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RECOMBINANT F1-V FUSION PROTEIN PROTECTS BLACK-FOOTED FERRETS (*MUSTELA NIGRIPES*) AGAINST VIRULENT *YERSINIA PESTIS* INFECTION

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Abstract: Black-footed ferrets (*Mustela nigripes*) are highly susceptible to sylvatic plague, caused by the bacterium *Yersinia pestis*, and this disease has severely hampered efforts to restore ferrets to their historic range. A study was conducted to assess the efficacy of vaccination of black-footed ferrets against plague using a recombinant protein vaccine, designated F1-V, developed by personnel at the U.S. Army Medical Research Institute of Infectious Diseases. Seven postreproductive black-footed ferrets were immunized with the vaccine, followed by two booster immunizations on days 23 and 154; three control black-footed ferrets received a placebo. After the second immunization, antibody titers to both F1 and V antigen were found to be significantly higher in vaccinates than controls. On challenge with 7,800 colony-forming units of virulent plague by s.c. injection, the three control animals died within 3 days, but six of seven vaccinates survived with no ill effects. The seventh vaccinate died on day 8. These results indicate that black-footed ferrets can be immunized against plague induced by the s.c. route, similar to fleabite injection.

Key words: Black-footed ferret, *Mustela nigripes*, plague, vaccination, *Yersinia pestis*.

INTRODUCTION

Sylvatic plague, caused by the bacterium *Yersinia pestis*, is primarily a disease of wild rodents that is transmitted by fleas and can afflict humans as well as other mammals. The plague bacterium was probably introduced into the United States through seaports in the early 1900s and quickly spread into native rodent populations, particularly in the western states.[3] The disease was first observed in prairie dogs (*Cynomys* spp.) in the 1930s in Arizona and New Mexico[6] and since then has had devastating effects on many prairie dog populations, often killing 90–100% of individuals in affected colonies.[9] Today, sylvatic plague has spread throughout the range of Gunnison's prairie dogs (*Cynomys gunnisoni*) in Arizona, Utah, New Mexico, and Colorado. It also persists in white-tailed prairie dogs (*C. leucurus*) in Wyoming and Colorado, in Utah prairie dogs (*C. parvidens*) in Utah, and in black-tailed prairie dogs (*C. ludovicianus*) in Colorado, New Mexico, Texas, Oklahoma, and Montana.[2,5]

The endangered black-footed ferret (*Mustela nigripes*) is also highly susceptible to plague and may suffer high mortality rates on infection.[10] Other carnivores may become infected with *Y. pestis* and seroconvert, but with the exception of felines, they are somewhat resistant to the disease. This includes domestic ferrets (*Mustela putorius furo*) that have survived experimental injections of large numbers of the bacteria.[10] Black-footed ferrets depend primarily on prairie dogs for both food and shelter and thus may be exposed to the bacteria either by consumption of plague-infected prey or by fleabite. Once thought to be extinct, a captive breeding and recovery program was established for black-footed ferrets in 1987 after an outbreak of canine distemper nearly decimated the last known wild colony that was discovered 6 yr earlier. The occurrence of plague in prairie dog populations and its potentially devastating effect on black-footed ferret reestablishment is a major impediment to this recovery program.[3]

Although attempts have been made, with limited success, to control plague in prairie dog colonies by dusting burrows with insecticides after the onset of an epizootic or through population reduction, neither of these methods are appropriate for an endangered species such as the black-footed ferret. Recent studies have shown that multiple doses of a recombinant vaccine, consisting of two fused plague antigens, F1 and V (F1-V fusion protein), protect mice against the bubonic or pneumonic form of plague.[8] Our objectives were to determine whether F1-V antigen administered through s.c. injection could protect black-footed ferrets from virulent plague challenge.

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MATERIALS AND METHODS

Experimental animals

Black-footed ferrets (five males and five females) were obtained from the U.S. Fish and Wildlife Service's Black-footed Ferret Recovery Program and transported to the U.S. Geological Survey National Wildlife Health Center (NWHC), Madison, Wisconsin, where they were placed in a Biosafety Level 3 animal-holding facility. The black-footed ferrets came from multiple captive breeding facilities including the National Zoological Park's Conservation Research Center, Louisville Zoological Gardens, the Cheyenne Mountain Zoo, and the National Black-footed Ferret Conservation Center. All animals were 3–5 yr of age, had been surgically sterilized, and were vaccinated against rabies and canine distemper.

