INFECTION AND IMMUNITY, July 2006, p. 4048–4057 Vol. 74, No. 7
0019-9567/06/$08.00+0 doi:10.1128/IAI.01787-05

Evaluation of Protection Afforded by *Brucella abortus* and *Brucella melitensis* Unmarked Deletion Mutants Exhibiting Different Rates of Clearance in BALB/c Mice

M. M. Kahl-McDonagh and T. A. Ficht*

Department of Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843-4467

Received 3 November 2005/Returned for modification 27 January 2006/Accepted 14 April 2006

Research for novel *Brucella* vaccines has focused upon the development of live vaccine strains, which have proven more efficacious than killed or subunit vaccines. In an effort to develop improved vaccines, signature-tagged mutant banks were screened to identify mutants attenuated for survival. Mutants selected from these screens exhibited various degrees of attenuation characterized by the rate of clearance, ranging from a failure to grow in macrophages after 24 h of infection to a failure to persist in the mouse model beyond 8 weeks. Ideal vaccine candidates should be safe to the host, while evoking protective immunity. In the present work, we constructed unmarked deletion mutants of three gene candidates, *manBA*, *virB2*, and *asp24*, in both *Brucella abortus* and *Brucella melitensis*. The Δ*asp24* mutants, which persist for extended periods in vivo, are superior to current vaccine strains and to other deletion strains tested in the mouse model against homologous challenge infection after 12, 16, and 20 weeks postvaccination. The Δ*asp24* mutants also display superior protection compared to Δ*manBA* and Δ*virB2* mutants against heterologous challenge in mice. From this study, a direct association between protection against infection and cytokine response was not apparent between all vaccine groups and, therefore, correlates of protective immunity will need to be considered further. A distinct correlation between persistence of the vaccine strain and protection against infection was corroborated.

Brucella species are small, facultative, gram-negative, intracellular coccobacilli that are classified phylogenetically within the α-2 subdivision of *Proteobacteria*. Several species of *Brucella* are the etiologic agents of brucellosis, a disease affecting numerous animal species that is also a zoonotic disease for humans (4, 15, 17, 29, 39). Animal exposure most commonly occurs through inhalation, in utero exposure from infected mothers to calves, ingestion of infected fetal tissues, or in some cases may be sexually transmitted (29). Acute infections in animal hosts are observed after *Brucella* organisms invade the chorionic trophoblast cells of the placenta, resulting in abortion of the fetus. Persistence is then classified by the organism's ability to reside in reproductive tissues and the mammary gland and lymph nodes, chronically shedding into the milk (18). Animal brucellosis can be a serious cause of economic loss due to abortion and infertility and also represents a public health threat (22, 40).

In the United States, *Brucella abortus* infection in cattle has been controlled by the combined use of vaccination, testing for exposure, and slaughter of seropositive animals, but the disease has not been successfully eradicated in wildlife species. Commonly, S19 and RB51 have been used for vaccination, but these vaccines are not ideal for all wildlife species at risk. S19 may cause abortion when administered to pregnant animals, and RB51 fails to protect most wildlife species from challenge infection (12, 14). As a result, improved vaccines need to be designed that will combine safety and efficacy to all species at risk, including domestic herds and wildlife. The scope of *Brucella* research has also expanded to include the demand for better protection and reaction of the United States against potential bioterrorist threats. Superior vaccine strains are therefore in demand for improved protection of agricultural and wildlife reservoirs as well as direct intervention in human infection.

Numerous attenuated mutants have been identified in *Brucella* species via signature-tagged mini-Tn*5* mutagenesis to identify *Brucella* genes encoding factors or products vital for survival and virulence within the host (2, 17, 25, 32). Mutants defective for genes required early in infection are rapidly cleared from both the mouse model and from macrophages in culture. Mutants defective for genes required later in infection are retained longer in the mouse model and, depending on the defect, may or may not show any difference in the macrophage assay (24). Since mice infected via intraperitoneal injection with wild-type *Brucella abortus* retain bacteria within the spleen for up to 24 weeks postinfection, mutant persistence in the spleen can therefore be compared to wild-type persistence in the spleen to determine the degree of attenuation of the mutant (25). Mutants may be classified according to their rate of clearance from the mouse.

