Live Attenuated Mutants of *Francisella tularensis* Protect Rabbits against Aerosol Challenge with a Virulent Type A Strain

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*Francisella tularensis*, a Gram-negative bacterium, is the causative agent of tularemia. No licensed vaccine is currently available for protection against tularemia, although an attenuated strain, dubbed the live vaccine strain (LVS), is given to at-risk laboratory personnel as an investigational new drug (IND). In an effort to develop a vaccine that offers better protection, recombinant attenuated derivatives of a virulent type A strain, SCHU S4, were evaluated in New Zealand White (NZW) rabbits. Rabbits vaccinated via scarification with the three attenuated derivatives (SCHU S4 ΔguaBA, ΔaroD, and ΔfipB strains) or with LVS developed a mild fever, but no weight loss was detected. Twenty-one days after vaccination, all vaccinated rabbits were seropositive for IgG to *F. tularensis* lipopolysaccharide (LPS). Thirty days after vaccination, all rabbits were challenged with aerosolized SCHU S4 at doses ranging from 50 to 500 50% lethal doses (LD50). All rabbits developed fevers and weight loss after challenge, but the severity was greater for mock-vaccinated rabbits. The ΔguaBA and ΔaroD SCHU S4 derivatives provided partial protection against death (27 to 36%) and a prolonged time to death compared to results for the mock-vaccinated group. In contrast, LVS and the ΔfipB strain both prolonged the time to death, but there were no survivors from the challenge. This is the first demonstration of vaccine efficacy against aerosol challenge with virulent type A *F. tularensis* in a species other than a rodent since the original work with LVS in the 1960s. The ΔguaBA and ΔaroD SCHU S4 derivatives warrant further evaluation and consideration as potential vaccines for tularemia and for identification of immunological correlates of protection.

Tularemia (also known as rabbit fever) is a disease caused by *Francisella tularensis*, a Gram-negative coccobacillus (1). Human disease has been noted primarily with two subspecies, *Francisella tularensis* subsp. *tularensis* (type A) and *Francisella tularensis* subsp. *holarctica* (type B), of which *F. tularensis* subsp. *tularensis* is considered the more virulent. Transmission can occur by contact with infected animals or tissues, ingestion, arthropod vectors, or inhalation. In humans, pneumatic tularemia is marked by fever and influenza-like symptoms, rapidly progressing to septicemia and potentially death if untreated. Because of the low infectious dose when inhaled and the severity of disease, *F. tularensis* is considered a tier 1 select agent by the CDC.

Initial attempts to develop a tularemia vaccine focused on a killed vaccine, which was ineffective (2, 3). Subsequently, an attenuated type B strain of *F. tularensis* obtained from the former Soviet Union was passaged *in vitro* and evaluated as a vaccine. Dubbed the live vaccine strain (LVS), this vaccine was safe and immunogenic in both animals and humans (4–13). Further, LVS provided protection in nonhuman primates and humans against aerosol challenge with a virulent type A strain, SCHU S4, although this was vaccine and challenge dose dependent (7, 14–16). Because LVS was attenuated by passage in culture, there is uncertainty regarding the potential for reversion. There have been studies evaluating inactivated LVS combined with antibody or adjuvants, which have shown protection with mouse models (17, 18). Killed and subunit vaccine approaches are considered less desirable because it can be more difficult to generate strong immune responses and the responses are typically less durable, requiring more frequent boosting. For these reasons, a more defined live attenuated vaccine candidate conferring a high level of efficacy is strongly desired.

Recent efforts to develop a tularemia vaccine have focused on live attenuated vaccines using site-specific deletion of virulence genes (3, 19–27). Studies have identified a number of mutations that attenuate *F. tularensis* to various degrees. Some of these mutants have been evaluated as vaccines in mice for protection against virulent strains. In general, these mutants have been successful in protecting mice against parenteral challenge and low-dose respiratory challenge. We report here the first efforts to advance these potential vaccines into use with the rabbit model, which we have previously shown is a relevant model of human pneumonic tularemia (28).

