The efficacy of a recombinant plague vaccine (rF1V) was evaluated in cynomolgus macaques (CMs) to establish the relationship among vaccine doses, antibody titers, and survival following an aerosol challenge with a lethal dose of *Yersinia pestis* strain Colorado 92. CMs were vaccinated with a range of rF1V doses on a three-dose schedule (days 0, 56, and 121) to provide a range of survival outcomes. The humoral immune response following vaccination was evaluated with anti-rF1, anti-rV, and anti-rF1V bridge enzyme-linked immunosorbent assays (ELISAs). Animals were challenged via aerosol exposure on day 149. Vaccine doses and antibody responses were each significantly associated with the probability of CM survival ($P < 0.0001$). Vaccination also decreased signs of pneumonic plague in a dose-dependent manner. There were statistically significant correlations between the vaccine dose and the time to onset of fever ($P < 0.0001$), the time from onset of fever to death ($P < 0.0001$), the time to onset of elevated respiratory rate ($P = 0.0003$), and the time to onset of decreased activity ($P = 0.0251$) postinfection in animals exhibiting these clinical signs. Delays in the onset of these clinical signs of disease were associated with larger doses of rF1V. Immunization with ≥12 μg of rF1V resulted in 100% CM survival. Since both the vaccine dose and anti-rF1V antibody titers correlate with survival, rF1V bridge ELISA titers can be used as a correlate of protection.

**Plague** is caused by the Gram-negative bacterium *Yersinia pestis*. While natural outbreaks of disease occur in wildlife populations, humans are most often incidental victims in the life cycle of the bacterium in rodents. Human disease may also result from contact with blood or tissues of infected animals or exposure to aerosolized droplets containing bacteria (1, 2). Three forms of the disease exist and are believed to be dependent upon whether the bacteria enter the lymph nodes (bubonic), the bloodstream (septicemic), or the lungs (pneumonic). Primary pneumonic plague is caused by inhalation of *Y. pestis*. It is the least common form of the disease (2%); however, it is the most serious form because of the high fatality rate and the potential for direct human-to-human transmission via the spread of respiratory droplets (3). Because of the potential person-to-person transmission of pneumonic plague, the Centers for Disease Control and Prevention (CDC) considers this disease to be a serious potential threat and has classified *Y. pestis* as a category A (tier 1) bioterrorism agent (4).

Aerosol dissemination represents the most likely modern-day scenario for the use of *Y. pestis* as a biological weapon, with a battlefield scenario or terrorist attack likely resulting in a significant number of pneumonic plague fatalities. In 1970, the World Health Organization modeled an attack scenario that predicted that the airborne release of 50 kg of *Y. pestis* over a city of 5 million would result in 150,000 cases of plague, 36,000 of which would be fatal (5).

No licensed vaccines against plague are available for human use in the United States. The previously available U.S. Pharmacopeia (USP) vaccine was a killed, whole-cell (KWC) vaccine. Although its efficacy was never confirmed in controlled clinical studies, observations of vaccinated humans and a number of animal studies suggested that the USP vaccine was effective against bubonic plague but had limited efficacy against pneumonic plague (6–10). Further, this vaccine produced a number of moderate-to-severe side effects, ranging from mild headache to severe malaise and fever (9, 10). Live-attenuated vaccines have been used in several countries but were not licensed in the United States because of their reactogenicity (11). To overcome the limited efficacy and reactogenicity associated with KWC and live-attenuated plague vaccines, respectively, the *Y. pestis* F1 and V antigens were identified as promising components of a new generation of recombinant protein vaccines.

Early proof-of-concept studies investigated the immunogenicity and efficacy afforded by vaccination with the individual F1 and V antigens and found that each provides some level of protection from a challenge. However, the combined use of the F1 and V antigens was found to have an additive protective effect and was more effective than single-antigen vaccines in mouse models of pneumonic plague (7, 8, 12).

