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IMMUNIZATION OF BLACK-TAILED PRAIRIE DOG AGAINST PLAGUE THROUGH CONSUMPTION OF VACCINE-LADEN BAITS

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ABSTRACT: Prairie dogs (*Cynomys* spp.) are highly susceptible to *Yersinia pestis* and, along with other wild rodents, are significant reservoirs of plague for other wildlife and humans in the western United States. A recombinant raccoon poxvirus, expressing the F1 antigen of *Y. pestis*, was incorporated into a palatable bait and offered to three groups ($n=18$, 19, and 20) of black-tailed prairie dogs (*Cynomys ludovicianus*) for voluntary consumption, either one, two, or three times, at roughly 3-wk intervals. A control group ($n=19$) received baits containing raccoon poxvirus without the inserted antigen. Mean antibody titers to *Y. pestis* F1 antigen increased significantly in all groups ingesting the vaccine-laden baits, whereas the control group remained negative. Upon challenge with virulent *Y. pestis*, immunized groups had higher survival rates (38%) than the unimmunized control group (11%). The mean survival time of groups ingesting vaccine-laden baits either two or three times was significantly higher than that of animals ingesting vaccine-laden baits just one time and of animals in the control group. These results show that oral immunization of prairie dogs against plague provides some protection against challenge at dosages that simulate simultaneous delivery of the plague bacterium by numerous (3–10) flea bites.

Key words: Black-tailed prairie dogs, immunization, sylvatic plague, vaccine, *Yersinia pestis*.

INTRODUCTION

Plague, caused by the bacterium *Yersinia pestis*, is a disease of wild rodents that can afflict humans as well as other mammals (Perry and Fetherston, 1997; Koornhof et al., 1999) and is well-known as a disease that has devastated human and animal populations throughout history. In the past century, plague caused severe epidemics in many parts of the world, resulting in human deaths and severe economic losses (Titball and Leary, 1998). Human cases of plague in the US, while not numerous, are largely the result of contact with infected rodents (Gage et al., 1992). Devastating outbreaks in prairie dogs in New Mexico, Arizona, and Colorado, from 1982–1984, coincided with the large number of human plague cases in 1983 and 1984 (Gage et al., 1992; Craven et al., 1993).

Ground-dwelling rodents, like the prairie dogs in the western US, are particularly susceptible to plague, with >90% mortality in afflicted colonies (Barnes, 1993). Prairie dogs in some locations are considered a

“keystone” species, serving a critical role in maintaining the biotic diversity and integrity of the western grasslands that stretch from southern Canada to northern Mexico (Miller et al., 1994). The black-tailed prairie dog (once the most abundant mammal in North America) has declined to less than 2% of its former population due to habitat loss, intentional poisoning, and introduced disease, and was identified as a potential candidate for federal listing as a threatened species by the US Fish and Wildlife Service in 2000 (Graber et al., 1998; USDI FWS, 2000). Sylvatic plague was specifically identified as the most serious threat to the continued existence of this species over significant areas of its range. The disease has extirpated prairie dogs in some areas of North America (Lechleitner et al., 1962; Fitzgerald, 1993) and often causes local extinctions and population reductions, followed by partial recovery (Cully et al., 1997; Roach et al., 2001).

Many animals, including badgers, canids, hawks, and owls (Miller et al., 1994) use prairie dogs as food resources, but the

species most dependent on prairie dogs is the endangered black-footed ferret (*Mustela nigripes*; Sheets et al., 1971), thought to be extinct until 1981. A captive breeding and recovery program was established for ferrets in 1987 after disease outbreaks nearly eradicated the last known wild population. Because black-footed ferrets rely almost exclusively on prairie dogs for food, and on prairie dog burrows for shelter, their management and recovery is tightly linked to prairie dog survival and management (Truett et al., 2006). Plague in prairie dog towns significantly impacts black-footed ferret survival by destroying their primary prey and habitat base. In addition, the black-footed ferret is also highly susceptible to plague (Williams et al., 1994) and may suffer high mortality rates upon infection (Godbey et al., 2006), even though other predators and closely related species, such as the domestic ferret, appear to be resistant to the disease. Plague in prairie dogs is a major impediment to the ongoing ferret recovery programs of the US Fish and Wildlife Service, the National Park Service, the Bureau of Land Management, and numerous state agencies (Barnes, 1993; Godbey et al., 2006; Lockhart, 2006).