In order to evaluate protection as a function of vaccine persistence, mutants exhibiting different rates of clearance were selected for further study. Rough mutants were selected due to overall interest in their use, such as the approved rough vaccine strain RB51, due to a lack of interference with diagnostic tests (34, 43, 45, 46, 49). Reliance on O-antigen for serological diagnosis of brucellosis is based on potent antibody responses directed against this cell surface antigen, and interest in the use of rough organisms as vaccines stems from the ease of detection of anti-O-antigen antibodies (34, 46). The rapid clearance of rough organisms, however, is often consid-

* Corresponding author. Mailing address: Texas A&M University, Department of Veterinary Pathobiology, MS 4467, College Station, TX 77843-4467. Phone: (979) 845-4118. Fax: (979) 862-1088. E-mail: tficht@cvm.tamu.edu.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
B. abortus strains		
2308	Wild type	B. Deyoe
Strain 19	Vaccine strain	NVSL
BAΔ*asp24*::kan	Δ*asp24*::Km	This work
BAΔ*asp24*	Δ*asp24*	This work
BAΔ*virB2*::kan	Δ*virB2*::Km (polar)	This work
BAΔ*virB2*	Δ*virB2* (nonpolar)	This work
BAΔ*manBA*	Δ*manBA*	This work
B. melitensis strains		
16M	Wild type	Ficht lab
Rev 1	Vaccine strain	M. Banai
BMΔ*asp24*::kan	Δ*asp24*::Km	This work
BMΔ*asp24*	Δ*asp24*	This work
BMΔ*virB2*::kan	Δ*virB2*::Km (polar)	This work
BMΔ*virB2*	Δ*virB2* (nonpolar)	This work
BMΔ*manBA*::kan	Δ*manBA*::Km	This work
BMΔ*manBA*	Δ*manBA*	This work
E. coli strains		
DH5α	F^- ϕ80*dlacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_k^- m_k^+) *phoA supE44* λ *thi-1 gyrA96 relA1*	Invitrogen
Top10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ*Δ*M15* Δ*lacX74 recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ*Δ*M15* Δ*lacX74 recA1 endA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *nupG*	Invitrogen
Plasmids		
pBluescript KS	ColE1, *bla*	Stratagene
pKD4	FLP/FRT, Km^r	B. Wanner
pEX18Ap	*sacB bla*	H. Schweizer
pMMKB	TAF101/TAF104 cloned into pEX18Ap	This work
pMMK8	TAF101/TAF104 cloned into pBluescript	This work
pMMK16	pMMK8 separated by TAF300/TAF301 (kanamycin resistance)	This work
pMMK29	TAF356/TAF359 cloned into pBluescript	This work
pMMK31	TAF356/TAF359 cloned into pEX18Ap	This work
pMMK33	pMMK29 separated by TAF204/205 kanamycin resistance gene	This work
pAV2.2	Plasmid to make marked *virB2* deletion	13
pAS1.1	Plasmid to make unmarked *virB2* deletion	13

ered a weakness of rough vaccine strains, preventing a long-term protective immune response (34). Furthermore, the immunity induced by RB51 appears to vary from host to host and is ineffective in several wildlife species (12, 14).

Because of the controversy surrounding the use of rough vaccines, we chose to evaluate another highly attenuated but smooth organism, a *virB* mutant. The *virB* type IV secretion system forms a complex serving to secrete or export macromolecules (5, 7). Mutations in this operon attenuate *Brucella* survival in macrophages and in the mouse model, suggesting that a completely functional VirB system is necessary for the establishment and maintenance of infection (5–8, 13, 30). It is a logical step to construct a knockout of this gene, since it is part of a macrophage/host cell-specific virulence mechanism identified in *Brucella* species.

Mutants that are not rapidly cleared in the mouse model have been shown to exhibit superior protective immunity; thus, it was desirable to correlate vaccine efficacy with survival of the vaccine strain (24). Based on preliminary evidence demonstrating persistence in macrophages and delayed attenuation in the mouse model, the *asp24* gene was selected as a deletion candidate for this study (GenBank accession number AE009508). The gene encoding Asp24 had never been identified in mutant screens, consistent with extremely late or reduced attenuation.