A recombinant plague vaccine (rF1V) is currently in advanced development by the U.S. Department of Defense (DoD) to provide preexposure prophylaxis to military personnel 18 to 55 years old against battlefield exposure to aerosolized *Y. pestis* (13). The vaccine contains both the F1 and V antigens fused into a single protein that is adsorbed to aluminum hydroxide adjuvant to enhance the immunogenicity of the rF1V protein. The efficacy of rF1V cannot be determined directly in humans because of the
ethic implications of conducting *Y. pestis* inhalational challenge studies. In addition, the incidence of pneumonic plague in the general population is extremely low, making field studies impractical. Therefore, licensure of rF1V will rely upon immunogenicity and efficacy data obtained in nonclinical studies, immunogenicity and safety data from clinical studies, and adherence to the requirements of the Animal Rule (14). The CM was chosen as a suitable nonhuman primate model of pneumonic plague on the basis of previous studies that demonstrated that CMs exhibit a clinical course of disease similar to that described for humans (15). Further, proof-of-concept model development studies indicated that CMs responded to plague vaccines and were protected from disease following a lethal aerosol challenge with *Y. pestis* (15, 16).

We initially developed and characterized the CM model of pneumonic plague (17) by following the recommendations in reference 18. The results of this initial model development study showed that a postexposure rise in temperature, loss of the temperature diurnal rhythm, bacteremia, and increased heart and respiratory rates, followed by a decrease in activity, strongly correlated with a lethal outcome. The pathology in the lungs of all CMs that died included pneumonia, pulmonary congestion, fibrinous pleuritis, and neutrophil infiltration. This is consistent with the pathology described for human pneumonic plague (16).

The vaccination and challenge studies described here were designed to further develop the CM model. These studies were designed to establish the relationships among rF1V vaccine doses, bridge enzyme-linked immunosorbent assay (ELISA) titers, and the survival of CMs with the goal of assessing the utility of the bridge ELISA as a correlate of protection, which is an important component of bridging clinical and nonclinical data to predict clinical benefit. Additionally, the effect of the rF1V vaccine dose on disease progression following an aerosol challenge was evaluated. These studies complete the planned development studies characterizing the CM model of pneumonic plague. The results indicate the CM is an appropriate model for demonstrating the efficacy of rF1V according to the Animal Rule.

**MATERIALS AND METHODS**

**Animals.** Indonesian-origin CMs, 80 male and 41 female, were procured from a licensed USDA-approved vendor and weighed at least 2 kg at the time of vaccination. All of the animals tested negative for the presence of preexisting titers of antibody to the rF1V vaccine antigen. They were also tuberculin test negative and seronegative for simian T-cell leukemia virus, simian immunodeficiency virus, simian retroviruses (SRV-1, SRV-2, SRV-3, and SRV-5 via PCR) and herpes simian B virus. This study was approved by the Battelle Institutional Animal Care and Use Committee and the Animal Care and Use Review Office of the U.S. Army Medical Research and Materiel Command and conducted according to the principles set forth in reference 19.

**Test and control articles.** Current good manufacturing practices grade rF1V drug product (160 μg/ml), formulated in 5 mM Na/K phosphate buffer containing 150 mM NaCl and 1.3% (wt/vol) Alhydrogel, was diluted in sterile phosphate-buffered saline (PBS) without additional adjuvant to prepare vaccine doses ranging from 0.016 to 20 μg per CM. Vaccine dilutions were prepared on each day of vaccination. All of the animals received sterile PBS.

**Study design.** Three CM studies were planned and conducted in a stagewise approach such that results from one study allowed for the design and selection of vaccine doses for subsequent studies. The first two studies were dose-ranging studies (*n* = 21 and 44) that were designed to generate a range of survival outcomes to evaluate the relationships among the vaccine dose, the antibody response, and survival. The third study (*n* = 56), an expanded efficacy study, was conducted to further evaluate these relationships with the goal of increasing statistical confidence in the study data. Group sizes and vaccine doses were selected for each study on the basis of statistical analysis of the cumulative results and on the basis of a probabilistic simulation of the most likely subsequent outcomes. For all studies, CMs were randomized into study groups according to body weight and age, with males and females randomized separately. On days 0, 56, and 121, CMs were administered vaccine or saline in a 0.5-ml volume by the intramuscular route. To control for bias, vaccine and control materials were labeled to identify each individual CM and included the vaccination day and date but not vaccine dose or study group information. Technicians performing CM vaccinations, observations, sample collections, and gross necropsies were blind to the vaccination status of the CMs. Blood samples were collected to evaluate antibody titers on days 0, 14, 28, 56, 70, 84, 98, 121, 135, and 149. Blood samples drawn on the day of vaccination and the day of challenge were collected before those activities were conducted. A target challenge dose of 229 50% lethal doses (LD_{50}) of *Y. pestis* Colorado 92 (CO92) (5.5 × 10^{6} CFU) was selected for these studies on the basis of an earlier model development study that established an LD_{50} of 24 CFU (17). Animals were challenged by aerosol inhalation on day 149 and observed for 21 days postchallenge.