**MATERIALS AND METHODS**

**Biosafety and regulatory information.** All work with live *F. tularensis* was conducted at biosafety level 3 (BSL-3) in the University of Pittsburgh Regional Biocontainment Laboratory (RBL). For respiratory protection, all personnel wore powered air-purifying respirators (3M GVP-1 PAPR with L-series bumpcap) or used a class III biological safety cabinet. Vespene II se detergent (1:128 dilution; Steris Corporation, Erie, PA) was used to disinfect all liquid wastes and surfaces associated with the agent. All solid wastes, used caging, and animal wastes were steam sterilized. Animal carcasses were digested via alkaline hydrolysis (Peerless Waste Solutions, Holland, MI). The University of Pittsburgh Regional Biocon-
Tularemia Laboratory is a registered entity with the CDC/USDA for work with *F. tularensis*.

**Rabbits.** Young female New Zealand White (NZW) rabbits were housed in the University of Pittsburgh Regional Biocontainment Laboratory (RBL) at animal biosafety level 3 (ABSL3) for the duration of the studies. Prior to vaccination, IPTT-300 temperature/ID chips (BioMedic Data Systems, Seaford, DE) were implanted subcutaneously. Body weight was recorded once in the morning, and body temperature was recorded twice daily. Temperature was read using a DAS-7000 reader (BioMedic Data Systems). All studies were approved by the University of Pittsburgh’s Institutional Animal Care and Use Committee.

**Bacteria.** For aerosol exposures, virulent *F. tularensis* strain SCHU S4 was grown as previously described using brain heart infusion (BHI) broth (28, 29). After the exposures were completed, nebulizer and all-glass impinger (AGI) contents were quantified on cysteine heart agar (CHA). Stocks of attenuated mutants of SCHU S4 and LVS were generated previously and stored at −80°C. SCHU S4 Δ*aroD* contains an unmarked deletion in the FFTT1103 gene, as described previously (30, 31). SCHU S4 Δ*guaBA* and SCHU S4 Δ*aroD* contain unmarked complete deletions and were constructed by allelic exchange as described previously (23).

**Vaccination.** Rabbits were vaccinated by scarification. Rabbits were anesthetized by subcutaneous injection of ketamine (80 mg/kg of body weight) and xylazine (8 mg/kg); once anesthesia was confirmed, the rabbit was bled and a small area of the dorsal surface was shaved. Approximately 0.1 ml of bacteria at a concentration of 1 × 10^9 CFU/ml were placed in a drop on the shaved area, and a bifurcated needle (Becton, Dickinson) was jabbed through the drop of bacteria into the skin 17 times. The drop was allowed to absorb into the skin, after which the xylazine was reversed by intramuscular (i.m.) injection of 0.2 to 1 mg/kg yohimbine. The scarification site was monitored daily for the first 7 days after vaccination.

**ELISA.** Enzyme-linked immunosorbent assays (ELISA) were performed using standard ELISA procedures. Briefly, dilutions of rabbit sera were incubated for 1 h at 37°C on 96-well plates coated with purified LPS from *Francisella tularensis* LVS (BEI Resources, Manassas, VA). After washing with PBS-Tween, secondary goat anti-rabbit IgG-horseradish peroxidase (HRP) (Fitzgerald Industries, Acton, MA) was added to the plates and incubated for 1 h at 37°C, after which the plates were washed again with phosphate-buffered saline (PBS)-Tween, and BM chemiluminescence ELISA substrate (Roche Applied Sciences, Indianapolis, IN) was added to the plates. Plates were then read on an Lmax plate reader ( Molecular Devices, Sunnyvale, CA). Endpoint titers were determined as the inverse of the highest dilution with a luminescence reading above 3 times the standard dilution of control sera.

**Aerosol challenge.** Aerosols were conducted inside a class III biological safety cabinet (Baker Co., Sanford, ME) located inside the RBL as previously described (28). Briefly, rabbits were exposed two at a time for 20 min in a nose-only exposure chamber (CH Technologies, Westwood, NJ) using a 3-jet Collison nebulizer controlled by the AeroMP exposure system (Baera Technologies, Hagerstown, MD) while plethysmography data were collected in real time using Buxco XA software (Buxco Research Systems, Wilmington, NC) during the exposure. The aerosol concentration at the exposed (presented) dose were determined as described previously (28, 32).

**Statistical methods.** Data were collected and organized using spreadsheets in the software program Microsoft Excel 2007; graphing and statistical analyses, including one-way analysis of variance (ANOVA) and *t* tests, were conducted using the GraphPad Prism 6 program.