The animals were housed individually in stainless steel rabbit cages (76 × 51 × 41 cm). Cage doors were covered with Plexiglas® acrylic sheets to prevent escape from the cage. Food and water were provided ad lib. in stainless steel boxes and sipper bottles. Nest boxes (31 × 15 × 23 cm; Rubbermaid Home Products, 1147 Akron Road, Wooster, Ohio 44691, USA) containing shredded paper, 20-cm polyvinyl chloride elbows, 30-cm corrugated drainpipe, and plastic balls were placed into each cage. On arrival at NWHC, the animals were weaned from a raw meat diet (Toronto Carnivore Mix Diet, Milliken Meat Products, 3347 Kennedy Road #1, Scarborough, Ontario M1V3F1, Canada) and acclimated to a pelleted ferret diet (Totally Ferret®, Performance Foods Inc., 3001 Industrial Lane, Unit 4, Broomfield, Colorado 80020, USA) over a 4- to 6-wk period. Additional food items included Iams® canned cat food (Iams Company, 7250 Poe Avenue, Dayton, Ohio 45414, USA; 15 ml, 1–3 times per week) and thawed mice or pinkies (1–4 times per week).

Prophylactic treatment with Albon® (sulfadimethoxine; Pfizer Inc., 235 East 42nd Street, New York, New York 20017, USA; p.o., 55 mg/kg) was administered at shipment, followed by 22.5 mg/kg once daily for 10 days after shipment to prevent coccidiosis. An additional course of treatment with trimethoprim/sulfa (Tribrisen® Schering-Plough Corporation, World Headquarters, 2000 Galloping Hill Road, Kenilworth, New Jersey 07033, USA; 30 mg/kg, p.o. for 14 days) was required during the acclimation period. For handling and restraint, black-footed ferrets were chamber induced with isoflurane (AErrane®, Baxter, Deerfield, Illinois 60015, USA) administered at 5% with a flow rate of 2 L oxygen/min. Once induced, the animals were maintained in a state of anesthesia with 2–3% isoflurane. Blood samples were collected from jugular veins using 23 ga needles with 3-cc syringes.

Vaccine preparation

The F1-V plague vaccine has been described.[8] Before administration to black-footed ferrets, the antigen was diluted in modified Dulbecco medium (Sigma Chemical Co., P.O. Box 14508, St. Louis, Missouri 63178, USA) to provide a concentration of 400 μg/ml. This solution was mixed 1:1 with an adjuvant, 0.2% Alhydrogel (United Vaccines, 7819 Airport Road, Madison, Wisconsin 53562, USA), that was also diluted in modified Dulbecco medium. The resulting vaccine–adjuvant mixture was rocked gently overnight at 4°C.

Yersinia pestis preparation and isolation

The strain, *Y. pestis* CO92, a wild-type isolate of human origin, was provided by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID, Fort Detrick, Frederick, Maryland 21792, USA). Seventy-five microliters of the isolate was spread on blood agar plates (Remel, 12076 Sante Fe Drive, Lenexa, Kansas 66215, USA) and incubated for 48 hr at 28°C. A heavy culture was obtained by scraping the growth, inoculating 200 ml heart infusion broth (Difco Laboratories, Detroit, Michigan 48201, USA) containing 2% xylose, and incubating for 48 hr at 28°C. A stock suspension was prepared by adding sterile glycerol (20% v/v) to the broth culture, mixing thoroughly, and aliquoting 1 ml volumes. Aliquots were stored frozen at −80°C.

Concentration, purity, and virulence of our *Y. pestis* stocks were determined by numerous plate counts and mouse tests. In each trial, serial 10-fold dilutions of a 1-ml aliquot were made in 0.85% saline. Concentration was determined by plating 100 μl per dilution onto blood agar plates and incubating for 48 hr at 28°C. Stock concentration was based on an average of the plate counts trials, which were consistent and pure. Virulence was determined by s.c. injection of outbred ICR female mice (7–12 wk old; Harlan Sprague Dawley Inc., P.O. Box 29176, Indianapolis, Indiana 46229, USA); four mice were inoculated with 0.2 ml of each dilution. The Reed–Muench method was used to calculate a mouse 50% lethal dose (LD50) of 20 colony-forming units (cfu) for our challenge stock.

The challenge inoculum for the black-footed ferrets was prepared by diluting an entire 1-ml aliquot to the desired concentration in sterile saline. Serial dilutions of the inoculum were spread on blood agar plates to confirm the actual cfu. Plates were

incubated for 48 hr at 28°C. Mouse tests were performed to confirm virulence of the challenge inoculum.

