Development and Characterization of Mouse Models of Infection with Aerosolized *Brucella melitensis* and *Brucella suis*[▽]

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There is a need to identify vaccines that can protect against *Brucella*, a potential bioterrorism agent. We have developed mouse models of infection with aerosolized *Brucella melitensis* and *Brucella suis* and demonstrated their utility for the evaluation of vaccines using the model live *B. melitensis* vaccine strain Rev.1.

Brucellosis is a zoonotic disease that is caused by *Brucella* species. Four species, *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, and *Brucella canis*, are currently known to be pathogenic to humans (1, 5, 14). In animals, brucellosis can have a huge economic impact, since infection can lead to abortions, stillbirths, and the loss of fertility in livestock. In comparison, brucellosis in humans is a debilitating disease characterized by fever, sweats, and aches. In approximately 5% of cases it can be fatal when complications, usually endocarditis, arise (4). The illness can last a number of weeks, and even with antibiotic treatment, relapses can occur. Brucellosis is common in developing countries and areas without effective animal disease control policies. In these countries, the microorganisms are usually transmitted through ingestion, inhalation, or direct skin contact. Unpasteurized milk is a common source of infection, as is inhalation from carcasses among abattoir workers (5, 11). There are several live attenuated vaccines licensed for use in animals. Of these, the most widely used are *B. melitensis* Rev.1 and *B. abortus* S19 or RB51 (reviewed in reference 17). These vaccines are unsuitable for use in humans since they are insufficiently attenuated and still cause disease (1, 17).

Brucellosis is one of the most-common laboratory-acquired infections; it is readily aerosolized and highly infectious. *Brucella* species have been considered potential biological warfare agents and are classed as category B threat agents (15). *B. suis* was the first agent weaponized by the United States, in 1952 (6). Furthermore, there are also claims that *Brucella* bacteria were used by the Japanese Manchuria Unit and were developed by the former Soviet Union Biopreparat offensive biological weapon program (15). Since it is possible that a bioterrorist attack with *Brucella* bacteria would result in aerosolized bacteria causing inhalational infection, there is a requirement to develop and utilize appropriate animal models of aerosolized *Brucella* infection in order to evaluate the efficacy of vaccines or therapies for human brucellosis.

A small-rodent model of brucellosis offers advantages over the use of larger animals for preliminary studies, including the relative ease of use, ethical acceptability, and cost. Here we describe the development and characterization of mouse models of infection with aerosolized *B. melitensis* and *B. suis* bacteria and demonstrate the utility of these models in evaluating vaccines and therapeutics for inhalational brucellosis using the model live attenuated *B. melitensis* Rev.1 vaccine. A mouse model of intranasal (i.n.) infection with *B. melitensis* has previously been described (12), and since we started this work, two other laboratories have described studies of infection of mice with aerosolized *Brucella* bacteria. Kahl-McDonagh et al. described aerosol infection of BALB/c mice with *B. abortus* 2308 and *B. melitensis* 16 M and the use of these models for the evaluation of protective efficacy of deletion mutants (10). Olsen et al. similarly described the infection of BALB/c mice with aerosolized strain 16 M or 2308 and demonstrated that vaccination with the live attenuated animal vaccine *B. abortus* RB51 provided protection against intraperitoneal but not aerosolized *B. abortus* challenge (13). Our findings both support and extend the data generated in these studies.

Infection of mice with aerosolized *B. melitensis* 16 M bacteria. In all experiments described in this study, mice were handled in accordance with the Animal (Scientific Procedures) Act (1986). In order to establish the optimal dose of aerosolized *B. melitensis* 16 M bacteria required for infection in BALB/c mice, groups of 12 animals (6 to 7 weeks old; Charles River Laboratories) were exposed to retained doses of 10^2, 10^3, 10^4, or 10^5 CFU of aerosolized *B. melitensis* 16 M bacteria. For aerosolization, *B. melitensis* 16 M bacteria (obtained from the culture collection at the Veterinary Laboratories Agency, Weybridge, United Kingdom) were grown to stationary phase in *Brucella* broth at 37°C. Bacteria were aerosolized by using a Collison atomizer and conditioned within a Henderson apparatus (2, 9). Mice were exposed for 10 min, and impinger samples were collected for 1 min during each exposure, enabling viable counts following serial dilution and routine culture on chocolate agar plates. Calculations were used to determine the retained dose of bacteria inhaled by each mouse (7, 8). Thirty days before exposure, six animals per group were immunized via the subcutaneous (s.c.) route with the model live attenuated vaccine *B. melitensis* strain Rev.1. After 14, 21, and 28 days, immunized and nonimmunized mice were culled and the bacterial loads in the spleens were determined. Spleens were homogenized in phosphate-buffered saline using the barrel of