**RESULTS**

**Response to vaccination.** Over the course of three experiments, groups of rabbits were inoculated by scarification either with PBS (mock-vaccinated controls), LVS, or a recombinant derivative of SCHU S4 (SCHU S4 Δ*guaBA*, SCHU S4 Δ*aroD*, or SCHU S4 Δ*fpB* [ΔFFT1103]). All vaccines were well tolerated by the rabbits. A spike in body temperature was seen 1 day after vaccination, with an average temperature of 40.1°C among vaccinated rabbits (*n* = 36), compared to 38.5°C for mock-vaccinated controls (*n* = 17) (Fig. 1). The average day 1 fever was highest for rabbits that received the Δ*aroD* (40.5°C) vaccines, followed by the LVS group (40.2°C), the Δ*guaBA* strain group (39.5°C), and the Δ*fpB* strain group (39.4°C). The difference in temperature on day 1 for vaccinated rabbits was significantly different (*P* < 0.05) from that for mock-vaccinated rabbits for all groups except the Δ*fpB* strain group (Fig. 1F). Subsequent temperatures for vaccinated rabbits were not significantly different from those for mock-vaccinated rabbits throughout the first 14 days of the postvaccination period.

In humans, development of an erythematous eschar at the vaccination site is considered a good “take” with LVS (33). One week after vaccination, the site was examined (Fig. 2). All vaccinated rabbits developed a notable eschar at the vaccination site, while the site on mock-vaccinated rabbits appeared normal. Fourteen days after vaccination, no visible signs of the eschar remained and the rabbits appeared otherwise healthy (data not shown). No weight loss or changes in behavior or food and water consumption were observed after vaccination for any rabbit (data not shown).

Rabbits were bled at 21 days postvaccination and evaluated for serum levels of IgG to *F. tularensis* LVS endotoxin (*F. tularensis* LPS) (Fig. 3A and Table 1). Only a selected subset of each group was evaluated for serum antibody. Out of seven mock-vaccinated animals evaluated, one had a positive titer of IgG to *F. tularensis* LPS (1:165; positive was defined as an endpoint titer of >1:50) at 21 days after vaccination, while all vaccinated rabbits evaluated (*n* = 20) had serum IgG titers of >1:500, with a geometric mean of 1:3,660 across all vaccines tested. Rabbits vaccinated with the Δ*guaBA* strain (1:4,963) or the Δ*aroD* (1:4,656) strain had the highest geometric mean IgG titers among vaccine groups, although the differences noted between vaccine groups were not significant.

**Response to challenge.** Thirty days after vaccination, all of the rabbits were aerosol challenged with virulent *F. tularensis* strain SCHU S4 at doses between 1,000 (~40 50% lethal doses [LD₅₀]) and 10,000 CFU (~400 LD₅₀) depending on the experiment. Survival of rabbits did not correspond with the challenge dose (data not shown), so the data for all three studies are grouped together. While mock-vaccinated rabbits succumbed to the challenge between days 4 and 6, rabbits vaccinated with LVS or the Δ*fpB* strain survived longer (average time to death of 7.0 or 6.4 days, respectively) (Fig. 3B and Table 1), although all ultimately succumbed, with the last LVS-vaccinated rabbit dying on day 10 after challenge. Rabbits vaccinated with the Δ*aroD* or Δ*guaBA* strain showed a prolonged survival time (6.7 and 7.3 days, respectively), but unlike the case with LVS or the Δ*fpB* strain, a portion of the rabbits that received those vaccines (27 to 40%) survived to 28 days postchallenge. For all of the vaccine groups except the Δ*fpB* strain group, the average time to death was significantly different from that for the mock-vaccinated controls as determined by one-way ANOVA using Dunnett’s test (Table 1).

In mock-vaccinated rabbits, fever began on day 2, peaked on days 3 and 4 after challenge, and then started to decline on days 5 and 6 (Fig. 4). For rabbits vaccinated with the Δ*guaBA* strain, the onset of the febrile response was similar to that for mock-vaccinated controls, but at days 3 and 4 postchallenge, the febrile response was lower than that for mock-vaccinated rabbits; fever peaked on day 5 and gradually declined to within normal limits on day 12 (Fig. 4A). A similar response was seen in the Δ*aroD* strain.
vaccination group (Fig. 4B). In the ΔfipB strain group, rabbits developed a fever slightly before mock-vaccinated controls, although the peak fever response was not until days 5 and 6, after that seen in mock-vaccinated controls (Fig. 4C). LVS-vaccinated rabbits had a fever onset and peak very similar to those of mock-vaccinated rabbits, although the fever did not decline prior to LVS-vaccinated rabbits succumbing to infection (Fig. 4D). Although the temperature profiles suggested a lower overall febrile