Isolation of *Y. pestis* from tissue of challenged animals was accomplished by direct plating on blood agar. The liver, lung, and a nasal swab from each animal were cultured for *Y. pestis*. From some animals, the spleen, heart, kidney, and skin tissue at the site of injection were also cultured depending on gross observations. Tissue impressions of cut surfaces were made on blood agar plates and streaked for isolation. Plates were incubated at 28°C for 48 hr. Subcultures of presumptive *Y. pestis* were tested and identified with bacteriophage strips obtained from the Centers for Disease Control and Prevention (Fort Collins, Colorado 80522, USA). Subcultures of other bacteria were identified using the API 20E system (bioMerieux Vitek Inc., 595 Anglum Drive, Hazelwood, Missouri 63042, USA).

Experimental design

This study was reviewed and approved by the 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 full-face 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).

Seven black-footed ferrets received 0.5-ml F1-V vaccine–adjuvant preparation (100 μg of antigen) by s.c. injection between the scapulae. Three control animals received a placebo of 0.5-ml Dulbecco medium. On day 23, all animals were immunized again with the same doses of vaccine and placebo, as described above, and a third immunization was administered on day 154. Three weeks later (day 176), the animals were challenged with 7,800 cfu of *Y. pestis* (at least 10 LD50) administered in 0.2-ml sterile saline by s.c. injection in the right caudal thigh. Blood samples were taken from animals before first vaccination and on days 23, 50, and 169. Animals were monitored daily for signs of illness, and day of death was noted; severely debilitated animals were euthanized. Ferrets surviving to 26 days after challenge were bled and then euthanized by intracardiac injection of euthanasia solution (Euthasol®; Delmarva Laboratories Inc., 1500 Huguenot Road, Suite 106, Midlothian, Virginia 23113, USA). Dead or euthanized animals were frozen at −20°C and later necropsied on a selected day to minimize human exposure to *Y. pestis*. Selected tissues were collected for bacterial isolation.

Serology

Blood samples were collected in sterile glass serum separator tubes and centrifuged. Serum was transferred to 2-ml polypropylene tubes and frozen at −20°C until assayed. Antibody to F1 was analyzed at both NWHC and USAMRIID using an enzyme-linked immunosorbent assay (ELISA). Selected samples were also analyzed for antibody to V antigen at USAMRIID by ELISA. Briefly, 96-well polystyrene plates (Polysorp Nunc-Immuno plates; Nalge Nunc International, 75 Panorama Creek Drive, P.O. Box 20365, Rochester, New York 14602, USA) were coated overnight at 4°C with 0.7-μg F1 antigen (USAMRIID) in a 100-μl volume or 0.1 μg of V antigen (USAMRIID) in a 50-μl volume. Serum samples serially diluted from 1:50 to 1:12,800 in TRIS-buffered saline with Tween 20 were added to wells in 50 μl volumes and incubated at 37°C for 1 hr. Goat anti-domestic ferret immunoglobulin G (IgG) conjugated to horseradish peroxidase (KPL, 2 Cessna Court, Gaithersburg, Maryland 20879, USA) and diluted 1:80 was added to wells (50 μl/well) and incubated at 37°C for 1 hr. Plates were washed four times in TRIS-buffered saline with Tween 20 after each of these steps. Peroxidase (ABTS Peroxidase Substrate®, KPL) was used as the substrate and 1% sodium dodecyl sulfate as the stop solution. Optical absorbance was measured at 405 nm using an EL800 plate reader (Bio/Tek Instruments Inc., P.O. Box 998, Winooski, Vermont 05404, USA).

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 using a one-tailed Mann–Whitney test[11] at $P = 0.05$. Difference in survivorship between groups was tested at $P = 0.05$ using the Fisher Exact test,[11] and days to death were compared using a one-tailed Mann–Whitney test at $P = 0.10$.

RESULTS

Of the seven black-footed ferrets immunized with F1-V, six developed an increased level of anti-F1 IgG antibodies on immunization compared with prevaccination baseline (Fig. 1); one animal did not

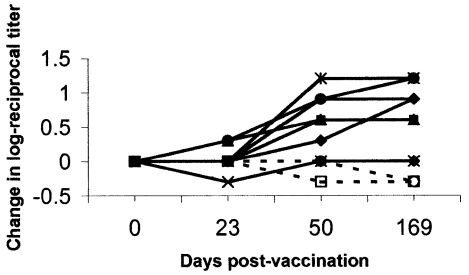

Figure 1. Change in $\log_{10}$ reciprocal anti-F1 immunoglobulin G antibody titer in response to vaccination. Solid lines represent responses of vaccinated animals; dashed lines represent responses of unvaccinated animals. The vaccinate that died is indicated by the solid circle.