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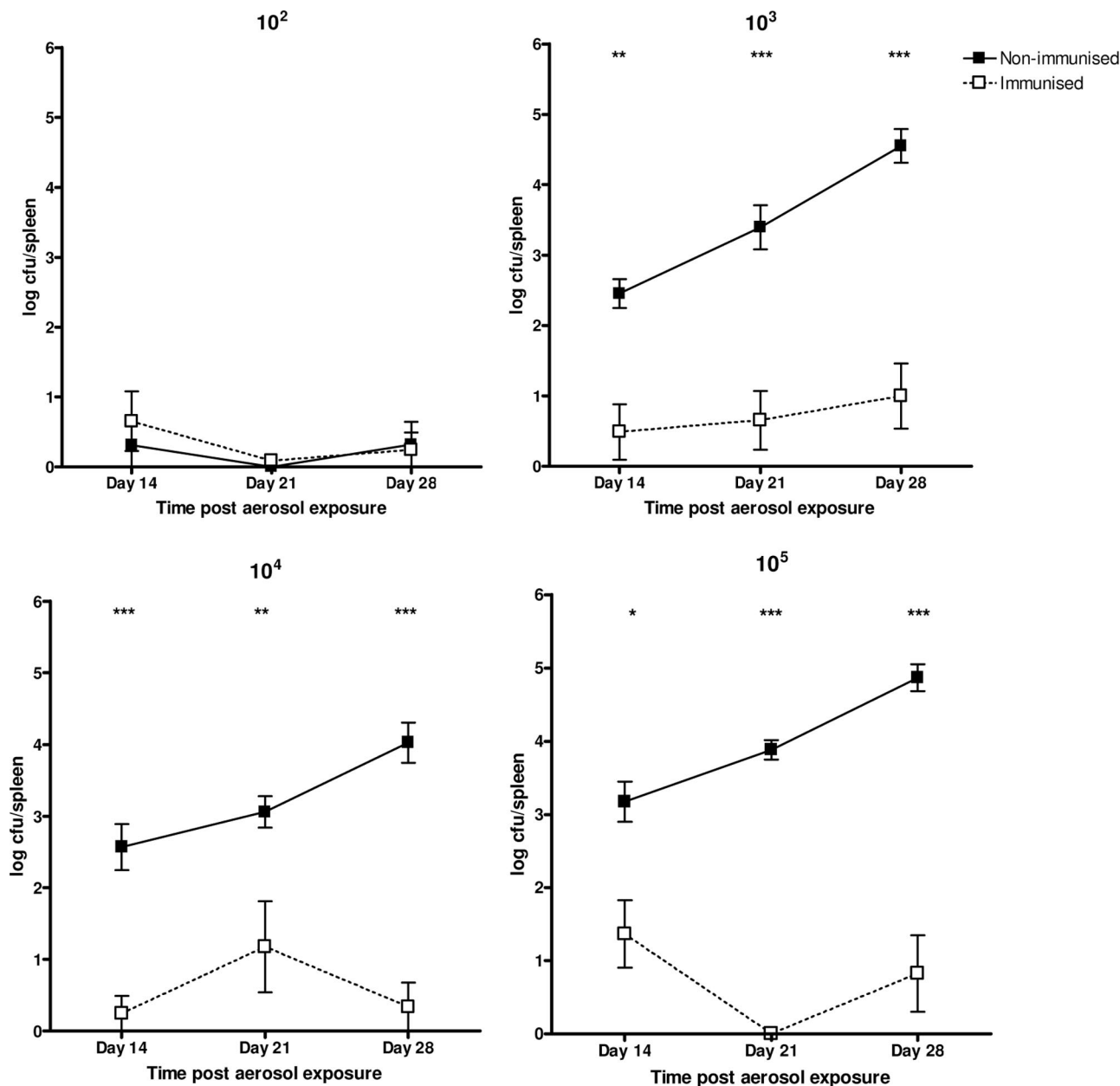
FIG. 1. Kinetics of splenic colonization in BALB/c mice following exposure to aerosolized *B. melitensis* 16 M bacteria. Groups of 12 mice were exposed to retained doses of 10^2, 10^3, 10^4, or 10^5 CFU of *B. melitensis* 16 M as indicated. Half of each group of mice were immunized s.c. with the live vaccine *B. melitensis* Rev.1 30 days prior to the exposure to *B. melitensis* 16 M. The remaining mice were not immunized. At 14, 21, and 28 days postchallenge, all mice were culled, spleens were homogenized, and recovered *B. melitensis* 16 M bacteria were enumerated. One-way ANOVA was used to compare log-transformed bacterial numbers of *B. melitensis* 16 M in the spleens of Rev.1-immunized mice to the numbers in the spleens of nonimmunized mice. Error bars show standard deviations. Statistical significance values are as follows: $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$.