Lowering the incidence of plague infections in prairie dogs and other wild rodents would likely reduce zoonotic transmission of the disease to humans and enhance the recovery potential of the black-footed ferret. One method for controlling disease in free-ranging wildlife is to prevent infection through a targeted oral immunization program. We evaluated raccoon poxvirus (RCN) as a vaccine delivery system for the fraction 1 (F1) capsular antigen of *Y. pestis*, first in mice (Osorio et al., 2003) and then orally in black-tailed prairie dogs (Mencher et al., 2004). Vaccinated mice were protected from lethal plague challenge with up to 8×10^7 LD₅₀ of *Y. pestis* (Osorio et al., 2003). In a subsequent experiment, vaccine (herein designated RCN-F1) was presented to prairie dogs in the form of

oral bait that was voluntarily consumed (Mencher et al., 2004). The majority of vaccinated animals (56%) survived challenge with 130,000 bacteria; a severe, but realistic, dose of *Y. pestis* that animals might receive via numerous flea bites in a natural outbreak of plague (Engelthaler et al., 2000; Eisen et al., 2006). However, the majority of nonvaccinated controls did not survive challenge. In the study described here, we provide further evidence that black-tailed prairie dog consumption of baits containing RCN-F1 provides significant protection against plague. We also determined that optimal protection from plague requires consumption of vaccine-laden baits during at least two time intervals; three time intervals did not improve survival rates significantly, and one was insufficient.

MATERIALS AND METHODS

Experimental Animals

Adult black-tailed prairie dogs captured from wild colonies in June 2004 near Wall, South Dakota, USA (43°59'33"N, 102°14'28"W) were transported to the US Geological Survey National Wildlife Health Center (NWHC, Madison, Wisconsin, USA). At the time of capture, plague was not considered endemic in this region. The animals were dusted with carbaryl prior to shipment and, upon arrival at NWHC, they were inspected for external parasites (none were found), injected with an anthelmintic (200 ug/kg Ivermectin, Merck & Co., Inc., West Point, Pennsylvania, USA), and treated with 200 ul of Advantage flea control (Imidacloprid; Bayer HealthCare, Animal Health Division, Shawnee Mission, Kansas, USA) via external application to the skin on the back of the neck. Electronic microchip identification units (Avid Identification Systems, Inc., Folsom, Louisiana, USA) were inserted into each animal, between the scapulae, via subcutaneous injection. Prairie dogs were group-housed in isolation rooms with approximately 16.72 square m of floor space. Beta chips (Northwestern Productions Corporation, Warrensburg, New York, USA) covered the floor, and custom-made stainless steel nest boxes with connecting lengths of PVC pipe were used for shelter. An alfalfa-based pelleted food (approximately 50 g per animal per day) and fresh

TABLE 1. Percent survival of black-tailed prairie dogs (*Cynomys ludovicianus*) after *Y. pestis* challenge in relation to vaccine treatment. Treatment groups refer to the number of times that RCN-F1 vaccine-laden baits were consumed by prairie dogs (1, 2, or 3 times; C = control). Percent survival in groups not sharing a letter are significantly different at $P<0.05$.

| Treatment groups | Treatment | | | n | % Survival |
|------------------|---------------------|---------------------|---------------------|----|--------------------|
| | Day 0–1 | Day 20–21 | Day 42–43 | | |
| C | RCN-TK ⁻ | RCN-TK ⁻ | RCN-TK ⁻ | 19 | 10.5 ^a |
| 1 | RCN-TK ⁻ | RCN-TK ⁻ | RCN-F1 | 18 | 22.2 ^{ab} |
| 2 | RCN-TK ⁻ | RCN-F1 | RCN-F1 | 19 | 42.1 ^b |
| 3 | RCN-F1 | RCN-F1 | RCN-F1 | 20 | 45.0 ^b |

vegetables (broccoli, carrot, green beans, and sweet potato chunks) were provided once daily. Water was available ad libitum.

