INFECTION AND IMMUNITY, Sept. 2011, p. 3653–3658 Vol. 79, No. 9
0019-9567/11/$12.00 doi:10.1128/IAI.05330-11
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Protective Efficacy and Safety of *Brucella melitensis* 16MΔ*mucR* against Intraperitoneal and Aerosol Challenge in BALB/c Mice▽

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Received 3 May 2011/Returned for modification 21 May 2011/Accepted 14 June 2011

Brucellosis is a zoonosis of nearly worldwide distribution. Vaccination against this pathogen is an important control strategy to prevent the disease. Currently licensed vaccine strains used in animals are unacceptable for human use due to undesirable side effects and modest protection. Substantial progress has been made during the past 10 years toward the development of improved vaccines for brucellosis. In part, this has been achieved by the identification and characterization of live attenuated mutants that are safer in the host but still can stimulate an adequate immune response. In the present study, the identification and characterization of the *mucR* mutant (BMEI 1364) as a vaccine candidate for brucellosis was conducted. BALB/c mice were vaccinated intraperitoneally at a dose of 10^5 CFU with the mutant to evaluate safety and protective efficacy against intraperitoneal and aerosol challenge. All animals vaccinated with the vaccine candidate demonstrated a statistically significant degree of protection against both intraperitoneal and aerosol challenge. Safety was revealed by the absence of *Brucella* associated pathological changes, including splenomegaly, hepatomegaly, or granulomatous disease. These results suggest that the 16MΔ*mucR* vaccine is safe, elicits a strong protective immunity, and should be considered as a promising vaccine candidate for human use.

Brucella spp. are a diverse group of pathogens causing disease in many hosts, including humans. As the cause of brucellosis, *Brucella abortus* and *B. melitensis* are of particular importance to human health, with an estimated annual incidence of 500,000 human cases worldwide but predominantly in developing countries (31). Vaccination against brucellosis is an important control strategy to prevent the disease (29). Currently licensed vaccine strains implemented in the veterinary field are unacceptable for human use due to the undesirable side effects, antibiotic resistance, and lack of characterization (33, 37). Increased knowledge of protective immune responses to *Brucella* infection, along with the elucidation of the complete genomic sequence have provided better opportunities to understand, develop, and improve new vaccines against brucellosis.

Previous studies performed in this laboratory using signature-tagged mutagenesis permitted the identification of *in vitro* and *in vivo* virulence genes (1, 16, 40). Among these, a *mucR* (BMEI 1364) mutant was attenuated for survival in the mouse and macrophage model (40). The role of MucR protein in *Brucella* is unknown, but the function of the *mucR* gene has recently been identified in soil and plant bacteria such as *Agrobacterium tumefaciencis* and *Sinorhizobium meliloti* (19, 28). MucR is a transcriptional regulator that coordinates a diverse set of bacterial behaviors, including the control of exopolysaccharide production, which is important not only in bacterium-plant symbiosis but also in biofilm formation (4, 5).

In the present study, we conducted a series of experiments designed to characterize *B. melitensis* 16MΔ*mucR* as a potential candidate vaccine against intraperitoneal and aerosol *B. melitensis* 16M challenge. Vaccination with the mutant did not induce systemic or local adverse reactions and significantly protected BALB/c mice against intraperitoneal and aerosol challenge.

MATERIALS AND METHODS

Mice. Six- to eight-week-old female BALB/c mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and acclimated for 1 week prior to infection or vaccination. All experimental procedures and animal care were performed in compliance with the institutional animal care guidelines.

Bacterial strains. Strains used in these experiments include *B. melitensis* 16MΔ*mucR* (engineered for the present study and used as the vaccine candidate) and the virulent strain *B. melitensis*16M biovar 1(originally obtained from the American Type Culture Collection and reisolated by this lab from an aborted goat fetus) (24). Bacteria were grown on tryptic soy agar (TSA; Difco/Becton Dickinson) or Farrell's medium (TSA supplemented with Oxoid *Brucella* supplement) at 37°C with 5% CO_2. For construction of the *B. melitensis* 16MΔ*mucR* knockout, the medium was supplemented with kanamycin (100 μg/ml) or carbenicillin (100 μg/ml). Sucrose medium was utilized for *sacB* counterselection and unmarked gene deletion selection as previously described (25). *Escherichia coli* cultures utilized for the construction of the 16MΔ*mucR* mutant were grown on Luria-Bertani (LB) (Difco/Becton Dickinson) plates or in LB broth overnight at 37°C with or without ampicillin (100 mg/liter) or kanamycin (100 mg/liter).