**Telemetry.** Animals in the two dose-ranging studies (*n* = 65) were implanted with telemetry transmitter units (D70-PCT; Data Sciences International) for collection of body temperature, activity, and respiration data. The cardiac lead was not used and was trimmed and tied off according to the manufacturer’s instructions. Implantation surgery was performed as previously described (17). An approximately 5-week recovery period was allowed before the animals were placed in the study. To conserve battery life, telemetry units were not turned on during the vaccination period. Animals in the expanded efficacy study were not implanted with telemetry units.

**Immune response.** Bridge ELISAs were performed as previously described (20) to determine the levels of anti-rV, anti-rF1, and anti-rF1V antibodies elicited by vaccination. The bridge ELISA uses an assay format that allows direct comparison across clinical and nonclinical samples and is therefore considered species neutral. Antibody binding to the rF1V antigen coating the plate is detected by a biotinylated rF1V antigen rather than a biotinylated species-specific antibody. The assay is considered semiquantitative because the standard curve is generated with chicken IgY antibodies to allow measurement of polyclonal antibodies to *Y. pestis* antigens in serum. Results are calculated by using a four-parameter logistic curve fit. The assay’s limits of detection are 5 and 320 U/ml, with limits of quantitation between 10 and 160 U/ml.

**Challenge material.** Four days before a challenge, a vial of CO92 Working Cell Bank was thawed and streaked for isolation on Congo red agar (CRA). The plate was incubated at 26 ± 2°C for 36 to 72 h. Several CRA-pigmented colonies were selected and suspended in sterile heart infusion broth (HIB) until the optical density at 600 nm (OD_{600}) of the suspension was 0.200 ± 0.05. The suspension was diluted 1:100 into sterile HIB, and the culture was incubated at 26 ± 2°C with shaking for approximately 24 h. Bacteria were collected by centrifugation, washed, and resuspended in PBS containing 0.05% (wt/vol) gelatin and 9.7% (wt/vol) α-α-trehalose (PBSGT) to an OD_{600} of 2.56 ± 0.2, which corresponds to a reference concentration of approximately 2.05 × 10^{8} CFU/ml. The bacteria were further diluted in PBSGT to the appropriate nebulizer concentration required to achieve the target aerosol challenge.

Challenge material was characterized for titer, phenotype, purity, and Gram staining. Titer and phenotype were determined by plating dilutions of the challenge material in triplicate on tryptic soy agar (TSA) and CRA, respectively. The plates were incubated at 28 ± 2°C for 36 to 96 h. Colony counts were performed to determine titer and percent pigmentation. Purity was evaluated by streaking a sample for single-colony isolation on the following medium types: TSA with 5% sheep blood (TSAB), MacConkey agar (MAC), phenylethyl alcohol agar (PEA), and cefsulodin-irgasan-novobiocin (CIN) agar. A set of plates consisting of each medium type...
were incubated at three different temperatures, 28 ± 2°C, 37 ± 2°C, and 23 ± 2°C. Colony morphology on plates incubated at the three temperatures was recorded daily for 3 days (72 ± 2 h), with the exception of the MAC plates, which were discarded after 48 h. After 72 h, the sets of plates incubated at 28 ± 2°C and 37 ± 2°C were also discarded. The remaining set of TSAB, PEA, and CIN plates was incubated at 23 ± 2°C for an additional 11 days, and the colony morphology was recorded at 7 and 14 days. Gram staining was performed by using routine procedures.

**Aerosol challenge.** CMs were challenged on study day 149 by head-only aerosol exposure. Before exposure, each CM was anesthetized with tiletamine HCl and zolazepam HCl (Telazol; 2 to 6 mg/kg), moved into a step exposure form. Before exposure, each CM was anesthetized with tiletamine HCl and zolazepam HCl (Telazol; 2 to 6 mg/kg), moved into a step exposure form.