a syringe and a 40-μm cell strainer (Falcon). The homogenates were serially diluted in phosphate-buffered saline and plated onto chocolate agar and tryptose soy agar containing 10 U/ml penicillin (which does not support *B. melitensis* Rev.1) to enumerate and differentiate the viable bacteria (Fig. 1). Protection was assessed by comparison of bacterial numbers of *B. melitensis* 16 M in the spleens of immunized and nonimmunized mice, and the results were analyzed by one-way analysis of variance (ANOVA) to determine differences at the 95% confidence

level using Bonferroni's multiple-comparison test (GraphPad PRISM version 4.0 for Windows). At the lowest retained dose of 10^2 CFU, there was no difference in the splenic loads of immunized or nonimmunized mice at any time. However, following retained doses of 10^3, 10^4, or 10^5 CFU, there were significant differences in splenic colonization in the immunized and nonimmunized animals, indicating that protection against infection could be measured in the model (Fig. 1). *B. melitensis* Rev.1 was not detected in the spleen, indicating that the vac-

cine strain had been cleared by 2 weeks postchallenge. Overall, these results suggest that a retained dose of at least 10^3 CFU of aerosolized *B. melitensis* 16 M bacteria is suitable for the initiation of a significant infection in BALB/c mice, confirming findings described by Kahl-McDonagh et al. (10). Additionally, we have demonstrated that this model is suitable for the evaluation of protection against *B. melitensis* infection using, as a model vaccine candidate, the live attenuated animal vaccine *B. melitensis* Rev.1.

A subsequent experiment was performed to confirm and further characterize this mouse model of aerosolized *B. melitensis* 16 M infection. Groups of 12 Rev.1-immunized or nonimmunized mice received a retained dose of 10^4 CFU aerosolized *B. melitensis* 16 M. Animals were culled on days 7, 14, 21, and 28 postinfection, and spleens, livers, lungs, and uteruses were aseptically removed. Organs were homogenized for enumeration as described above. High levels of *B. melitensis* 16 M bacteria were found in the lungs of nonimmunized mice at each time point studied (data not shown). While the result was not significantly different, Rev.1-immunized mice harbored lower numbers of *B. melitensis* 16 M bacteria in their lungs (data not shown). Nonimmunized and immunized mice harbored *B. melitensis* 16 M in their livers from days 7 and 21, respectively. *B. melitensis* 16 M bacteria were recovered from the uteruses of a high proportion of nonimmunized mice at all times but were found in the uteruses of immunized mice only on day 28 postexposure. Importantly, there were significant differences in the numbers of viable *B. melitensis* 16 M bacteria recovered from the spleens of immunized and nonimmunized mice at 14, 21, and 28 days postexposure, confirming the data from our initial experiment. In immunized mice, *B. melitensis* Rev.1 was detected in the liver up to 14 days postinfection and in the spleen on day 7 postexposure only. No *B. melitensis* 16 M bacteria were recovered from blood samples cultured in *Brucella* broth at any time.

Using a retained dose of 10^4 CFU of *B. melitensis* 16 M, a further experiment was performed to determine the bacterial loads in livers, lungs, and spleens at 2, 5, and 8 weeks postchallenge. The results of this experiment showed that bacterial loads in the spleen were at their highest at 5 weeks postchallenge (data not shown). In the lungs there was a decrease in bacterial load over time, and in the livers there was a slight increase over time (data not shown). Again, these findings are consistent with those of Kahl-McDonagh et al. (10) and with those of Mense et al. (12), who followed the course of infection in BALB/c mice administered *B. melitensis* 16 M via the i.n. route. In addition, our data showed consistent protection afforded by Rev.1 at 2 to 4 weeks after exposure to *B. melitensis* 16 M, demonstrating a time frame during which the protective efficacy of a treatment can be evaluated in this model.