Vaccine administration

The raccoon pox-vectored recombinant plague vaccine RCN-IRES-tPA-YpF1 (designated as RCN-F1 in this paper) was produced as previously described (Osorio et al., 2003) and stored at -70°C in 2-ml aliquots until bait production. Briefly, the vaccine was created by replacing the thymidine kinase (TK) gene of RCN with the gene for *Y. pestis* F1 and associated regulatory elements (a poxvirus promoter, an internal ribosome entry site, and the secretory leader from tissue plasminogen activator). Virus stocks were thawed and diluted to 5×10^7 median tissue culture infective doses (TCID₅₀)/ml in Hank's medium (Gibco BRL, Carlsbad, California, USA) supplemented with 5% glycerin (Sigma, St. Louis, Missouri, USA) immediately before use. Preparation of sweet potato baits containing the vaccine, and the validation of vaccine titer, was described in Mencher et al. (2004). Placebo baits were prepared in a similar manner, but contained RCN without F1 and with the TK gene deleted (RCN-TK⁻).

Adult prairie dogs were randomly assigned to each of four isolation rooms, based on treatment. Treatment groups included one negative control group (Group C; $n=19$) that received placebo baits, and three oral vaccine groups (Groups 1, 2, and 3) that received RCN-F1 vaccine baits during one ($n=18$), two ($n=19$), or three ($n=20$) time intervals (Table 1). The groups were not matched for sex or age. Two additional animals were placed in the room with the unvaccinated control group to act as uninfected contact controls for the plague challenge.

Animals were prepared for bait administration by withholding fresh vegetables for 48 hr and food pellets for 12–18 hr. All animals were

then identified by microchips and individually placed in pet carriers with a small food dish containing either a single vaccine-laden bait or a single placebo bait, depending upon the experimental group and time interval. After 2–4 hr, all animals were released and bait consumption was recorded for each individual. This process was performed again the next day to ensure bait consumption at three time intervals; days 0 and 1, days 20 and 21, and days 42 and 43 (Table 1). Placebo baits were given to Group C at all 3 time intervals and to those animals not receiving vaccine-laden baits at that particular interval (Table 1).

***Yersinia pestis* challenge**

Three weeks after the final vaccine administration, all animals were challenged with the CO92 wild-type isolate of *Y. pestis* (provided by the United States Army Medical Research Institute of Infectious Diseases [USAMRIID], Fort Detrick, Maryland, USA), with the exception of the two uninfected contact controls housed with Group C. Stock aliquots of the bacteria, prepared and quantified as previously described (Osorio, et al., 2003), were diluted 1,000-fold in sterile saline. A volume of 0.2 ml of this solution was administered to each prairie dog by subcutaneous injection in the right hip region. Plate counts of the challenge inoculum indicated a dose of 65,202 colony forming units (3,260 mouse intradermal median lethal doses [LD₅₀s]) were given to each prairie dog, and concurrent mouse tests confirmed its virulence. Several attempts were made, prior to this experiment, to determine a LD₅₀ for our *Y. pestis* challenge inoculum in black-tailed prairie dogs (Rocke, unpubl. data). However, unlike inbred mice, reproducible results could not be achieved with prairie dogs. Prairie dogs were monitored for 28 day for signs of illness or death. Animals with obvious clinical signs (labored breathing, disinclination to move) were humanely

euthanized, as were all survivors at the end of the 28-day period. Carcasses were frozen for future necropsy.

Upon necropsy, tissue samples from lung, liver, spleen, and skin, or from any visible abscess at the site of inoculation, were cultured in brain heart infusion broth (Difco, Irvine, California, USA) and on blood agar plates (Becton-Dickinson, Franklin Lakes, New Jersey, USA) at 28 C for up to 72 hr. Plague-induced mortality was verified by isolation of *Y. pestis*-specific DNA sequences from tissue culture by polymerase chain reaction (PCR), using primers specific for the *Y. pestis* F1 gene (Heath, et al., 1998). DNA fragments were fractionated and directly visualized using standard techniques.

