologic and serologic values and body temperatures were compared using two-sample *t*-tests or one way analysis of variance (Minitab, Inc., State College, Pennsylvania, USA) with significance level of $P \leq 0.05$.

RESULTS

Clinical signs associated with vaccination were not observed in any of the hybrid ferrets. There were no significant differences in body temperatures between the vaccinated and control hybrid ferrets. Two control hybrid ferrets died of causes unrelated to the study but all other animals remained clinically healthy.

Mean total leukocyte counts for hybrid ferrets receiving the MLV vaccine declined below counts for the other groups and were significantly ($P < 0.05$) lower on days 35, 52, and 123 post-vaccination. Similarly, mean total lymphocyte counts of this group were significantly ($P < 0.05$) depressed below control values for 52 days following vaccination. The lowest mean lymphocyte count was 1392/μl on day 21 post-vaccination and the lowest individual lymphocyte count was 492/μl on day 7 post-vaccination. There was no significant difference in packed cell volumes between the hybrid ferret groups.

Virus neutralizing antibodies were detected in all animals that received MLV by 14 days post-vaccination and high levels were sustained until the end of the study (Fig. 1). A few individuals vaccinated with the inactivated vaccine had low antibody titers by 7 days post-vaccination and all had titers by 21 days post-vaccination (Fig. 1). Titers reached a peak about 7 days following the third dose and then declined until the animals received the booster vaccination. Antibody titers in this group increased from about day 524 post-vaccination until about day 700.

The control nonvaccinated hybrid ferrets developed typical CD by day 8 following challenge with virulent virus and were euthanized. Canine distemper-like paramyxovirus was detected in fecal samples on day 7 post-challenge and in small intestine samples from these animals examined by electron microscopy at necropsy. Lesions observed included hyperkeratosis

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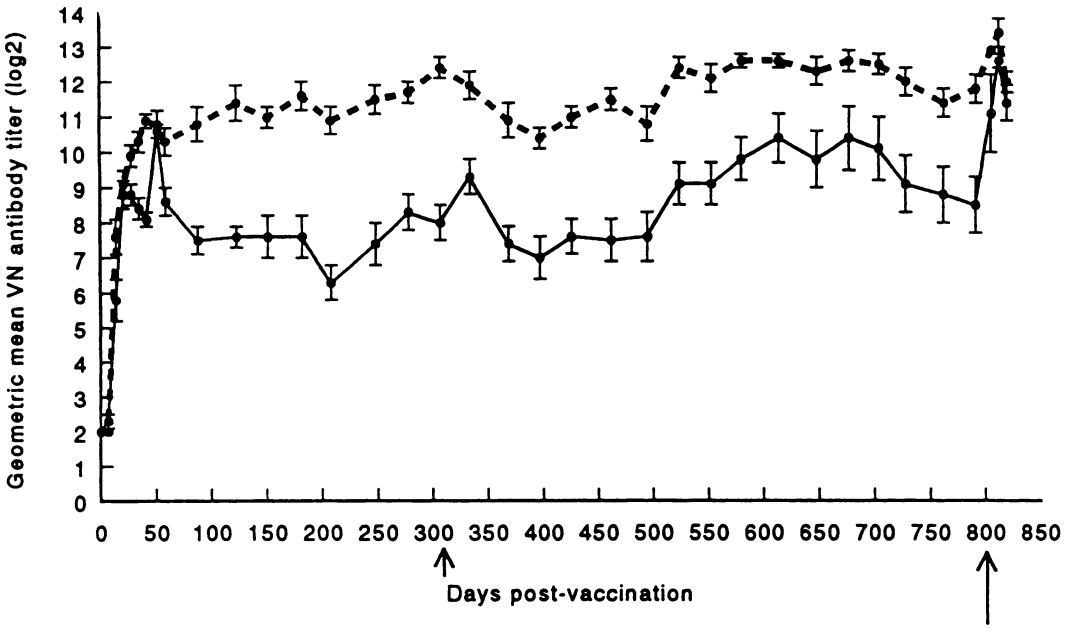