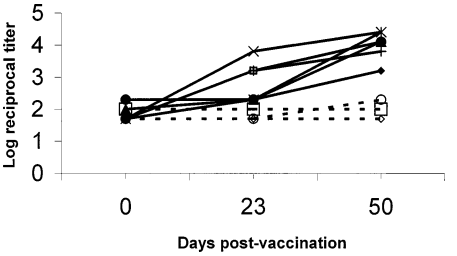

Figure 2. The $\log_{10}$ reciprocal anti-V immunoglobulin G antibody titer in response to vaccination. Solid lines represent responses of vaccinated animals; dashed lines represent responses of unvaccinated animals. The vaccinate that died is indicated by the solid circle.

develop F1 antibody until after the challenge. None of the negative controls showed an increase in titer over baseline at any point. The mean change in antibody titer was statistically significant between vaccinates and negative controls on day 50 ($P = 0.025$) and day 169 ($P < 0.025$) but not on day 23 ($P > 0.10$) after vaccination. Antibody to V antigen was detected in three vaccinates on day 23 and in all seven on day 50, 27 days after the second boost, and the mean change in titer between vaccinates and controls was significantly different both on days 23 and 50 ($P < 0.05$; Fig. 2). Anti-V titers were not run on day 169.

On challenge with virulent plague by s.c. injection, the three controls died on day 3 or 4, and *Y. pestis* was cultured from their tissues. In contrast, six of seven vaccinates survived plague challenge ($P = 0.033$), with the seventh animal dying on day 8. A mixed culture of *Y. pestis* and *Escherichia coli* was isolated from the lung, liver, and spleen of this animal. No *Y. pestis* or other significant bacteria were detected in the cultured tissues of the surviving vaccinates that were later euthanized.

On gross examination, control animals were in good flesh, but the lungs were mottled with pale and congested areas. Histopathologic examination revealed numerous bacilli in all tissues examined. The absence of an inflammatory response suggested an overwhelming peracute infection. In the one vaccinate that died, there was a 1-cm nodular, cyst-like structure in the liver, a 4 × 12 mm area of congestion in the stomach mucosa, and thick, reddish purple fluid in the gall bladder. Histopathology revealed that this animal did not have the extensive disseminated bacteremia observed in the controls and that an inflammatory response was evident in numerous tissues. Pneumonia and cholecystitis were the most severe lesions, but myositis, splenitis, hepatitis, and gastritis were also evident.

DISCUSSION

Black-footed ferrets vaccinated with three doses of F1-V antigen were protected against s.c. challenge with virulent *Y. pestis*. The one vaccinate that died on plague challenge survived longer (8 days after challenge) than corresponding controls (3–4 days after challenge). All the ferrets vaccinated with F1-V antigen developed a noticeable rise in anti-V antibody after immunization, and all but one also developed anti-F1 antibody; this animal survived infection. In mouse studies, the V antigen has been shown to be a determinant of virulence in *Y. pestis* and is also a protective immunogen,[1] whereas F1 antigen (capsular protein) does not appear to be directly involved in virulence of *Y. pestis*.[7] Because at least one of the surviving vaccinates in our experiment only had antibody to V and not F1, anti-V antibody may be a better indicator of protection in ferrets than anti-F1.

Black-footed ferrets are extremely sensitive to plague infection and in nature could be exposed either by fleabite or consumption of infected prey. In a previous study, s.c. injection of four black-footed ferrets with approximately 800 cfu of *Y. pestis* killed all the exposed animals (Williams, pers. comm.). The majority of our immunized ferrets survived s.c. injection of approximately 10 times that dose (7,800 cfu). Furthermore, in a subsequent pilot study, two of five ferrets immunized with F1-V survived consumption of a plague-infected mouse (Rocke, unpubl. data), a much more potent challenge with millions of bacteria. Although these results are promising, further studies with larger sample sizes are necessary to determine the maximum plague challenge dose that immunized animals can survive through different routes of exposure, to determine the duration of immunity after vaccination, and to develop an oral route of administration that can be used in the field. The animals used in this

study were postreproductive, had been moved from other facilities, and were 3–5 yr old. Several animals developed tumors or other problems during the course of our study. Higher antibody titers and an improved response to challenge may be obtained on vaccination of younger animals (1–3 yr) and animals housed in more natural environments.

Until other methods of plague control are developed, the F1-V vaccine might be useful in protecting animals 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 and killed for that purpose. However, the loss of numerous captive ferrets at one facility from ingestion of plague-infected meat demonstrated the hazard of this practice even with quarantine of the prairie dogs.[4] Vaccination of captive black-footed ferrets against plague could reduce this risk. Ferrets intended for release into the wild could be immunized with F1-V antigen several times before release and reimmunized on recapture. This might reduce mortality rates of ferrets during plague outbreaks. However, control of plague in black-footed ferrets and the ultimate recovery of the species will require control of the disease in their primary prey, prairie dogs.

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