**RESULTS**

**Challenge material.** Characterization of the challenge material for all three studies indicated that the average concentration of the overnight cultures was 1.65 × 10⁹ CFU (range, 8.83 × 10⁸ to 5.53 × 10⁹ CFU). Further, the cultures were Gram-negative, pure cultures of *Y. pestis* with >99% pigmented colonies.

**Aerosol exposures.** The mean estimated inhaled dose of *Y. pestis* across all three studies was 5.54 × 10⁸ CFU (range, 3.97 × 10⁸ to 2 × 10⁹ CFU) which represents 231 LD₅₀ (range, 17 to 833 LD₅₀). The mass median aerodynamic diameter of the particles across all aerosol exposures ranged from 1.65 to 2.87 μm. Aerosols with these particle sizes are capable of reaching the alveoli of the lungs, which was the target area for deposition in the lung (21).

**Antibody responses.** Data from all three studies were combined to evaluate the antibody responses following vaccination. Since the kinetics of the anti-rF₁, anti-rV, and anti-rF₁V antibody responses following vaccination were similar, the results presented here focus on the anti-rF₁V antibody response as the representative response (Fig. 1). Anti-rF₁V antibody titers were measured in CMs vaccinated with doses of ≥0.4 μg beginning 2 weeks following the first vaccination. The geometric mean concentration (GMC) of anti-rF₁V antibody decreased before administration of the second dose on day 56 but was above the baseline level. A robust antibody response was observed in all groups that received >0.4 μg of rF₁V 2 weeks (day 70) following the second vaccination, with approximately 2-log increases in the GMCs of anti-rF₁V antibody compared to those at day 56. A decrease from the peak antibody titers was observed by day 84; however, anti-rF₁V antibodies did not decline to the levels observed just before the second vaccination. Administration of the third vaccination on day 121 made the GMCs of anti-rF₁V antibody slightly higher than the second vaccination. Anti-rF₁V antibodies were not detected in CMs vaccinated with rF₁V doses of ≤0.08 μg or in sham-vaccinated control CMs.

**Survival.** A summary of vaccine dose, percent survival, mean time to death, and GMCs of anti-rF₁V antibody at the time of challenge is shown in Table 1. Administration of the rF₁V vaccine to CMs on a three-dose schedule with doses ranging from 0.0016 to 20 μg demonstrated antibody-dependent survival and elicited a range of survival outcomes following a lethal aerosol challenge. A significant association between vaccine doses and survival (*P < 0.0001*) was observed, with higher vaccine doses associated with higher probabilities of survival. The proportion of CM survival in groups vaccinated with ≥4 μg was significantly greater than that of the control group, in which 2 of 20 CMs survived the challenge. Further, a statistically significant (*P < 0.0001*) vaccine dose-dependent response was observed in all three bridge ELISAs (rF₁, rV, and rF₁V). Similarly, the association between bridge...
ELISA titers (anti-rF1, anti-rV, and anti-rF1V) on day 149 and survival was statistically significant (all \( P \) values of \(<0.0001\)). Logistic-regression curves illustrating these relationships, with anti-rF1V as the representative response, are shown in Fig. 2. Pairwise comparisons assessing the time to death and overall survival of the groups show significantly later deaths in groups vaccinated with \( \geq 4 \mu g \) than in the controls (Table 2).

**Clinical observations.** Clinical signs associated with pneumonic plague, such as fever, coughing, changes in respiration, lethargy, and hunched posture, were observed during the postchallenge period. There was a statistically significant difference between survivors and nonsurvivors \( (P < 0.05) \) in the frequency of clinical signs, as determined by Fisher’s exact test (Table 3). There was a trend toward an increased incidence of the clinical signs hunched posture and lethargy with smaller doses of rF1V (data not shown).

**Telemetry.** An increase in body temperature was the first telemetry parameter exhibiting a change following a challenge. Fever was identified as an increase in body temperature of \( \geq 1.5^\circ C \) above the animal’s baseline for \( \geq 2 \) h. Mean temperature changes from the baseline plotted over time postchallenge are presented for all vaccine dose groups in Fig. 3a. Of the 65 CMs in the dose-ranging studies, 33 (25 vaccinated and 8 control) exhibited fever and 29 (21 vaccinated and 8 control) succumbed to the challenge. The mean time to onset of fever was 63 h postchallenge (range, 46.2 to 155.3 h), with a mean time to death following the onset of fever of 55.7 h (range, 33.3 to 87.8 h). Statistically significant correlations between the vaccine dose and time to onset of fever \( (P < 0.0001) \) and the time from onset of fever to death \( (P < 0.0001) \) were observed. None of the CMs vaccinated with 16 or 20 \( \mu g \) of rF1V developed fever. Beginning 49 h postchallenge, group mean temperatures of control CMs and those vaccinated with \( \leq 0.4 \mu g \) of rF1V were elevated \( 2^\circ C \). A similar increase in temperature was delayed 24 h in CMs vaccinated with 2 \( \mu g \) of rF1V.

