Vaccination as a Potential Means To Prevent Plague in Black-footed Ferrets

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Abstract

This study was conducted to further assess the feasibility of vaccinating black-footed ferrets (*Mustela nigripes*) against plague (caused by the bacterium *Yersinia pestis*). On days 0 and 28, 17 postreproductive ferrets were immunized by subcutaneous injection with a recombinant fusion protein containing F1 and V antigens from *Y. pestis.* Another 17 animals received a placebo by the same route. Two weeks after the second immunization, mean antibody titers to *Y. pestis* F1 and V antigens were measured and found to be significantly higher in vaccinates than their preimmunization values ($P < 0.0001$) and significantly higher than the control values ($P < 0.0001$). Six months postimmunization, 16 vaccinates and eight controls were challenged with approximately 8,000 colony forming units of virulent plague by subcutaneous inoculation. Eleven of 16 vaccinates (69 percent) survived with no ill effects whereas all eight control animals died within 3–6 days. Two months later, the 11 surviving vaccinates were challenged again by ingestion of a plague-infected mouse. None of the animals showed any ill effects and all survived. In contrast, seven control ferrets fed infected mice died within 2–4 days, including one animal that did not actually ingest the mouse but was likely exposed to it. This study demonstrates that immunization of ferrets with the recombinant F1-V fusion protein can induce significant antibody responses and reduce their susceptibility to plague infection.

Keywords: black-footed ferrets, immunization, *Mustela nigripes*, sylvatic plague, vaccine, *Yersinia pestis*

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Introduction

Sylvatic plague, caused by the bacterium *Yersinia pestis*, is primarily a disease of wild rodents that is transmitted between mammals via flea (Insecta: Siphonaptera) bite, direct contact, ingestion, or inhalation. Since its introduction into the United States in the early 1900s, plague has become firmly established in native rodent populations throughout the West, causing frequent epizootics (Barnes, 1993). For many species of wildlife, plague mortality has become a serious conservation issue. Over half of the North American rodent species of conservation concern (Hafner and others, 1998), including several species of prairie dogs (*Cynomys* spp.), reside within the range of plague in western North America (Barnes, 1982). In addition, the endangered black-footed ferret (*Mustela nigripes*), which relies almost exclusively on prairie dogs for food and shelter, is highly susceptible to plague and suffers high mortality upon infection (Williams and others, 1994; Rocke and others, 2004).

Current methods to control plague in prairie dog colonies include dusting burrows with insecticides after the onset of an epizootic and population reduction. Although these methods have limited success in controlling outbreaks in rodents, they may be applied too late to be effective for ferrets, and population reduction is inappropriate for an endangered species. Recent studies have shown that multiple doses of a recombinant vaccine, consisting of two fused plague antigens, F1 and V (F1-V protein), protect laboratory mice against the bubonic or pneumonic form of plague (Heath and others, 1998). In a pilot study conducted at the U.S. Geological Survey's National Wildlife Health Center (NWHC) in Madison, Wis., six of seven ferrets that received a three-dose regimen of F1-V protein via subcutaneous injection survived challenge with 7,800 colony forming units (CFU) of *Y. pestis* 3 weeks after their last booster dose (Rocke and others, 2004). The objectives of the study described herein were to assess vaccine efficacy with a larger group of animals and with a longer duration between vaccination and challenge (6 months).

Methods

Thirty-four ferrets (23 females and 11 males) were selected for this study at the U.S. Fish and Wildlife Service,

National Black-footed Ferret Conservation Center (NBFFCC), Wheatland, Wyo. (now located near Wellington, Colo.), where the initial immunization and collection of baseline blood samples took place. All animals were 3–4 years of age and had been vaccinated previously against rabies and canine distemper. At the NBFFCC, animals were marked individually with subcutaneous embedded microchips (AVID® Microchip I.D. Systems, Folsom, La.) and housed individually in 2.5-cm wire-mesh cages (61 x 61 cm) with vinyl floors. Wooden nest boxes (45 x 22 x 28 cm) were attached to the exterior of the cages via 30-cm corrugated drain pipe. Bedding consisted of absorbent cellulose (ALPHA-dri™; Shepherd Specialty Papers, Watertown, Tenn.). The animals were fed 60–70 g of a raw horsemeat diet (Toronto Zoo Small Carnivore Diet; Milliken Meat Products, Ltd., Scarborough, Ontario, Canada) once daily. Water was provided ad libitum in ceramic bowls or sipper bottles.