FIG 1 Fever response to scarification with live attenuated strains of F. tularensis. NZW rabbits were vaccinated by scarification on the dorsal surface. Body temperature was recorded twice per day for 14 days (d) after vaccination. Graphs show average temperatures for rabbits vaccinated with the ΔguaBA (A), ΔaroD (B), of ΔfipB (C) strain or LVS (D) compared to those for mock-vaccinated controls. (E) Body temperatures 24 h after vaccination for individual rabbits in each group (symbols), along with the mean temperature for each group (solid line) and the standard deviations (error bars). Data from three experiments were combined.
response in vaccinated rabbits, there was no difference between peak temperatures seen in vaccinated rabbits and those in mock-vaccinated rabbits (Table 1). In contrast, when only day 4 body temperatures were compared, mock-vaccinated rabbits had a significantly higher fever than any of the vaccine groups except the LVS group. Surviving vaccinated rabbits did have a significantly lower peak temperature than either mock-vaccinated or nonsurviving vaccinated rabbits (data not shown).

All mock-vaccinated controls lost weight beginning on day 3 and continuing until they succumbed to the infection (Fig. 5). In the ΔguaBA and ΔaroD strain-vaccinated groups, rabbits lost weight more slowly and lost less weight overall than mock-vaccinated rabbits (Fig. 5A and B). In contrast, rabbits in the ΔfipB and LVS groups lost weight as rapidly as controls through at least the first 4 days after challenge (Fig. 5C and D). Vaccinated rabbits that ultimately succumbed to challenge lost more weight than mock-vaccinated rabbits (data not shown). This increased weight loss in nonsurviving vaccinated rabbits was likely a function of their prolonged survival relative to that of the mock-vaccinated rabbits (data not shown). There was no difference in maximum weight lost between vaccine groups and the mock-vaccinated rabbits, and weight loss on day 4 was significantly different from that of mock-vaccinated rabbits only for the ΔguaBA strain vaccination group (Table 1). Among vaccinated rabbits, weight loss was significantly less for surviving rabbits than for those that succumbed (data not shown).

**DISCUSSION**

There have been a number of efforts to develop vaccines for tularemia to replace LVS, although there are no reports in the published literature since the 1960s evaluating tularemia vaccines other than LVS with any model other than a rodent (3). This is the first report that we are aware of demonstrating any protection (defined as survival of ≥28 days after challenge) with the rabbit
model against aerosol challenge with SCHU S4, a virulent type A strain of \textit{F. tularensis} and the prototype strain for tularemia studies. The vaccines evaluated here had all demonstrated safety, immunogenicity, and protection with murine models prior to being evaluated for rabbits (23, 30, 34). Rabbits were chosen for these studies as an intermediary between murine and nonhuman primate studies; the disease was originally known as rabbit fever, and rabbits have been shown to be a relevant model of the human disease. LVS was included in these challenge studies because new vaccine candidates would need to be at least as safe as LVS while providing better protection than LVS to warrant further consideration as a human vaccine. LVS has been shown to be safe and immunogenic in rats, rabbits, nonhuman primates, and humans (3, 4, 7, 14, 15, 35–38). LVS is the only tularemia vaccine that was