To prepare organism for animal infections, *Brucella* were harvested from the surface of the plates after 3 days of incubation using phosphate-buffered saline (PBS; pH 7.2). The bacteria were pelleted and resuspended to a final concentration based on optical density readings using a Klett meter and a standardized curve. Actual viable counts were confirmed by serial dilution, plating, and enumeration.

Construction of the *B. melitensis* 16MΔ*mucR* deletion mutant. The mutant was constructed as previously described with some modifications (25). The sequence upstream of the *mucR* gene (BMEI1364) was amplified from *B. melitensis* 16M by using the primer pair 5′-GCTCTAGAGCCCATCAACAACAGGACAAACGG-3′ (contains an XbaI site) and 5′-GGCGGCGCGCCTGGTTGCTCCGAACTATGCTG (contains an AscI site). The sequence downstream of *mucR* was amplified with the primer pair 5′-CCAGGCGCGCCGCCGCTGCGTATTTCATAATC (contains an AscI site) and 5′-GCTCTAGAGCCTTTGCAGGTTTTCCGTATCTTT (contains an XbaI site). These two products were ligated to one another via overlapping PCR via the AscI site (New England Biolabs) engi-

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▽ Published ahead of print on 27 June 2011.

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neered between the two sequences. This overlap product was then ligated to pEX18Ap via the XbaI site (named pMMK40). A kanamycin resistance (Kan^r) gene was subsequently ligated within the vector by the unique AscI site (plasmid pMMK44). This construct was used for electroporation into *Brucella melitensis* 16M. Potential marked deletion mutants were Kan^r and carbenicillin sensitive (Carb^s) and were verified by PCR and Southern blotting; the confirmed mutant was named 16MΔ*mucR*::Kan. The unmarked deletion mutant was engineered by electroporation of pMMK40 into 16MΔ*muc*R::Kan and selected on TSA plus carbenicillin at 100 μg/ml. Cointegrants with the phenotypes Kan^r, Carb^r, and sucrose sensitive (Suc^s) were selected, indicating a cointegrant with a functional *sacB* gene. Bacteria were selected in the presence of sucrose for resolution of cointegration as previously described (25). All knockout candidates were verified by PCR and Southern blotting to demonstrate gene deletion and loss of the Kan cassette.

Evaluation of *B. melitensis* 16MΔ*mucR* attenuation in mice. Forty 6- to 8-week-old female BALB/c mice were used to evaluate the persistence and replication of the *B. melitensis* 16MΔ*mucR* mutant. Mice were inoculated intraperitoneally with either (i) 10^6 CFU in 100 μl of 16MΔ*mucR* or (ii) 10^6 CFU in 100 μl of the parental strain 16M. Groups of four mice were euthanized via carbon dioxide asphyxiation at 1, 3, 6, 9, or 12 weeks postinfection. At each time point, the spleens were harvested, weighed, and homogenized in 1 ml of peptone saline. Serial dilutions were prepared, and 100-μl aliquots of each dilution (including the undiluted organ) were plated in duplicate onto TSA plates. The levels of infection were expressed as means ± the standard errors of the mean (SEM) of individual log CFU/spleen values.

Immunization of mice for efficacy studies. Six- to eight-week-old female BALB/c mice were distributed into three treatment groups and inoculated intraperitoneally with a single dose of *B. melitensis* 16MΔ*mucR*. Treatment groups included doses of 10^5 CFU/mouse, 10^6 CFU/mouse, and PBS only as a control. Mice were housed for 20 weeks postvaccination under ABSL-3 conditions.

Vaccination efficacy against intraperitoneal challenge. At 20 weeks postvaccination, mice ($n = 5$ per group) were challenged intraperitoneally using 6×10^5 CFU of *B. melitensis* 16M/mouse. At 1 week postchallenge, the mice were euthanized via CO_2 asphyxiation. Spleens, lungs, and livers were collected, weighed, and homogenized in 1 ml of PBS and plated onto TSA or Farrell's medium plates to determine the total CFU/organ. Serial dilutions were performed, and aliquots were plated. The levels of infection were expressed as means ± the SEM of the individual $\log_{10}$ CFU/spleen, $\log_{10}$ CFU/liver, and $\log_{10}$ CFU/lung values.