Serology

Blood samples (300 μ l) were collected from the medial saphenous vein of each prairie dog on day 0, before the priming vaccination, and on day 64 prechallenge; blood samples were also obtained from survivors post-challenge. Serum was collected and stored at -20 C until analyses.

Antibody titers to *Y. pestis* F1 antigen were determined using enzyme-linked immunosorbent assay (ELISA), with F1 antigen supplied by USAMRIID, as described previously (Mencher et al., 2004) but with some modifications. Briefly, serum samples were serially diluted 4-fold from 1:160 to 1:40,960; test samples were run in duplicate. Each plate also contained four replicates of a negative-control serum sample and two replicates of a positive-control serum sample. A horseradish-peroxidase-labeled anti-prairie dog IgG, custom-prepared by Bethyl Laboratories (Montgomery, Texas, USA), was diluted 1:100 and used as the secondary antibody. Titers <160 were considered negative and recorded as 40. The highest dilution that was positive (exceeded the mean of four negative control samples by three standard deviations) was considered the endpoint, and its reciprocal value was recorded as the titer.

Antibody titers to another *Y. pestis* protein, V antigen, were measured similarly by ELISA, in samples collected prechallenge and from post-challenge survivors, using V protein provided by USAMRIID. The samples were serially diluted 4-fold from 1:160 to 1:10,480 and tested in duplicate as described above.

Statistical Analyses

Antibody titers were transformed by calculating the \log_{10} of the titer in order to symmetrize the data and improve the accuracy

of confidence interval coverage. All statistical analyses were performed using SAS software (SAS, 2005). For comparing antibody titers between groups, we used a robust linear models approach that first involves transforming the data to ranks and then applying the appropriate linear models tests: a *t*-test for two groups or a one-way analysis of variance for more than two groups. This approach is equivalent to the nonparametric Wilcoxon and the Kruskal-Wallis tests, respectively (Conover and Iman, 1981). For comparing antibody titers pre- and post-vaccination or pre- and post-challenge, a matched-pairs analysis was used. The Kaplan-Meier survival analysis was used to calculate survival curves, followed by log rank tests to determine significance. Contingency table analysis was used to compare the 28-day survival rate among and between treatment groups, followed by Fisher's exact test (2-tailed) for comparisons between specific groups.

RESULTS

All animals consumed at least one of the two baits offered during each time interval, and most consumed both. After plague challenge, animals in every treatment group became sick and died (Table 1). One of the two uninfected contact control animals, cohoused with Group C, also died. *Yersinia pestis* was cultured and verified by PCR from the liver of all animals that died, including the one contact-control animal that died. Two animals that received placebo baits in Group C survived challenge. Interestingly, *Y. pestis* was isolated from a lesion at the site of inoculation, which was discovered upon necropsy of one of the surviving vaccinates euthanized on day 28, but was not isolated from any other tissues tested from survivors.

Kaplan-Meier analysis showed that survival of vaccinates (regardless of group) was significantly different than survival of Group C (Log rank $\chi^2=4.430$, $df=1$, $P=0.035$), and significant differences among the 4 groups (log rank $\chi^2=9.437$, $df=3$, $P=0.024$) were detected. Survival was higher in Groups 2 and 3 (Fig. 1) compared to Group C (log rank $\chi^2=5.084$, $df=1$, $P=0.0242$ and $\chi^2=6.98$, $df=1$,

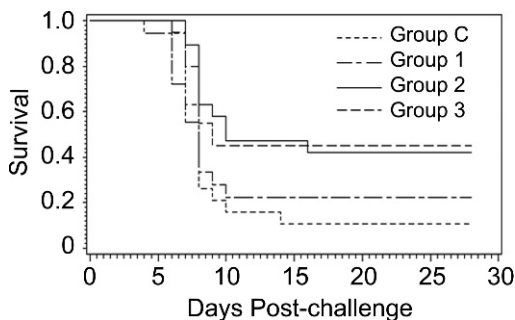


FIGURE 1. Kaplan-Meier survival curves of black-tailed prairie dogs (*Cynomys ludovicianus*) after *Y. pestis* challenge in relation to vaccine treatment. Treatment groups refer to the number of times that RCN-F1 vaccine-laden baits were consumed by prairie dogs (one, two, or three times; C = control).