For challenge experiments, all ferrets were transported to the NWHC where they were placed in a Biosafety Level 3 animal holding facility. Upon arrival, the animals were treated prophylactically for coccidiosis and housed individually in stainless steel cages as described previously (Rocke and others, 2004). The animals were fed Toronto Zoo Small Carnivore Diet or Dallas Crown Carnivore Diet (Dallas Crown, Inc., Kaufman, Tex.) when the Toronto Zoo Small Carnivore Diet was unavailable. Methods of anesthesia and blood sampling were described in Rocke and others (2004).

This study was reviewed and approved by NWHC's Animal Care and Use Committee and Biosafety Committee. All personnel handling plague-infected animals or carcasses were required to wear powered, air-purifying (Hepa-filtered) respirators with fullface shields, rubber aprons and boots, and double surgical gloves. In addition, personnel collecting and handling animals and conducting necropsies were required to take prophylactic antibiotics (as prescribed by occupational health physicians).

On days 0 and 28, 17 ferrets at NBFFCC received 0.5 mL F1-V vaccine-adjuvant preparation (100 µg of antigen) by subcutaneous injection between the scapulae. The F1-V fusion protein and our methods of preparing the vaccine have been described previously (Heath and others, 1998; Rocke and others, 2004). Seventeen control animals received a placebo of 0.5 mL of Dulbecco's Medium (Sigma Chemical Co., St. Louis, Mo.). One control animal was euthanized due to disease unrelated to vaccination; the rest were transported to NWHC the 12th week postvaccination where they were held in isolation for several months prior to plague challenge. During this period, two other animals (one vaccinate and one control) were euthanized due to disease issues unrelated to vaccination. The control animal had severe abscessation and edema of the neck region from which *Streptococcus zooepidemicus* was isolated. The vaccinate experienced acute, medically nonresponsive hind limb paresis. Upon histological examination, both animals were found to have kidney lesions (tubular nephrosis and glomerulopathy).

Six months postvaccination (day 178), six vaccinates and eight controls were challenged with 7,800 CFU of our *Y. pestis* challenge stock (CO92) described previously (Rocke and others, 2004); the bacteria were administered in 0.2 mL sterile saline by subcutaneous injection in the scapular region. Blood samples were taken from animals prior to first vaccination and on days 28, 42, and 167. Animals were monitored daily for signs of illness, and day of death was noted; severely debilitated animals were euthanized by CO_2 asphyxiation.

To determine if survivors were protected from further plague infection, the 11 vaccinated ferrets surviving 2 months after the initial subcutaneous challenge were bled to determine titers to plague antigens, and each was then orally challenged with a single plague-infected mouse; seven unvaccinated ferrets each fed a single infected mouse served as controls. For the oral challenge, 6-week-old mice were inoculated with a 0.1-mL volume of >4,000 CFU *Y. pestis* by intradermal injection. Upon death within 3 days after challenge, the mice were placed in the cage of each ferret. Any carcasses or parts of carcasses not ingested by ferrets within 3–4 hours were removed and discarded. Any ferrets surviving the second challenge were bled to determine antibody titers after 4 weeks and then euthanized by intracardiac injection of euthanasia solution (Euthasol; Delmarva Laboratories, Midlothian, Va.). In both experiments, dead or euthanized ferrets were immediately necropsied. Selected tissues were collected for bacterial isolation (Rocke and others, 2004) and histology.

Serology

Blood samples were collected in sterile glass serum separator tubes from all animals prior to immunization, boost, and challenge. Survivors were also bled after challenge. After centrifugation of blood samples, the serum was transferred to 2-mL polypropylene tubes and frozen at -20ºC for future analyses. Antibodies against F1 and V antigens were measured by using an enzyme-linked immunosorbent assay (ELISA) as previously described (Rocke and others, 2004).

Statistical Analysis

Antibody titers were transformed by calculating the $\log_{10}$ of the reciprocal titer value. Change in titer was then calculated by subtracting an individual animal's transformed preinoculation anti-F1 or anti-V titer from the transformed titer of each of that same animal's subsequent blood samples. Statistical difference in change of titer between groups was tested separately at each blood sampling period by using a one-tailed Mann-Whitney test at $P = 0.05$ (Zar, 1999). Difference in survivorship between groups was tested at $P = 0.05$ by using the Fisher Exact test (Zar, 1999), and days to death were compared by using a one-tailed Mann-Whitney test at $P = 0.10$.