Vaccination efficacy against aerosol challenge. At 20 weeks postvaccination, groups of five mice were challenged with an aerosol chamber dose of 5×10^9 CFU of B. *melitensis* 16M/ml. At 4 weeks postchallenge, the mice were euthanized, and the lungs, livers, and spleens were removed, weighed, homogenized in 1 ml of PBS, serially diluted, and plated onto Farrell's medium to determine total CFU/organ. The levels of infection were expressed as means ± the SEM of the individual $\log_{10}$ CFU/spleen, $\log_{10}$ CFU/liver, and $\log_{10}$ CFU/lung values. A group of three mice were euthanized directly after aerosol exposure to quantify the CFU/lung inhaled.

Histopathology. Tissues from mice were assessed to determine the degree of pathology associated with aerosol challenge. Mice were either vaccinated with 16MΔ*mucR* and subsequently aerosol challenged (2 weeks postchallenge) or not vaccinated (i.e., naive) and aerosol challenged (2 weeks postchallenge). The animals were euthanized by CO_2 asphyxiation, and the spleens, lungs, livers, kidneys, and hearts were harvested, fixed in 10% buffered formalin, paraffin embedded, and stained with hematoxylin and eosin. The histological changes were assessed between groups. Slides were analyzed by a board-certified veterinary pathologist.

Statistical analysis. Bacterial burden from mutant clearance and efficacy studies are expressed as the mean CFU ± the standard errors (SE) and are presented graphically as the $\log_{10}$ CFU of *Brucella* recovered per organ. Culture-negative organs were assigned a value of 4 CFU, which is below the limit of detection of 5 CFU/organ. Spleen weight data from kinetics studies are plotted as the mean spleen weights in mg ± the SE.

For the survival of 16MΔ*mucR* in mice, a Student *t* test was performed to compare splenic colonization and weight of the knockout strain to the wild-type control group at each time point. Efficacy studies compared vaccinated and subsequently challenged mice to mice receiving PBS as a vaccine control that were challenged with the wild-type organism. In the intraperitoneal challenge study, statistical significance of differences between vaccinates were analyzed by analysis of variance (ANOVA) for each organ separately, followed by Tukey's honestly significant (HSD) post test comparing all groups to one another. In the aerosol protection studies a Student *t* test was performed for each organ separately to compare the vaccinees to naive mice. For all statistical analyses, *P* values of <0.05 were considered statistically significant.