$P=0.0082$, respectively), but there was no difference between Group 1 and Group C ($\chi^2=0.028$, $df=1$, $P=0.866$). Group 2 survival was higher than Group 1 ($\chi^2=3.90$, $df=1$, $P=0.048$), but there was no difference between Group 3 and Group 1 ($\chi^2=3.16$, $df=1$, $P=0.075$). There was also no difference in survival between males and females among the vaccinated animals in Groups 1, 2, and 3 ($\chi^2=1.654$, $df=1$, $P=0.1984$) or among controls in Group C ($\chi^2=0.052$, $df=1$, $P=0.820$).

Overall, prairie dogs that consumed vaccine-laden baits (Groups 1, 2, and 3 combined) had a higher 28-day survival rate after challenge (38%) than the Group C animals that consumed placebo baits (11%), as analyzed via Fisher's exact test ($df=1$, $P=0.043$). An analysis by group (Table 1) revealed that Group 2 and 3 animals had a higher 28-day survival rate than Group C ($df=1$, $P=0.048$ and $P=0.031$, respectively), but there was no significant difference between Groups 1 and C ($df=1$, $P=0.405$). Differences in survival rates between Groups 2 and 3 ($df=1$, $P=1.0$), Groups 1 and 2 ($df=1$, $P=0.295$), and Groups 1 and 3 ($df=1$, $P=0.182$) were insignificant.

Animals that consumed vaccine-laden baits (Groups 1, 2, and 3 combined) had a

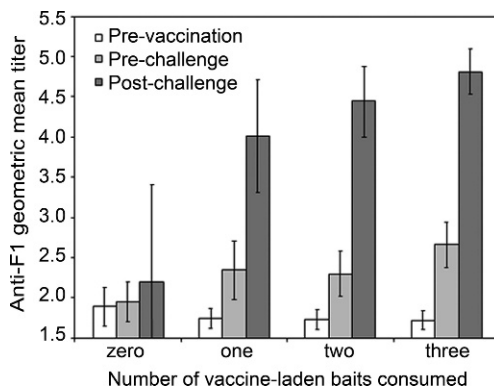


FIGURE 2. Geometric mean anti-F1 antibody titers in black-tailed prairie dogs (*Cynomys ludovicianus*) that consumed RCN-F1 vaccine-laden baits in all animals prior to challenge (prechallenge) and in survivors post-challenge. Mean prechallenge titers of all vaccinated groups (1, 2, and 3) were significantly higher ($P<0.05$) than their mean prevaccination titers and significantly higher ($P<0.05$) than the prechallenge titers of group C. Error bars indicate 2 standard errors of the mean.

significantly higher mean antibody titer (273) to F1 antigen post-vaccination compared to their prevaccination levels (54; t -ratio = -6.775 , $df=56$, $P<0.0001$). Likewise, vaccinates had higher mean prechallenge antibody titers (273) than controls (89) in Group C ($\chi^2=14.722$, $df=1$, $P<0.0001$). No significant differences were detected ($\chi^2=3.609$, $df=2$, $P=0.165$) in the mean prechallenge antibody titers among Groups 1, 2, and 3 (Fig. 2). However, mean prechallenge antibody titers were significantly higher ($\chi^2=9.898$, $df=1$, $P=0.0017$) in vaccinated animals that survived challenge (640) compared to prechallenge titers of those that died (166), regardless of treatment group. There was no difference in mean antibody titer among vaccinates with regard to sex ($\chi^2=2.227$, $df=1$, $P=0.1356$). In vaccinates that survived challenge, antibody titers to F1 antigen increased significantly (t -ratio = -9.192 , $df=20$, $P<0.0001$) in all 21 animals, from a mean prechallenge titer of 640 to a mean post-challenge titer of 3,148. Anti-F1 antibody was also detected in one of the two surviving control animals (640). Antibody to V antigen was detected in 10 of 21 surviving

vaccinates, with a range in titer from 640 to 10,240, but was not detected in either of the surviving controls.