FIGURE 1. Geometric mean (±SE) serum virus neutralizing antibody titers against canine distemper virus in vaccinated hybrid ferrets. Titers of hybrid ferrets vaccinated with modified-live virus vaccine are shown by the dashed line. Titers of hybrid ferrets vaccinated with inactivated vaccine are shown by the solid line. Control ferrets remained seronegative throughout the study. Hybrid ferrets vaccinated with inactivated vaccine were given boosters on day 307 (short arrow) and all vaccinated hybrids were challenged with virulent canine distemper virus on day 789 (long arrow).

of lips, eyelids, and footpads, and typical eosinophilic intracytoplasmic inclusion bodies in epithelial cells of multiple organs. One hybrid ferret with a prechallenge antibody titer of 1:32 in the inactivated vaccine group was euthanized with clinical CD. Canine distemper-like paramyxovirus was detected in feces and in mucosa of small intestine and lesions typical of CD were found in this animal. Two other hybrid ferrets in the inactivated vaccine group had depression, cutaneous hyperemia, and lymphopenia but recovered from infection. Antibody titers prior to challenge in these animals were 1:64 and 1:256. Five hybrid ferrets in the inactivated vaccine group with antibody titers of 1:256 to 1:1024 remained clinically normal. Paramyxovirus was not detected in feces of any challenged hybrids with the exception of those animals that died of CD. Mean total lymphocyte count in the inactivated vaccine group was significantly ($P < 0.05$) lower than that of the MLV vaccine group on day 7 post-challenge. Antibody titers increased greatly in all hybrid ferrets in the inactivated vaccine group that survived challenge (Fig. 1). All eight hybrid ferrets that received MLV vaccine remained clinically normal following challenge; there was no apparent alteration in hematologic values and antibody titers peaked 7 days post-challenge (Fig. 1).

One domestic ferret in the control group died 7 days following the start of the study due to causes unrelated to the trial. All other domestic ferrets remained clinically normal during the course of the study. There was no significant difference between vaccinated and control groups in total leukocyte counts. However, lymphocytes declined following vaccination and were significantly reduced from control values on day 7 ($P < 0.05$) and depressed through approximately 21 days post-vaccination. The lowest mean lymphocyte

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count was 1181/μl of blood and one animal had a lymphocyte count of 466/μl on day 7. All vaccinated domestic ferrets developed antibody titers comparable to hybrid ferrets.

DISCUSSION

Virus neutralizing antibody titers produced in hybrid ferrets following MLV vaccination were higher and of longer duration than those induced by the inactivated vaccine. This was expected based on evidence of long duration of antibody titers produced by MLV vaccines in other species (Appel, 1987). Based on these data, we believe that the MLV vaccine produced prolonged immunity in hybrid ferrets; this was supported by complete protection provided by this vaccine in animals challenged more than 2 yr post-vaccination.

The MLV vaccine caused prolonged post-vaccinal leukopenia and lymphopenia providing evidence of immunosuppression secondary to vaccine virus infection. Though clinical disease was not evident, these animals could be more susceptible to opportunistic or other pathogens. Immunosuppression has been documented in domestic dogs secondary to CD (Mangi et al., 1976; Krakowka, 1982), in domestic ferrets following MLV vaccination (Kauffman et al., 1982), and has been suspected in deaths of red pandas (Montali et al., 1983) and domestic ferrets (Kauffman et al., 1982) vaccinated with MLV vaccines. Subsequent studies of the immune response following vaccination of hybrid ferrets with the MLV vaccine have been conducted, and in addition to alterations in hematology, there was suppression of lymphocyte blastogenesis responses to nonspecific mitogens (List, 1994), giving further evidence for alteration of lymphocyte function. Immunosuppression must be considered as an important and possibly fatal side effect of vaccination of black-footed ferrets, considering their susceptibility to some MLV CD vaccine viruses.