### Table 1: Survival of rabbits after aerosol challenge with SCHU S4

<table>
<thead>
<tr>
<th>Vaccination group</th>
<th>No. of survivors/total</th>
<th>% survival</th>
<th>Time to death (days)</th>
<th>D4 PC temp (°C)</th>
<th>Peak PC temp (°C)</th>
<th>D4 PC wt loss (%)</th>
<th>Peak PC wt loss (%)</th>
<th>D21 ELISA</th>
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<tr>
<td>Mock</td>
<td>0/17</td>
<td>0.0</td>
<td>4.8</td>
<td>41.0</td>
<td>41.2</td>
<td>−8.0</td>
<td>−8.9</td>
<td>1.6</td>
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<tr>
<td>(\Delta\text{guaBA} \text{strain})</td>
<td>3/11</td>
<td>27.3</td>
<td>7.3**</td>
<td>40.3**</td>
<td>40.8</td>
<td>−4.1*</td>
<td>−9.3</td>
<td>3.7**</td>
</tr>
<tr>
<td>(\Delta\text{aroD} \text{strain})</td>
<td>4/11</td>
<td>36.4</td>
<td>6.7*</td>
<td>40.3**</td>
<td>41.0</td>
<td>−4.9</td>
<td>−11.0</td>
<td>3.7**</td>
</tr>
<tr>
<td>(\Delta\text{fipB} \text{strain})</td>
<td>0/5</td>
<td>0.0</td>
<td>6.4</td>
<td>40.2*</td>
<td>40.9</td>
<td>−6.0</td>
<td>−15.6</td>
<td>3.4</td>
</tr>
<tr>
<td>LVS</td>
<td>0/9</td>
<td>0.0</td>
<td>7.0**</td>
<td>40.9</td>
<td>41.4</td>
<td>−8.1</td>
<td>−13.8</td>
<td>3.4**</td>
</tr>
</tbody>
</table>

\(**\), significantly different from results for controls as determined by one-way ANOVA (\(P < 0.05\)); \(*\), highly significantly different from results for controls as determined by one-way ANOVA (\(P < 0.0001\)). PC, postchallenge.

**Time to death does not include survivors.

**D4 PC, day 4 postchallenge or day 34 postvaccination.

**Endpoint mean log10 titer of anti-LPS.

### Figure 4: Fever response after aerosol challenge

Thirty days after vaccination, rabbits were challenged by aerosol exposure to \textit{F. tularensis} strain SCHU S4. Body temperature was recorded twice per day for 14 days after challenge. Graphs show average temperatures for rabbits vaccinated with the \(\Delta\text{guaBA}\) (A), \(\Delta\text{aroD}\) (B), or \(\Delta\text{fipB}\) (C) strain or LVS (D) compared to those for mock-vaccinated controls. Data from three experiments were combined.
formally evaluated in human efficacy trials conducted in the 1960s (7, 8, 10, 13, 14, 39). LVS was found to provide at least partial protection against aerosol exposure to SCHU S4, although the level of protection was dependent on the dose and route of vaccination as well as the challenge dose.

In addition to concerns about protection against aerosol challenge, the potential for reversion has been a concern raised against LVS as a human vaccine (3). All of the vaccines tested here, including LVS, were attenuated in rabbits, producing at most a mild fever response no more than 1 day in duration and a small eschar at the site of vaccination but no other clinical signs indicative of disease. Attenuation, the potential for reversion, and subsequently safety are significant concerns with any live vaccine. It should be noted that other efforts at evaluating inactivated LVS combined with antibody or adjuvant as well as subunit vaccines have shown efficacy in mice (17, 18, 40). While killed and subunit vaccines are safer, they typically do not generate as robust an immune response (as live vaccines), requiring high doses to achieve equivalent protection, and more frequent booster immunizations are required to maintain protective immunity. Further, the abject failure of the killed Foshay vaccine to protect against tularemia has created a perception that killed or subunit vaccines will not provide sufficient protection against tularemia (4).

All of the vaccines proved to be immunogenic as determined by IgG titers against the LVS endotoxin in an ELISA. While this assay demonstrated that the rabbits responded to the vaccine, it did not distinguish between rabbits that survived challenge and those that succumbed. Some studies have shown that humoral immune responses can play a role in protecting against tularemia, including protection against virulent type A strains (35, 40, 41). Antibody may also be useful as a correlate of protection even if it is not the primary mechanism of protection. We continue to refine this assay in the hopes of identifying antibody responses that do correspond with survival. Based on the fever responses seen in surviving rabbits, these vaccine candidates did not confer sterilizing immunity, which would likely be antibody mediated. Cell-mediated immune (CMI) responses are considered to be of prime importance in protection against tularemia, including production of interleukin 6 (IL-6), IL-12, and gamma interferon (IFN-γ) (42–46). However, it is unclear how CMI responses confer protection against tularemia, and the antigens important for generating good CMI are not clear. The lack of reagents makes studying rabbit cellular immune responses difficult but not impossible. In studies under way, we are evaluating proliferation as a potential surrogate of cellular immune responses. We are also pursuing evaluation of other antigens as

FIG 5 Weight loss after aerosol challenge. Thirty days after vaccination, rabbits were challenged by aerosol exposure to F. tularensis strain SCHU S4. Body weight was recorded daily for 14 days after challenge. Graphs show average daily change in body weight for rabbits vaccinated with the ΔguaBA (A), ΔaroD (B), or ΔfipB (C) strain or LVS (D) compared to that for mock-vaccinated controls. Data from three experiments were combined.
targets in ELISAs. Further study is needed to better understand the mechanism that protect against pneumonic tularemia and potential correlates of protection.