Results

All 17 F1-V vaccinated ferrets developed significant antibody titers to both F1 and V antigens after immunization. In contrast, antibody titers of control animals remained negative. Geometric mean titers in anti-F1 and anti-V antibody increased significantly after the initial dose of vaccine was administered ($P < 0.0001$) and increased to even higher levels (means of 1:25,000 and 1:40,000, respectively) after the second dose, or boost ($P < 0.0001$) (fig. 1). Within 6 months, the mean anti-F1 and anti-V titers of vaccinates declined significantly ($P = 0.0004$ and $P < 0.0001$, respectively), although they were still significantly higher than their prevaccination titers ($P < 0.0001$) and the unvaccinated controls prior to challenge ($P < 0.0001$).

Eleven of the 16 vaccinated ferrets that were inoculated with *Y. pestis* survived the subcutaneous challenge and showed no signs of illness. The other five vaccinates became sick and died with an average time to death of 9.4 days. The first vaccinate died on day 4 with unusual gross lesions, including bloody diarrhea, multifocal hemorrhage throughout the intestines, and swollen kidneys. *Yersinia pestis* was isolated in low numbers from the spleen, and *S. zooepidemicus* was also isolated from the retropharyngeal lymph node. The three vaccinates that died on days 7 and 9 had gross lesions more consistent with unvaccinated controls (enlarged and slightly hemorrhagic lymph nodes, enlarged spleen, mottled lungs), and *Y. pestis* was isolated from numerous tissues from all three carcasses. The last vaccinate died on day 18 postchallenge. No *Y. pestis* was isolated from any tissue, but *S. zooepidemicus* was found in the spleen, lymph nodes, liver, lungs, heart, esophagus, and an abscessed region on the neck. In comparison, all eight unvaccinated controls inoculated with *Y. pestis* died within 3–6 days of challenge, with an average time to death of 4.3 days. All had gross lesions consistent with plague infection, and large numbers of *Y. pestis* were isolated from the tissues of all animals. Including the animals that had *S. zooepidemicus,* the survival rate of vaccinates was significantly higher than that of controls ($P = 0.02$), and time to death was significantly longer ($P = 0.02$). At the time of subcutaneous challenge, the mean anti-F1 titer of vaccinates that survived (9,030) was not significantly higher ($P = 0.165$) than that of vaccinates that died (5,580). The mean anti-V titer was significantly higher ($P = 0.035$), however, in surviving vaccinates (16,950) compared to those that died (9,030).

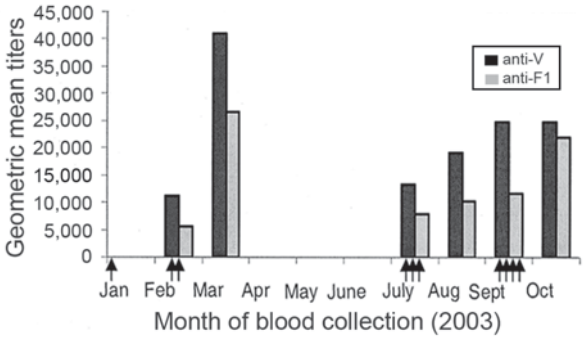

Figure 1. Geometric mean anti-F1 and anti-V antibody titers in black-footed ferrets (*Mustela nigripes*) immunized with F1-V protein. The dates of the first and second vaccinations (prime and boost), first subcutaneous challenge with *Yersinia pestis*, and second challenge via ingestion of infected mice are indicated with 1 arrow, 2 arrows, 3 arrows, and 4 arrows, respectively.

Two months after the subcutaneous challenge, the 11 surviving vaccinates received a second plague challenge via consumption of a plague-infected mouse. Each of them consumed an entire infected mouse, and all survived with no apparent clinical signs. In contrast, the seven control animals presented with infected mice all died within 2–4 days, including one animal that did not ingest its mouse but presumably licked or sniffed it; this animal died on day 4. *Yersinia pestis* was isolated from most of the controls, with the exception of one that died on day 2 that had an overwhelming infection of *S. zooepidemicus*.