Infection of mice with aerosolized *B. suis* 1330. In order to extend our study to inhalational brucellosis caused by *B. suis*, groups of 12 mice were immunized with the model vaccine Rev.1 via the s.c. route (6 animals) or left nonimmunized (6 animals) and then received retained doses of 10^2, 10^3, or 10^4 CFU of aerosolized *B. suis* 1330 (obtained and cultured as described for *B. melitensis* 16 M). After 2, 3, and 4 weeks, animals were culled and viable counts were performed on spleen, lung, and liver homogenates to determine bacterial loads (Fig. 2). The homogenates were plated onto chocolate agar and blood agar base media containing 2.5 mg/liter erythromycin (does not support *B. suis* 1330) to enumerate and differentiate the viable bacteria. Following *B. suis* challenge, the *B. melitensis* Rev.1 live vaccine strain could still be recovered from spleens and lungs at 4 weeks postexposure (8 weeks after immunization). Specifically, 255 CFU of *B. melitensis* Rev.1 was recovered from the spleen of one mouse after exposure to 10^2 CFU of *B. suis*; 43 to 500 CFU of *B. melitensis* Rev.1 was present in the spleens of three mice, and 3,000 CFU of Rev.1 was found in the lung of a single mouse after exposure to 10^3 CFU of *B. suis*; 187 and 268 CFU of *B. melitensis* Rev.1 were recovered from the spleens of two mice after exposure to 10^4 CFU of *B. suis*. Protection was afforded by the vaccine in this infection model (Fig. 2). There were significantly lower bacterial loads in the spleens at all time points when mice received a retained dose of 10^2 or 10^3 CFU of *B. suis*. Bacterial loads were significantly lower in the lungs of immunized mice than in those of nonimmunized mice at all time points after a retained dose of 10^2 CFU and at 3 and 4 weeks after a retained dose of 10^3 CFU of *B. suis*. Protection in the liver varied (Fig. 2). However, at the highest retained dose of 10^4 CFU of *B. suis* 1330, protection afforded by the vaccine was observed in the spleen only at 2 and 3 weeks postchallenge, in the liver only at 2 weeks postchallenge, and in the lungs only at 3 weeks postchallenge. At 4 weeks postchallenge, no protection was observed against a retained dose of 10^4 CFU *B. suis*. *B. suis* bacteria were also isolated from the uteruses of nonimmunized mice at each time point.

The isolation of *B. suis* and *B. melitensis* bacteria from the uteruses of infected animals demonstrates the tropism that the species has for this tissue and supports the suitability of the mouse model, reflecting the findings that *Brucella* bacteria are often identified in the reproductive systems of ruminant animals and have also been isolated from the uteruses of seals and otters (3). In comparison, *Brucella* bacteria were isolated from the blood of infected mice only on one occasion during this study (*B. suis* at 2 weeks postexposure to the highest retained dose), reflecting the finding that bacteremia in brucellosis is transient and can often go undetected (16). Positive blood cultures in human cases can vary from 10% to 90%, indicating that blood culturing is not a reliable method to indicate infection.