Four vaccinated CMs developed transient fever postchallenge. Two of the CMs received vaccine doses of 0.016 and 0.4 \( \mu g \), and onset of fever occurred at 54.5 and 47.4 h, respectively, postchallenge. The other two CMs were vaccinated with 12 \( \mu g \) and developed fever at 82.3 and 155.3 h postchallenge. The body temperatures of three of the four CMs returned to the baseline by day 6 postchallenge. The fever of the fourth CM (155.3 h) resolved to the

### TABLE 1 Summary of GMCs determined by rF1V bridge ELISA and associated survival

| Group | Vaccine dose (\( \mu g \)) | GMC (U/ml) of rF1V (SD)\(^a\) | Mean time to death (h) | % Survival (no. of survivors/total) | \( P \) value (survivors vs controls) |
|-------|--------------------------|-------------------------------|------------------------|------------------------------------|-------------------------------------|
| 1     | 20                       | 11,880 (1.168)                | NA\(^b\)               | 100 (4/4)                          | 0.0071\(^c\)                        |
| 2     | 16                       | 8,090 (1.306)                 | NA                     | 100 (6/6)                          | 0.0009\(^c\)                        |
| 3     | 12                       | 5,262 (1.552)                 | NA                     | 100 (9/9)                          | <0.0001\(^c\)                      |
| 4     | 8                        | 4,156 (2.060)                 | 81.3                   | 88 (22/25)                         | <0.0001\(^c\)                      |
| 5     | 4                        | 2,027 (2.264)                 | 102.8                  | 63 (15/24)                         | 0.0023\(^c\)                       |
| 6     | 2                        | 886 (2.542)                   | 125.2                  | 29 (6/21)                          | 0.5375                             |
| 7     | 0.4                      | 50 (9.164)                    | 111.1                  | 20 (1/5)                           | 1.0000                             |
| 8     | 0.08                     | <10 (NA)                      | 118.8                  | 0 (0/3)                            | 1.0000                             |
| 9     | 0.016                    | <10 (NA)                      | 102.5                  | 25 (1/4)                           | 1.0000                             |
| 10    | Control                  | <10 (NA)                      | 88.7                   | 10 (2/20)                          | NA                                 |

\(^a\) Anti-rF1V antibody titers determined by bridge ELISA.

\(^b\) NA, not applicable.

\(^c\) Survival is significantly greater than that of the control group, as determined by Bonferroni-Holm adjusted one-sided Fisher exact test \((P \) value of \(<0.05)\).
null
by day 3 postchallenge and succumbed were statistically significantly lower than the proportion of control CMs (8/8) with positive blood cultures (all P values of <0.05). Blood cultures collected from survivors at the end of the 21-day postchallenge observation period were all negative.

All CMs that succumbed to the challenge had >10^3 CFU/g in lung, spleen, liver, and kidney tissue samples. The lungs consistently exhibited the largest quantities of bacteria, followed by the spleen, liver, and kidneys (Table 4). The differences in tissue bacterial burdens between vaccinated and control CMs that succumbed were not statistically significant, as determined by t test (P = 0.8612).