Discussion

In this study, the majority (69 percent) of vaccinated ferrets survived subcutaneous plague challenge 6 months post-immunization in contrast to the unvaccinated controls that all died of the infection. These results are similar to those of our previous pilot study in which six of seven (86 percent) vaccinated ferrets survived subcutaneous challenge with the same dose of *Y. pestis* (Rocke and others, 2004). In that study, however, ferrets received an extra boost of F1-V just 3 weeks prior to challenge in a three-dose regimen whereas in the present study, the animals received only two doses and were not challenged with the bacteria until 6 months later. Mean anti-F1 and anti-V antibody titers of immunized animals increased significantly after vaccination, particularly after the boost; however, they decreased over the next several months to nearly preboost titers. Vaccinates that survived subcutaneous challenge had a slightly higher mean anti-V titer than those vaccinates that succumbed to the same challenge.

In nature, ferrets are likely exposed to plague by several means. They may be bitten by infected fleas as they navigate through burrows or as they feed on prairie dogs. It is also highly likely that ferrets contract plague while feeding on infected prairie dogs through either direct contact or inhalation of the bacteria. The one unvaccinated ferret in our study that contracted plague and died within 4 days even though it declined to consume the infected mouse is evidence of their extreme susceptibility to the bacteria via this route. Interestingly, in this study vaccinated ferrets that survived an initial subcutaneous challenge with *Y. pestis* all survived ingestion of an infected mouse 2 months later. This result suggests

that flea-bite exposure of vaccinated ferrets in nature could potentially boost their immune response enough to ward off further plague infection via consumption of infected prey. We suspect that some vaccinated ferrets would also survive an initial oral challenge with infected mice. In a previous pilot study, two of five vaccinated ferrets survived after ingestion of infected mice as an initial challenge (T. Rocke, unpub. data, 2001). These results are promising but insufficient, so we are currently exploring methods for boosting mucosal immunity in vaccinates.

At least four ferrets in this study were found to have *S. zooepidemicus* infections, one prior to challenge and three after challenge. In addition, three other ferrets had kidney lesions (glomerulonephritis) visible upon histologic examination of tissues that may have resulted from a previous infection (T. Rocke, unpub. data, 2003). Kidney damage is a reported sequela to *S. zooepidemicus* infection in humans (Barnham and others, 1983; Francis and others, 1993; Pinto and others, 2001) and horses (Divers and others, 1992). Raw horsemeat has been a documented source of *S. zooepidemicus* for other small carnivorous mammals, including short-nosed bandicoots (*Isoodon macrourus*) and shrews (*Tupaia glis* and *Elephantulus rufescens*) (Shaw and others, 1984) and several primate species (Schiller and others, 1989). In our study, ferrets were fed raw horsemeat diets from two different sources, both at NBFFCC and NWHC. Samples of the meat were cultured after the infection was diagnosed, but the bacterium was not isolated. Even though the source of infection is still unknown, we believe many of our study animals may have had underlying *S. zooepidemicus* infections or were recovering from an infection. This bacterium may have significantly impacted the ability of vaccinated ferrets to withstand challenge to *Y. pestis*.

Summary

The results of this study suggest that two doses of the F1-V protein are sufficient to reduce ferret mortality from subcutaneous injection of plague for at least 6 months postimmunization, even in the face of a chronic, underlying *Streptococcus* infection. We suspect that vaccination of younger animals (<1 year old) and animals that are less stressed would result in even higher antibody titers, better resistance to the disease, and longer duration of immunity. Until other methods of plague control are developed, the F1-V vaccine could protect ferrets in captive breeding facilities and animals intended for release programs. Black-footed ferret kits and dams in captive breeding programs are fed wild prairie dogs that are captured, quarantined, and killed for that purpose. However, the loss of numerous captive ferrets at one facility from ingestion of plague-infected prairie dog meat demonstrated the potential hazard of this practice (Castle and others, 2001) even with disease precautions and quarantine of the prairie dogs. Vaccination of captive ferrets against plague could reduce this risk. Ferrets intended for release into the wild could be immunized with F1-V antigen several times prior to release and reimmunized upon recapture, preferably within 6 months to 1 year postrelease. This might reduce mortality rates of ferrets during plague outbreaks. However, because black-footed ferrets are completely dependent on prairie dogs for their survival and prairie dogs are likewise highly susceptible to plague, the ultimate recovery of ferrets will require maintenance of stable prey populations and thus prevention of plague in prairie dogs.

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