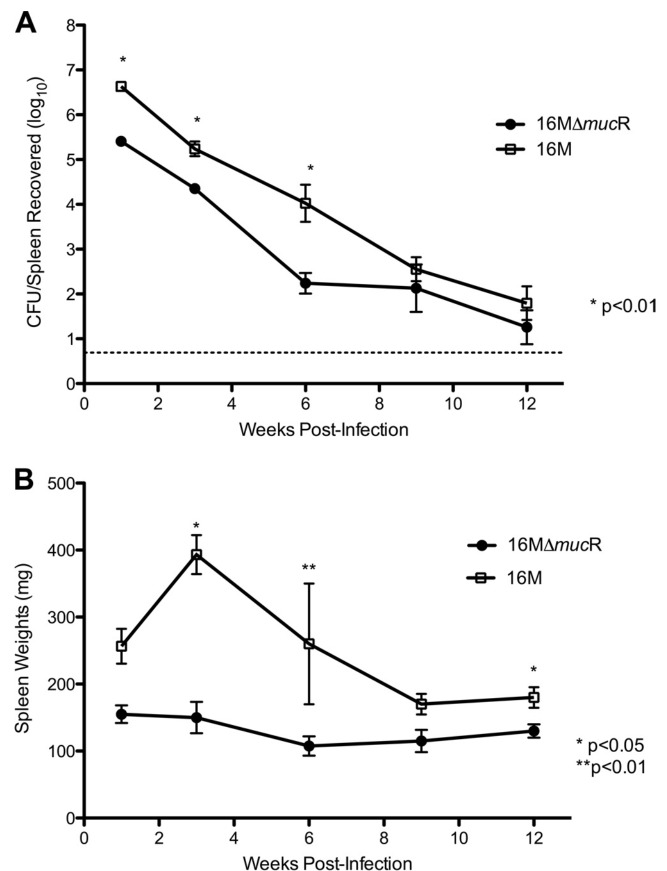

FIG. 1. Kinetics of clearance of 16MΔ*mucR* from mice. Forty 6- to 8-week-old female BALB/c mice were used to evaluate the persistence and replication of 16MΔ*mucR*. Mice were inoculated intraperitoneally with either (i) 10^6 CFU in 100 μl of 16MΔ*mucR* or (ii) 10^6 CFU in 100 μl of the parental strain 16M. Groups of four mice were euthanized via carbon dioxide asphyxiation at 1, 3, 6, 9, or 12 weeks postinfection. At each time point, the spleens were harvested, weighed, and homogenized in 1 ml of peptone saline. Serial dilutions were prepared, and 100-μl aliquots of each dilution (including the undiluted organ) were plated in duplicate on TSA plates. (A) The levels of infection were expressed as means ± the SEM of individual log CFU/spleen values. (B) The spleen weights were measured and used to compare the mutant strain to the wild-type organism. Statistical significance is based upon a Student *t* test comparing the deletion mutant to the wild-type strain. The solid line at 0.69 logs represents the lower limit of detection (≥5 CFU).

RESULTS

Attenuation of 16MΔ*mucR* in mice. To determine the effect of the *mucR* gene deletion *in vivo*, mice were infected intraperitoneally with 10^6 CFU of *B. melitensis* 16MΔ*mucR*/mouse. Compared to the wild-type strain 16M, the colonization of the spleen with 16MΔ*mucR* was significantly reduced at 1, 3, and 6 weeks ($P < 0.01$) but not at 9 or 12 weeks (Fig. 1A). Reduced splenic colonization by the 16MΔ*mucR* mutant correlated with reduced spleen weights (Fig. 1B), indicating a reduced inflammatory response by the mutant. The spleen weights of mice infected with wild-type 16M were consistently higher.

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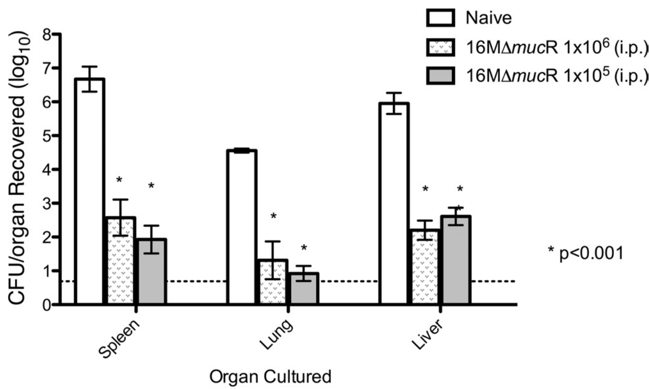

FIG. 2. Protection against homologous intraperitoneal 16M challenge. Groups of five female BALB/c mice were vaccinated with 16MΔ*mucR* at either 10^6 CFU/mouse or 10^5 CFU/mouse or left unvaccinated as naive controls. At 20 weeks postvaccination all animals were challenged with 6×10^5 CFU/mouse intraperitoneally. At 1 week postchallenge, mice were euthanized via CO_2 asphyxiation, and the spleens, livers, and lungs were collected. The data are reported as the $\log_{10}$ recovery of *Brucella* from organs. The solid line at 0.69 logs represents the lower limit of detection (≥5 CFU). Statistical analysis was performed by ANOVA for each organ separately, followed by a Tukey's HSD post test comparing all groups to one another.

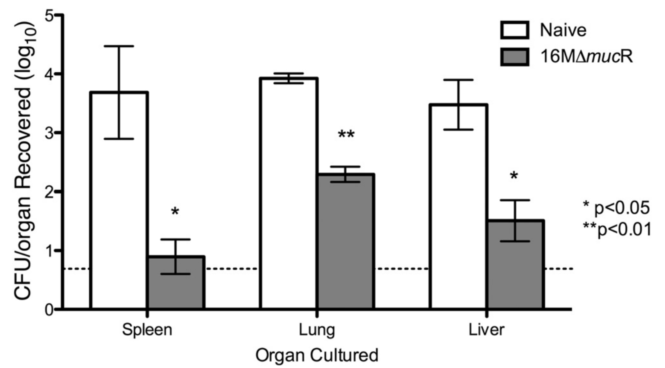

FIG. 3. Protection against homologous aerosol 16M challenge. Groups of five female BALB/c mice were vaccinated with 16MΔ*mucR* at 10^5 CFU/mouse or left unvaccinated as naive controls. At 20 weeks postvaccination, all of the animals were challenged with an aerosol chamber dose of 5×10^9 CFU of 16M/ml. At 4 weeks postchallenge, the mice were euthanized via CO_2 asphyxiation, and the spleens, livers, and lungs were collected. The data are reported as the $\log_{10}$ recovery of *Brucella* from organs. The solid line at 0.69 logs represents the lower limit of detection (<5 CFU). Statistical analysis was performed by using a Student *t* test comparing vaccinated to nonvaccinated mice for each organ separately.