**Necropsy and histopathology.** Gross lesions in animals that died following an aerosol challenge included mottled lung discoloration and/or discolored intrathoracic lymph nodes and were present regardless of treatment (vaccinated or control). These lesions correlated with microscopic observations of suppurative inflammation, fibrin exudation, hemorrhage, necrosis, and edema in the airways and interstitium of the lungs and within the intrathoracic lymph nodes, as well as intralesional rod-shaped bac-

![Telemetry data for body temperature (a), respiratory rate (b), and physical activity (c) are shown as group mean values over 8-h intervals. Each plot shows the mean change from the baseline; the zero ordinate represents no change from normal baseline values.](image-url)
TABLE 4 Concentrations of *Y. pestis* in tissues*\(^a\)

| Tissue  | 0 (control) | 0.016 | 0.08 | 0.4 | 2   | 4   | 8   |
|---------|-------------|-------|------|-----|-----|-----|-----|
| Spleen  | 5.15 \times 10^7 | 6.46 \times 10^7 | 6.76 \times 10^7 | 3.03 \times 10^7 | 3.74 \times 10^6 | 3.58 \times 10^6 | 1.41 \times 10^6 |
| Liver   | 1.79 \times 10^7 | 3.56 \times 10^7 | 5.86 \times 10^6 | 9.98 \times 10^6 | 1.42 \times 10^7 | 1.89 \times 10^7 | 3.04 \times 10^6 |
| Kidney  | 2.37 \times 10^6 | 8.73 \times 10^6 | 1.6 \times 10^5 | 1.68 \times 10^6 | 1.25 \times 10^6 | 6.86 \times 10^5 | 6.25 \times 10^5 |
| Lung    | 5.4 \times 10^6 | 1.69 \times 10^7 | 1.49 \times 10^6 | 3.18 \times 10^7 | 9.64 \times 10^6 | 1.03 \times 10^8 | 9.46 \times 10^8 |

*\(^a\) Mean concentrations of *Y. pestis* in tissues were determined at the time of necropsy.

TABLE 5 Incidence of microscopic lesions in lungs and lymph nodes

| Tissue  | No. of CMs with lesions/total (%) | 0 (control) | 0.016 | 0.08 | 0.4 | 2   | 4   | 8   |
|---------|----------------------------------|-------------|-------|------|-----|-----|-----|-----|
| Lung    | 18/20 (100)                      | 3/4 (75)    | 3/3 (100) | 4/5 (80) | 15/21 (71) | 9/24 (38) | 3/25 (12) |
| Lymph nodes |                        |             |       |      |     |     |     |     |
| Bronchial | 11/20 (55)                     | 1/4 (25)    | 1/3 (33) | 1/5 (20) | 12/21 (57) | 5/24 (21) | 1/25 (4)   |
| Mediastinal | 12/20 (60)                    | 0/4 (0)     | 0/3 (0)  | 0/5 (0)  | 8/21 (38)  | 5/24 (21)  | 1/25 (4)   |

*\(^a\) Animals vaccinated with \(\geq 12\) μg of rF1V survived a challenge, and lesions were not observed at the time of necropsy (end of the postchallenge observation period). Lesions were not observed in the two surviving controls.

DISCUSSION

The rF1V efficacy studies described here were conducted to further develop the CM model for use in testing the efficacy of rF1V according to the requirements of the FDA Animal Rule. Vaccination with rF1V elicited an immune response that provided protection from a lethal aerosol challenge with *Y. pestis*. Our study results are similar to those reported by other groups conducting proof-of-concept studies showing that rF1- and rV-based vaccines against plague elicit protective immune responses in CMs following an aerosol challenge (15, 22, 23). However, in contrast to these proof-of-concept studies, which mostly used only one vaccine dose, our studies evaluated a broad range of vaccine doses. This provided the opportunity to evaluate the relationship between vaccine dose and bridge ELISA titers across a range of survival outcomes and allowed us to identify statistically significant correlations among vaccine doses, anti-rF1V antibody titers, and the probability of survival. Combining all of our study data provided a statistically precise estimate of these relationships, supporting the use of rF1V bridge ELISA antibody titers as a correlate of protection.

Our earlier model development study (17) identified fever, as measured by telemetry, as the first clinical sign of disease following an aerosol challenge with CO92. Following the onset of fever, increased respiration and a decreased activity level (as measured by telemetry), a hunched posture, and bacteremia were observed in CMs that succumbed following a challenge. Therefore, in the present studies, we sought to determine whether rF1V vaccination delayed the onset or ameliorated signs of pneumonic plague in CMs.