The high serum neutralizing antibody titers that were initially elicited by the inactivated vaccine were of relatively short duration. However, antibody titers increased during the latter part of the study without booster vaccination. A similar trend was noted in the ferrets receiving the MLV vaccine; however, there was no change in the seronegative status of the control hybrids during the same time. The cause of this increase is not known. Titer of positive control serum in the virus neutralizing tests did not increase during this time. Possibly additional antigen was released due to chronic inflammation at the injection site, though no gross lesions were observed in these areas at necropsy. Chronic inflammation has been observed at vaccine injection sites in some black-footed ferrets (E. S. Williams, unpubl.). Increased lymphocyte counts occurred in all groups around day 500, and may be evidence of an unidentified immune stimulus that accounted for elevated antibody titers.

There was no evidence of clinical disease or significant hematologic alterations and we concluded that the inactivated vaccine is safe in hybrid ferrets. However, protection from virulent virus challenge was incomplete and morbidity and mortality occurred in this group. Those animals with the lowest antibody titers were clinically affected; a serum neutralizing antibody titer of $\geq$1:100 is generally considered protective in dogs (Montali et al., 1983) and essentially the same may be true in ferrets. Based on the apparent safety of this vaccine and the response to and relative ease of providing booster injections in captivity, we recommend using the inactivated vaccine to protect captive black-footed ferrets against CD in conjunction with quarantine procedures aimed at preventing exposure to virulent virus (Williams et al., 1992). Although the inactivated vaccine is useful in captivity, its use in the field is problematic because of the relatively short duration of antibody titers without boosters and the difficulty in catching and vaccinating free-ranging fer-

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rets. Additional vaccines, including recombinant paramyxovirus vaccines are currently being evaluated in black-footed ferrets.

The domestic ferret study was conducted to determine if they respond to vaccination similarly to hybrid ferrets. Many pet and research domestic ferrets are vaccinated with MLV avian-derived products in environments where they could come into contact with opportunistic and other potential pathogens; yet significant post-vaccinal disease in this species has not been reported. Hematologic changes following MLV vaccination were similar to those in hybrid ferrets but were of shorter duration. The cause of these species differences was not apparent. Black-footed ferrets are more susceptible to MLV infection than are domestic ferrets (Carpenter et al., 1976). Perhaps the F_1 hybrid ferrets are intermediate in susceptibility to vaccine virus.

The level of antibody production elicited by the avian-derived MLV vaccine in both hybrid and domestic ferrets was greater and of longer duration than that reported in domestic ferrets following vaccination with a canine cell origin multivalent MLV vaccine (Vangard DA_2PL, Norden Laboratories, Inc., Lincoln, Nebraska, USA) (Hoover et al., 1989). Use of this multivalent MLV vaccine failed to induce antibodies in captive river otters (*Lutra canadensis*) (Hoover et al., 1985) but the same avian-derived MLV used in our study caused seroconversion in three river otters (Petrini, 1992). Serologic response of our hybrid and domestic ferrets vaccinated with MLV was comparable to that of gray foxes (Halbrooks et al., 1981), bush dogs, maned wolves (*Chrysocyon brachyurus*), and fennec foxes (*Fennecus zerda*) (Montali et al., 1983) and somewhat greater than in American badgers (*Taxidea taxus*) (Goodrich et al., 1994) vaccinated with the same MLV vaccine.

Recovery of the black-footed ferret and establishment of free-ranging populations will require immunoprophylaxis against CD virus. Based on these trials, we believe that the MLV warrants additional study prior to use in black-footed ferrets. The prolonged duration of the antibody titers and complete protection to virulent challenge is important; however, the possibility of immunosuppression and its manifestations need to be better understood before exposing this endangered species to the MLV vaccine.

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