DISCUSSION

In this study, prairie dogs that consumed vaccine-laden baits one or more times were more likely to survive plague challenge (38%) than animals that consumed placebo baits (11%). The survival of animals that consumed vaccine-laden baits at two separate time intervals was greater than animals that ingested vaccine-laden baits at only one time interval, although there was no significant additional benefit in ingesting baits a third time. Interestingly, mean antibody titers were not significantly different among groups that received vaccine laden baits one, two, or three times. However, the mean prechallenge anti-F1 titer was significantly higher in vaccinated prairie dogs that survived than in vaccinates that died upon *Y. pestis* challenge. These results confirm the findings of our earlier study (Mencher et al., 2004), in which over half of the prairie dogs that consumed RCN-F1 vaccine-laden baits survived a severe plague challenge. In that study, we also reported a weak, but significant, relationship between anti-F1 antibody titer of vaccinated prairie dogs and survival against plague challenge.

Surprisingly, we recovered viable *Y. pestis* from one surviving vaccinate at 28 days post-challenge. A visible abscess at the site of inoculation had formed, and the exudate inside was culture-positive for *Y. pestis*; all other tissues from this animal were culture-negative. No anti-F1 antibody was detectable prior to challenge in this animal. After challenge, a significant antibody response to F1 was evident (10,240), but there was no response to V antigen. It appears as though the bacteria replicated at the site of inoculation, but was sequestered in the abscess. Although we examined and cultured the site of inoculation and tissues from the other 22

survivors, *Y. pestis* was not detected in any of them. This finding raises the possibility that some vaccinated animals could conceivably carry the plague bacterium for some period of time after exposure, and thereby remain a source of infection for other animals. We believe this scenario is unlikely, but will explore this possibility further in future studies. Furthermore, as in our previous study (Mencher et al., 2004), two unvaccinated prairie dogs survived plague challenge, despite the lack of antibody to F1 or V in these animals prior to challenge. This finding reaffirms our earlier suggestion that innate resistance to *Y. pestis* may occur in some prairie dogs. It is also consistent with observations of others (Pauli et al., 2006) who recently reported a 5% survival rate in a Wyoming black-tailed prairie dog population exposed to plague; half of the survivors in that case had demonstrable antibody to F1 antigen.

Interestingly, in our study, one of two contact control animals (that were not deliberately infected) housed in the same room as the unvaccinated, infected animals contracted plague and died, despite the apparent absence of fleas on the animals. No fleas were found on any of our experimental animals, before or after death. Although flea transmission is presumed to be the primary mode of plague transmission between individual prairie dogs (Cully et al., 2006), the colonial nature of prairie dogs and their proclivity for “kissing” and other high-contact social behaviors (Hoogland, 2006) would provide the ideal transmission mechanism for an infectious disease agent such as plague. In addition, we have observed that many of the plague-infected prairie dogs expel large numbers of bacteria in a bloody exudate upon death. After infection, the animals in our study were monitored very closely for morbidity and mortality, and visibly sick and dead animals were removed promptly to prevent cannibalism; therefore, ingestion of contaminated meat

was not the source of exposure in this animal. Thus, we believe this case is the first-reported evidence of direct contact transmission of plague between prairie dogs.

In summary, from this and our previous study (Mencher et al., 2004), it appears that consumption of RCN-F1 vaccine-laden baits, followed by a booster, provides approximately 40–50% protection against subcutaneous plague challenge. Because as many as 11,000–24,000 bacteria per bite may be regurgitated by flea vectors (Engelthaler et al., 2000), our challenge doses in this and the previous study simulated simultaneous delivery of the plague bacterium by numerous (3–10) flea bites. Actual dosages of plague delivered to prairie dogs by fleas during natural outbreaks are unknown, but exposure is likely to vary between individual animals and occur over a period of time, not all at once, as in our study. Thus, we believe our challenge doses may represent a worst-case scenario. Given this belief, we consider our results to be very encouraging, suggesting that targeted oral immunization may provide an important management tool to prevent plague in some species. More work is currently in progress to optimize the vaccine, to develop an appropriate delivery bait for field use, and to ultimately conduct field trials. Once those tasks are accomplished and regulatory issues are addressed, targeted vaccination of prairie dogs against plague may be attempted in specific locations in order to protect wildlife and human health.

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