Four vaccinated CMs in the dosage-ranging studies developed transient fever but survived a challenge. Two of these CMs received small vaccine doses (0.0016 and 0.4 μg) and were bactereemic on day 3 but tested negative on all subsequent days. At the time of the challenge, the CM vaccinated with 0.0016 μg of rF1V had no detectable anti-rF1V antibodies, whereas the CM vaccinated with 0.4 μg of rF1V had an anti-rF1V antibody titer of 127 U/ml. Given the low anti-rF1V antibody titer in one CM and the lack of antibodies in the other, the survival of these two CMs is believed to be associated with individual animal variability. The other two surviving CMs were vaccinated with 12 μg of rF1V and never developed bacteremia. The anti-rF1V antibody titers in these two CMs were 3,584 and 7,290 U/ml at the time of challenge, which suggests the immune response elicited by rF1V limited the infection and thus prevented bacteremia.

Postchallenge clinical observations were normal in two of the four CMs with transient fever, whereas the other two CMs exhibited diarrhea and/or occasional hunched posture. In the CM vaccinated with 0.4 μg, hunched posture was coincident with a transient fever. The occurrence of diarrhea in the other CM was observed periodically during the vaccination period, as well as the postchallenge observation period, and therefore not considered study related. This CM also occasionally exhibited a hunched posture postchallenge, but it was not associated with transient fever.

The relevance of these clinical observations in the four surviving CMs is not known. Hunched posture, most often observed just before death, was accompanied by other clinical signs such as respiratory distress, lethargy, cough, or not eating and was consistent with observations in our model development study (23). Dose-dependent vaccine responses were associated with delays in other parameters measured by telemetry in the dosage-ranging studies, such as an elevated respiratory rate and decreased activity. This provides further support that rF1V vaccination can provide protection against pneumonic plague.
All of the CMs in the dosage-ranging and expanded efficacy studies that became bacteremic, with the exception of the two animals discussed above, succumbed to a challenge. There was no significant difference in the proportions of animals having positive blood cultures between the small-dose vaccine groups (<2 μg) and the control group, suggesting that these small vaccine dosages provided little benefit in ameliorating disease. Further, the concentrations of *Y. pestis* in the lungs, livers, spleens, and kidneys of vaccinated and control CMs that succumbed to a challenge were not different between these groups. This suggests that once the bacteria reach the bloodstream and disseminate to these organs, low-level vaccine dosages do not induce an immune response sufficient to combat infection.

The observed survival of 2 of 12 control CMs in the expanded efficacy study was surprising, given the estimated dosages of 272 LD$_{50}$ (6.52 × 10$^3$ CFU) and 301 LD$_{50}$ (7.22 × 10$^3$ CFU) inhaled by these animals and that survival of control animals was not observed in the two dosage-ranging studies. An examination of the aerosol exposure system parameters indicated that the system operated as expected during the challenge of these CMs. Neither animal had anti-rF1V titers at the baseline or at any of the collection points throughout the vaccination period. However, bridge ELISA titers measured at the end of the 21-day postchallenge observation period in these CMs yielded anti-rF1V antibody titers of 376 and 1,480 U/ml, indicating that the animals were exposed to *Y. pestis* and mounted an immune response following the challenge. Other groups have also reported observations of control survival during vaccine efficacy studies conducted with CMs. In a study by Cornelius et al. (22), one of four control CMs survived a challenge with an inhaled dose of 1.45 × 10$^4$ CFU (216 LD$_{50}$) of CO92, while three animals that succumbed to the challenge received inhaled doses ranging from 1.27 × 10$^4$ to 2.3 × 10$^4$ CFU (189 to 343 LD$_{50}$). Quenee et al. (24) reported 25 and 50% survival of control CMs following exposure to 250 and 50 LD$_{50}$ respectively. Individual challenge data and assessments of rF1V antibody titers pre- and postchallenge were not provided for all of the control animals in these studies, making interpretation of the data difficult. On the basis of these observations, designers of efficacy studies with CMs should consider low-level control survival in determining group size to ensure that the studies are appropriately powered to determine survival differences between vaccinated and control groups.

Our studies describe the further development of the CM model to evaluate rF1V vaccine efficacy. Data generated in these studies determined that bridge ELISA titers and vaccine dose were highly statistically significant predictors of protection (*P* < 0.0001) and support the use of anti-rF1V bridge ELISA titers as a correlate of protection. Vaccination with rF1V was shown to ameliorate disease in a dose-dependent manner. The data support the use of the CM model and provide important information that will be used to design future pivotal efficacy studies that will play an important role in vaccine licensure based on the FDA Animal Rule.

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