Evaluation of immune protection provided by 16MΔ*mucR* against intraperitoneal 16M challenge. In order to determine the vaccination efficacy elicited by the 16MΔ*mucR* mutant, the level of protection provided by the vaccine candidate was evaluated against intraperitoneal *B. melitensis* 16M wild-type challenge at 20 weeks postvaccination. Animals were euthanized at 1 week postchallenge because this time point corresponds to the highest bacterial load in the spleen based on previous studies (25). At 1 week postchallenge (21 weeks postvaccination), there was a statistically significant decrease in the splenic, hepatic, and pulmonary bacterial loads from the mice vaccinated with the 16MΔ*mucR* mutant relative to those of the naive mice regardless of the vaccination dose, with a 4.14- to 4.75-log reduction in bacterial burden in the spleen ($P < 0.001$), 3.24- to 3.34-log reduction in the liver ($P < 0.001$), and 2.54- to 3.64-log reduction in the lungs ($P < 0.001$) (Fig. 2).

Evaluation of immune protection provided by 16Δ*mucR* against aerosol 16M challenge. In order to determine the vaccination efficacy elicited by the 16MΔ*mucR* mutant against a natural route of exposure, the level of protection provided by the vaccine candidate was evaluated against aerosol *B. melitensis* 16M wild-type challenge at 20 weeks postvaccination. For aerosol exposure studies, the 4-week postchallenge time point was chosen as the time point of peak splenic colonization (23). Mice that were euthanized within 1 h of aerosol exposure inhaled an average of 2.1×10^4 CFU/lungs, as determined by plating their lungs and enumerating the bacteria recovered.

When mice were challenged by the aerosol route, the 16MΔ*mucR* vaccine candidate protected mice significantly in all organs plated. The mutant afforded a 2.79-log reduction in the bacterial burden in the spleen ($P < 0.05$), a 1.97-log reduction in the liver ($P < 0.05$), and a 1.63-log reduction in the lungs ($P < 0.01$) (Fig. 3).

Evaluation of histological changes in mice vaccinated with 16MΔ*mucR*. Histological analysis of the lungs, livers, and spleens of BALB/c mice inoculated with either 16MΔ*mucR* (Fig. 4D, E, and F) or naive PBS controls (Fig. 4A, B, and C) that were subsequently aerosol challenged with wild-type 16M and euthanized 2 weeks postchallenge was assessed to determine the degree of inflammation elicited by the challenge organism in animals that were vaccinated with the mutant. Histologically, the bronchiole-associated lymphoid tissue was prominent at 2 weeks postchallenge (Fig. 4A), and no significant changes were seen in mice vaccinated with the *mucR* mutant (Fig. 4D). Inflammatory foci in the liver of naive aerosol challenged mice were rare at 2 weeks postchallenge (Fig. 4B) and absent in mice vaccinated with the *mucR* mutant (Fig. 4E). Changes in the spleen of naive mice consisted of marked extramedullary hematopoiesis at 2 weeks postchallenge (Fig. 4C). There were no significant changes in the spleen of mice vaccinated with the *mucR* mutant (Fig. 4F). No significant changes were observed in the kidneys or hearts in vaccinated or aerosol exposed animals (data not shown).

DISCUSSION

Historically, the most efficacious vaccines against brucellosis have been live attenuated vaccines, as is the case for multiple currently licensed vaccine strains for animal use, including S19, Rev1, and RB51 (15, 37). Unfortunately, both S19 and Rev1 vaccines, which have been highly efficacious in controlling the disease in cattle and goats, respectively, have proven to be unsafe or have the capacity to cause adverse reactions in humans due to local and systemic reactions that in some cases resulted in the development of the disease (3, 38). Other vaccinology alternatives, including the use of subunit, recombinant proteins and DNA vaccines which might be safer for human use, though capable of eliciting both humoral and cellular immune responses to a certain degree, generally induce lower or no protection compared to the live attenuated vaccines in animal models (8–10, 14, 30, 32, 33, 36). As such, a live