This study has shown, to our knowledge for the first time, that aerosolized *B. suis* 1330 bacteria are able to cause a systemic infection in the BALB/c mouse, providing an additional mouse model of aerosolized *Brucella* infection. Since *B. suis* was the first agent weaponized in the United States (6, 15) and is considered a potential bioterrorism agent today, the development of this model is an important addition to the arsenal of models that could be used for testing vaccines or therapies for human brucellosis. In comparison to infection with *B. melitensis*, infection of mice with aerosolized *B. suis* bacteria appears to cause a more acute infection, with higher numbers of bacteria colonizing the spleens and lungs and bacterial loads peaking earlier. Unlike infection with *B. melitensis*, a low retained dose of 10^2 CFU of *B. suis* bacteria is sufficient to cause a significant infection, and protection afforded by the model Rev.1 vaccine can be measured against this low dose.
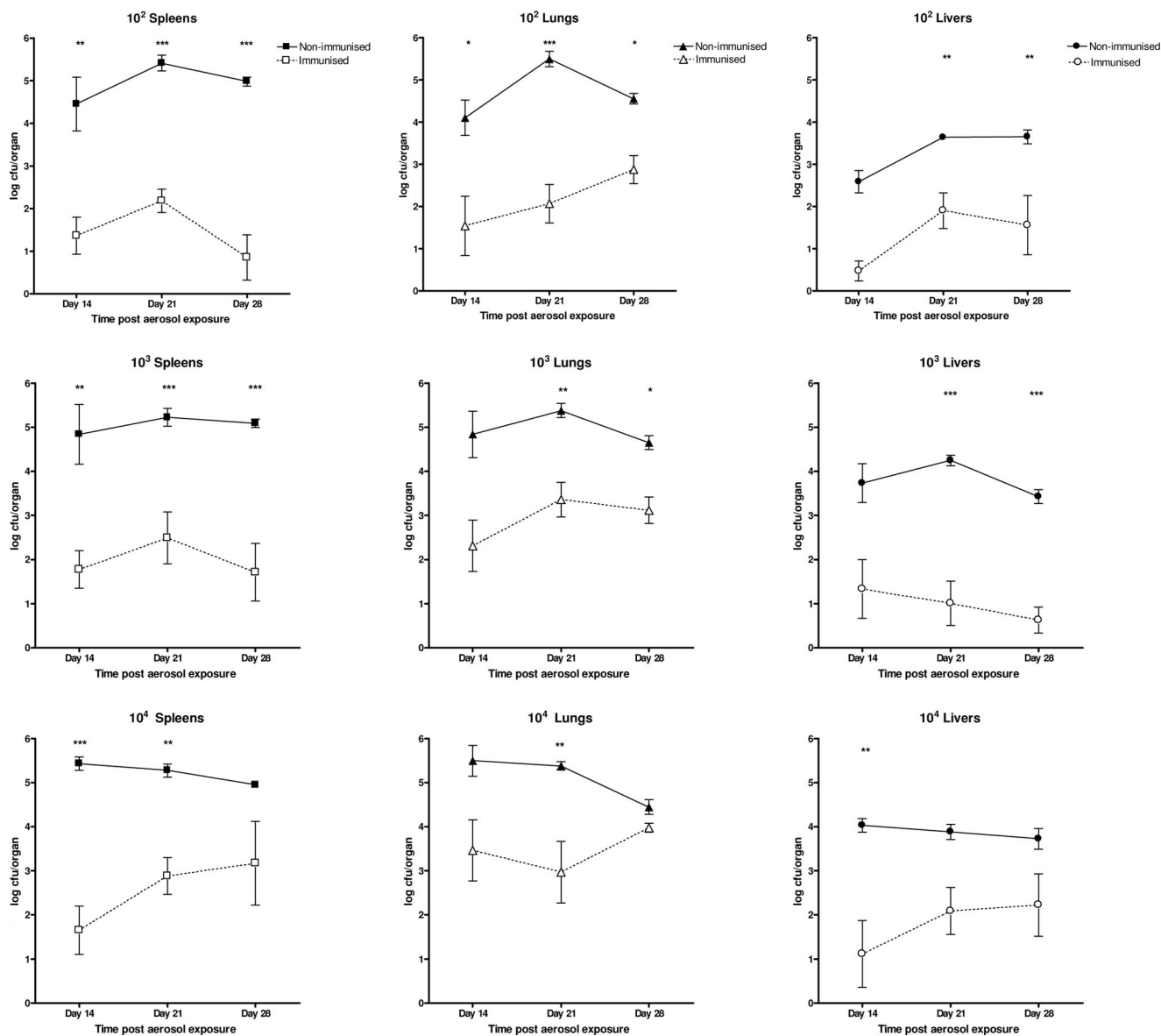
FIG. 2. Kinetics of infection in BALB/c mice following exposure to aerosolized *B. suis* 1330. Groups of mice were exposed to retained doses of 10^2 (top row), 10^3 (middle row), or 10^4 CFU (bottom row) of *B. suis* 1330. Half of the mice in each group were immunized s.c. with the *B. melitensis* Rev.1 live vaccine 30 days prior to challenge with *B. suis*. The remaining mice remained nonimmunized. At 14, 21, and 28 days postchallenge, all mice were culled, organs were homogenized, and recovered *B. suis* 1330 bacteria in spleens (first column), lungs (second column), and livers (third column) were enumerated. One-way ANOVA was used to compare bacterial numbers of *B. suis* 1330 in the organs of immunized mice to the numbers in the organs of nonimmunized mice. Error bars show standard deviations. Statistical significance values are as follows: ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