It has been suggested from findings of murine studies that a delayed inflammatory response to infection with F. tularensis might be partly responsible for the pathogenesis and virulence of the disease. It was interesting that in the rabbits, there was no difference between vaccinated and mock-vaccinated groups in terms of fever onset, but there were notable and significant differences in the fever peak and in how quickly the rabbits lost weight. These differences in clinical signs between vaccinated rabbits and mock-vaccinated rabbits suggest differences in the activation of the immune response to infection; in particular, the higher fever and weight loss on day 4 in mock-vaccinated rabbits suggest an overzealous proinflammatory response that might contribute to pathogenesis. More research is needed to understand the differences in the mechanisms underlying the observed clinical signs and the implications for pathogenesis and outcome of pneumonic tularemia.

All of the vaccines evaluated prolonged the time to death following challenge for rabbits relative to that for mock-vaccinated controls. The extension in time to death but not survival with LVS vaccination was not surprising, since this had been reported previously (47). What was surprising was that two of the three SCHU S4 attenuated strains did protect rabbits (27 and 36% survival with the ΔguaBA and ΔaroD strains, respectively) even against ~500 LD$_{50}$ of virulent SCHU S4. It should be noted that the challenge doses used in these studies (50 to 500 LD$_{50}$) were robust, especially compared to what is typically used in murine studies. Most murine studies that have demonstrated survival (instead of prolongation in time to death) after challenge with a type A strain have employed low challenge doses (in the range of 10 to 30 LD$_{50}$) (19–21, 27, 38). Studies evaluating tularemia vaccines in rats have used higher challenge doses, but rats are known to have a higher innate resistance to F. tularensis than either mice or rabbits (36, 48, 49). Based on our prior data and the data presented here, we feel that rabbits occupy a niche between mice and rats in terms of their susceptibility to F. tularensis. While rabbits are slightly more resistant to SCHU S4 challenge than mice (an LD$_{50}$ of 23 CFU for rabbits, compared to 3 CFU for mice), they are extremely resistant to LVS (LD$_{50}$ ≫ 10$^6$ CFU by aerosol; D. S. Reed, unpublished data), while the LD$_{50}$ for LVS in mice is ~1 × 10$^5$ CFU (29). In our opinion, the greater sensitivity of the rabbit model to type A strains but not to LVS makes the rabbit a useful model for evaluating safety and efficacy prior to nonhuman primate studies. The partial protection seen against the high challenge doses used in these studies warrants further consideration of these vaccines. Additional studies are needed, however, to elucidate which of these models is the most relevant to the human disease and the protection mediated by a vaccine.

The data reported in this study demonstrate that attenuated strains derived from a virulent F. tularensis type A strain can be safe and immunogenic and provide at least partial protection in a rabbit model against a robust aerosol challenge with SCHU S4. Studies in the 1960s with LVS demonstrated better protection against aerosol challenge with SCHU S4 in both men and nonhuman primates after oral or respiratory vaccination (6–8). Further evaluation of these attenuated strains is necessary to evaluate whether changes in the dose, route, and schedule of the vaccinations might improve the protection seen. Additional research is needed to understand the immunological mechanisms responsible for protection and potential correlates of protection, information that will be critical for supporting the advanced development of these vaccines, since pivotal efficacy studies will have to be done in animals under the auspices of the FDA’s Animal Rule (50, 51). These results, however, highlight the utility of this approach in generating a vaccine for pneumonic tularemia and demonstrate that a tularemia vaccine can protect against a high-dose challenge of aerosolized virulent F. tularensis.

ACKNOWLEDGMENTS

The research described herein was sponsored by the National Institutes of Health, grant U01 AI077909-01. We thank the Division of Laboratory Animal Resource’s veterinary technicians who work in the University of Pittsburgh and assisted with these studies.

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