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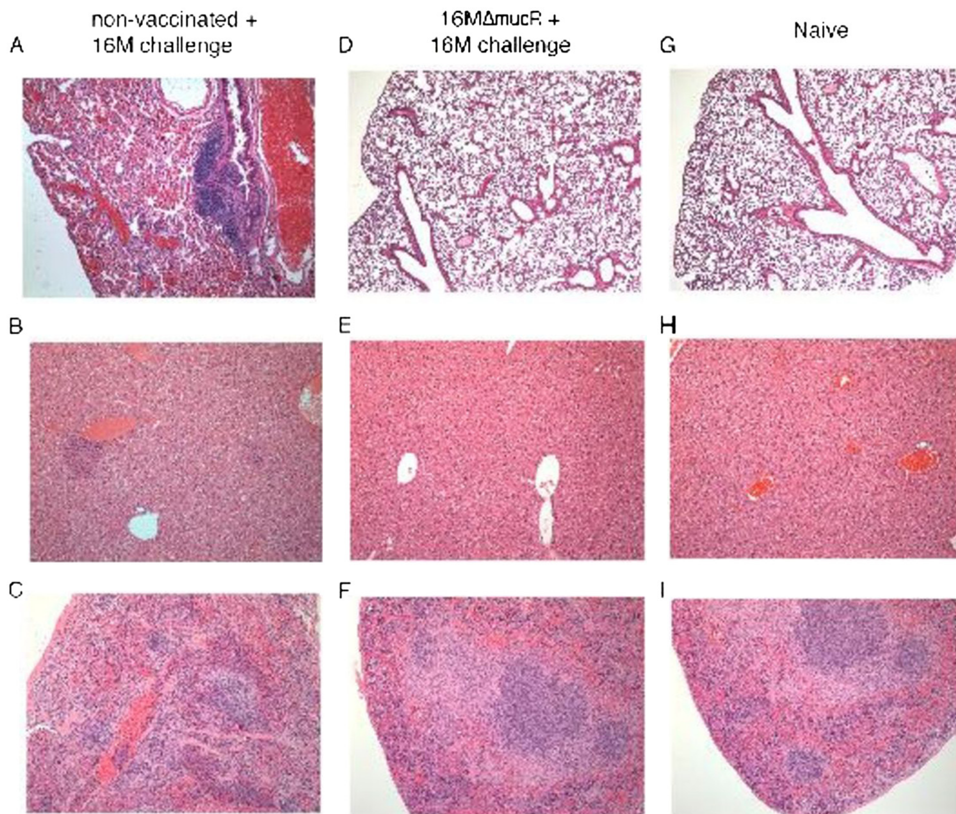

FIG. 4. Histological changes of organs associated with vaccination and challenge. BALB/c mice were vaccinated with 16MΔ*mucR* at 10^5 CFU/mouse or left unvaccinated as naive controls. At 20 weeks postvaccination, all of the animals were challenged with an aerosol chamber dose of 5×10^9 CFU of 16M/ml. At 2 weeks postchallenge, tissues were collected for histology. The histology of the nonvaccinated but challenged animals (A, B, and C) were compared to vaccinated but challenged animals (D, E, and F). The lungs (A and D), livers (B and E), and spleens (C and F) were compared to determine the reduction in pathology afforded by vaccination with the 16MΔ*mucR* mutant. Naive mice are depicted (G, H, and I) for comparison. Hematoxylin and eosin staining was used (×10 magnification).

attenuated organism has been utilized as the vaccine type of choice for the prevention of brucellosis. An ideal *Brucella* vaccine would be one that persists long enough to generate a robust immune response without eliciting the undesired side effects such as splenomegaly or clinical signs of disease.

Previous investigations using signature-tagged mutagenesis in this laboratory have identified multiple candidate mutants that are attenuated for virulence and survival in the mouse and macrophage models, among these is disruption of the *mucR* locus in *B. melitensis* 16M (40). We previously demonstrated an *in vitro* and *in vivo* role for the *mucR* transposon mutant. The organism was found to be significantly attenuated for survival *in vitro* and *in vivo* when the gene was interrupted. To further characterize the role of *mucR* in regard to survival, protective efficacy, and safety *in vivo*, an unmarked gene deletion was created.