Effect of the route of administration of *B. melitensis* Rev.1 live vaccine. Our initial experiments demonstrated protection against brucellosis afforded by s.c. administration of the model vaccine Rev.1, evidenced by a decreased bacterial load in the spleen compared with that in nonimmunized mice. We hypothesized that immunization by the i.n. route may be more effective in providing protection against inhalational brucellosis. Groups of eight mice were immunized with 2×10^5 CFU of *B. melitensis* Rev.1 administered by either the s.c. or i.n. route, and groups of eight mice remained nonimmunized. To immunize via the i.n. route, mice were lightly anesthetized before

administration to the nostrils. After 30 days, immunized and nonimmunized mice received a retained aerosol dose of 4×10^3 CFU of *B. melitensis* 16 M. Spleen, liver, and lung bacterial counts were taken at 2, 3, and 4 weeks postchallenge. Significantly lower bacterial loads were observed in the lungs of mice immunized via the i.n. route than in the lungs of nonimmunized mice at all times (Fig. 3). Reduced bacterial burdens were observed in the spleens and livers at 3 and 4 weeks postchallenge ($P < 0.05$; results not shown). Conversely, s.c. administration of Rev.1 led to protection in the spleen at all times ($P < 0.001$; not shown) but protection in the lungs (Fig.
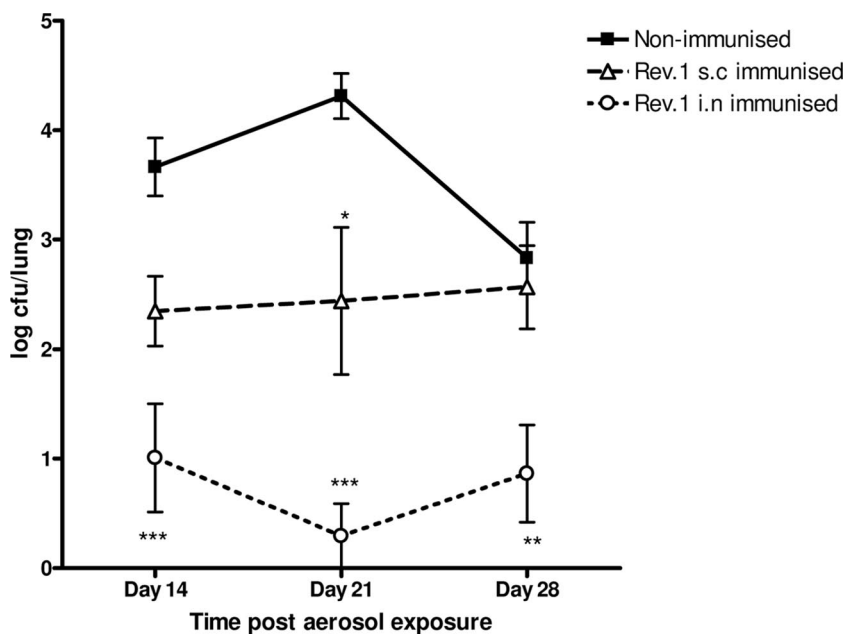
FIG. 3. Rev.1 vaccination by the i.n. route provides protection in the lungs of mice infected with *B. melitensis* 16 M. Groups of mice were challenged with a retained dose of 4×10^3 CFU of *B. melitensis* 16 M. Two groups had previously been immunized with the Rev.1 vaccine by either the s.c. or i.n. route. At 2, 3, or 4 weeks postchallenge, *B. melitensis* 16 M was recovered from the lungs of nonimmunized, s.c. Rev.1-immunized, and i.n. Rev.1-immunized mice. One-way ANOVA was used to compare bacterial numbers of *B. melitensis* 16 M in the lungs of nonimmunized mice to the numbers in the lungs of those immunized by the s.c. or i.n. route. Error bars show standard deviations. Statistical significance values for individual immunization routes are as follows: $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$.

3) and livers (not shown) at only one time point. The results of this experiment indicate that consideration of the route of administration may be important in developing therapeutics for inhalational brucellosis.

Overall, we have developed mouse models of infection with aerosolized *B. melitensis* 16 M or *B. suis* 1330 that may be applied to the evaluation of vaccines or therapeutics for brucellosis, and we now aim to undertake such studies in our laboratory.

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