In the Gram-negative soil bacterium *Sinorhizobium meliloti*, a clear role for the *mucR* gene has recently been established (28). MucR has been identified as a transcriptional regulator with multiple functions that help in the establishment of symbiosis, including a key role in the control of exopolysaccharide biosynthesis, which is necessary for biofilm formation (4, 5, 35). The biofilm provides bacteria with a physical barrier against antibiotics, innate defense mechanisms from the host, and environmental stress conditions, including UV radiation, pH changes, and osmotic shock among others (11, 12). In order to ensure a successful symbiotic association, exopolysaccharide production and biofilm formation are tightly regulated and partially controlled by the *mucR* gene. Deletion of *mucR* in *S. meliloti* therefore results in deficiencies in the invasion or the establishment of symbiosis. Other established roles of *mucR* in this organism include an induction of increased expression of multiple operons required for nitrogen fixation and respiration, as well as numerous type IV secretion systems and putative transport-related genes, all of which are necessary for a successful symbiosis (28).

In the case of *Brucella*, the role of *mucR* is less well understood. Preliminary studies from this laboratory using microarray technology suggests that the *mucR* gene regulates exopolysaccharide biosynthesis, as well as genes involved in iron sequestration and storage, nitrogen metabolism, and stress response mechanisms. (J. Weeks, unpublished data). Although preliminary and still under investigation, all of these putative roles of MucR in *Brucella* explain to a certain degree the attenuation of the mutant strain observed in J774A macrophages and in mice. Recently, it has been reported that *B. melitensis* 16M produces an exopolysaccharide; studies suggested that *Brucella* may indeed be capable of biofilm formation (17). It is possible that MucR may play a role in

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biofilm formation through the regulation of exopolysaccharide synthesis.

Protective efficacy as a function of persistence has been previously evaluated by this laboratory (25). The construction and characterization of multiple deletion mutants in *Brucella abortus* and *Brucella melitensis* has led to the conclusion that a vaccine candidate needs to persist in the host long enough in order to mount a strong protective immune response (15, 25). This observation is apparent here, as well with the *B. melitensis* *mucR* mutant. Interestingly, the *mucR* mutant persists for at least for 12 weeks in mice, similarly to the wild-type 16M, but the degree of colonization is significantly reduced compared to the parental strain during the acute phase of the infection. This difference in colonization properties may explain the lack of gross and microscopic changes associated with infection. Lack of hepatic granuloma formation or splenomegaly associated with vaccination suggests that immunization with the mutant is safe and therefore superior to many other *Brucella* vaccines, including licensed ones (12). Most importantly, protection against the most common microscopic changes associated with the disease in mice, such as granulomatous hepatitis, granulomatous splenitis, or splenomegaly, was not observed, indicating that vaccination with the *muc*R mutant not only reduced the bacterial burden in multiple organs but also prevents against the development of *Brucella*-associated pathological changes. Lack of splenomegaly associated with vaccination has been previously demonstrated as a safety parameter in other *Brucella* vaccine candidates (2).

Protection against intraperitoneal challenge, observing the output of bacterial colonization in the spleen of mice, has been historically used as a means of evaluating *Brucella* vaccine efficacy (6, 27, 34, 39). Although this vaccination or challenge location does not reflect a natural route of infection, it has been extremely useful in determining the potential efficacy of vaccine candidates against brucellosis. Most importantly, it provides a reproducible and invariable means of comparing multiple vaccine candidate strains that had been studied for the past 30 to 50 years. When 16MΔ*mucR* vaccinated mice were challenged against wild-type *B. melitensis* 16M, all of the animals demonstrated a statistically significant reduction in the bacterial burden in the spleen, lung, and liver regardless of the vaccination dose. The marked reduction in bacterial burden in the spleen conferred by the mutant is impressive and comparable to other live attenuated vaccine candidates tested by this laboratory and others (2, 20, 21, 25). Although an intraperitoneal challenge is of historical importance, a more logical approach is the use of an aerosol challenge route, not only because of the documented evidence of aerosol transmission of these organisms but also because of the potential threat of the use of *Brucella* as a bioterrorism agent (13, 18, 22, 26). It has been documented that 10 to 100 organisms are enough to cause disease in humans, and *Brucella* is therefore considered highly infectious when delivered by this route (7). Previous investigations performed by this laboratory have determined that BALB/c mice receiving an infectious dose of 5×10^9 CFU/ml added to the chamber nebulizer inhaled an average of 12,250 organisms per mouse (4.10 logs) and that tissue colonization reached a peak by 4 weeks postexposure (23). The high dose (100-fold more bacteria that actually needed to establish an infection) and the time postvaccination chosen to test efficacy provide us the means to evaluate the vaccine candidate efficacy under the most stringent conditions. Interestingly and importantly, the bacterial burdens in the spleen, liver, and lungs were markedly reduced in animals that received the vaccine, demonstrating the vaccine efficacy against an aerosol exposure. Gross and microscopic evaluation confirmed the protection against the pathological changes associated with the disease. As expected, the highest numbers of bacteria were isolated from the lung. It is possible that a diminished inflammatory response in the lungs masks the efficacy in reducing the bacterial colonization and, although high bacterial counts were observed, there were no significant gross or microscopic changes associated with the infection apart from an increased amount of bronchus-associated lymphoid tissue.

In the present study, the intraperitoneal vaccination with the live attenuated vaccine candidate 16MΔ*mucR* was able to markedly enhance the bacterial clearance in the spleen, lungs, and liver using two different challenge routes. Most importantly, vaccination conferred protection against *Brucella*-associated pathological changes. Future studies to determine the correlates of immune protection and the function of MucR are under way.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health grants R01048496 and U54AI057156.

We thank Gabriel Gomez and Alfredo Wong-Gonzalez for technical assistance.

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Editor: A. J. Bäumler

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INFECTION AND IMMUNITY, Dec. 2011, p. 5040 Vol. 79, No. 12
0019-9567/11/$12.00 doi:10.1128/IAI.05875-11
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AUTHOR'S CORRECTION

Protective Efficacy and Safety of *Brucella melitensis* 16MΔ*mucR* against Intraperitoneal and Aerosol Challenge in BALB/c Mice

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Volume 79, no. 9, p. 3653–3658, 2011. Page 3656: Figure 4 should appear as shown below.

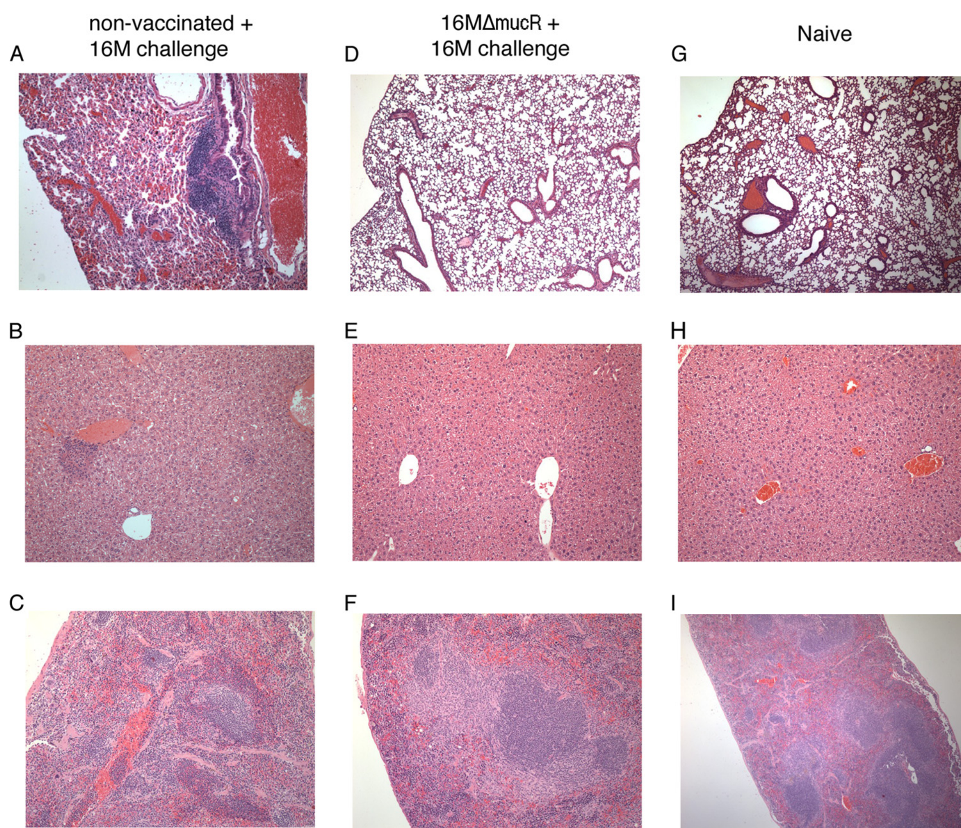

FIG. 4.