MATERIALS AND METHODS

Bacteria and bacterial cultures. *Escherichia coli* cultures were routinely grown on Luria-Bertani (Difco Laboratories) plates overnight at 37°C with or without supplemental kanamycin (100 mg/liter), carbenicillin (100 mg/liter), or chloramphenicol (50 mg/liter). All wild-type, vaccine, and unmarked deletion strains were routinely grown on tryptic soy agar (TSA; Difco Laboratories) at 37°C in an atmosphere containing 5% (vol/vol) CO_2. Virulent *B. abortus* strain S2308 was obtained from Billy Deyoe at the National Animal Disease Center in Ames, Iowa. *Brucella melitensis* bv. 1 (16M) was obtained from ATCC and reisolated by this lab from an aborted goat fetus. These strains were used as virulent challenge organisms and to generate vaccine candidates via deletion mutagenesis. Strain 19 was obtained from the National Veterinary Services Laboratory in Ames, Iowa. Rev 1 INRA was obtained from Menachem Banai. All bacterial strains were stored frozen at −80°C in medium supplemented with 50% (vol/vol) glycerol (Table 1).

Transfection of *Brucella* was performed via electroporation, using cultures prepared from the frozen stocks and grown to confluence on TSA at 37°C for 3 days. Bacteria were harvested into phosphate-buffered saline (PBS, pH 7.4; Gibco) to yield a final suspension containing approximately 4×10^{11} CFU/ml, as estimated turbidometrically using a Klett meter.

Inoculation doses used to infect mice were prepared the day of challenge following growth on solid medium, and the bacterial density was estimated using

TABLE 2. Primers used in this study

Primer name	Sequence (restriction enzyme engineered)	Fragment
TAF101	5′-GGAATTCGGCAAAGCGAGTGGGTGATTAG-3′(EcoRI)	*asp24* upstream
TAF102	5′-CGGGATCCTGAGCAAGTGCGGGAATAGC-3′(BamHI)	*asp24* upstream
TAF103	5′-CGGGATCCTGGGAATGGAGCGGCTTTAG-3′(BamHI)	*asp24* downstream
TAF104	5′-GCTCTAGATTTGAACACTTGGCGATAGCG-3′(XbaI)	*asp24* downstream
TAF300	5′-CGGGATCCCGCACGTCTTGAGCGATTGTGTAGG-3′(BamHI)	Kan cassette
TAF301	5′-CGGGATCCCGGGACAACAAGCCAGGGATGTAAC-3′(BamHI)	Kan cassette
TAF356	5′-CGGGATCCCTGGAGGAAAACAATCTGGG-3′ (BamHI)	*manBA* upstream
TAF357	5′-AAGACGGCGCGCCCGAACCTGTATCTGCCTG-3′(AscI)	*manBA* upstream
TAF358	5′-GTTCGGGCGCGCCGTCTTAACCCAAAACCGCTTCGTA-3′(AscI)	*manBA* downstream
TAF359	5′-GCTCTAGAGGGTTTTCTGATCGATCTGGTAGC-3′(XbaI)	*manBA* downstream
TAF204	5′-GGCGCGCCACGTCTTGAGCGATTGTGTAGG-3′(AscI)	Kan cassette
TAF205	5′-GGCGCGCCGGACAACAAGCCAGGGATGTAAC-3′(AscI)	Kan cassette

a Klett meter as described above. Inoculum doses were prepared, and serial dilution was performed retrospectively to accurately determine the number of organisms inoculated.

Recombinant plasmid construction. In order to construct vectors to eliminate genes of interest, primers were designed to amplify sequences flanking the genes. These flanking regions are referred to as the 5′ and the 3′ fragments and were joined to one another using specially designed PCR primers (Table 2). Importantly, the reverse primer of the 5′ fragment and the forward primer of the 3′ fragment include approximately 5 to 10 nucleotides of sequence complementary to the opposite fragment and a terminal restriction site. The 5′ and 3′ fragments were amplified in separate reactions, gel purified, and used as templates for a second round of PCR (38). The forward primer of the 5′ fragment and the reverse primer of the 3′ fragment were utilized in a second round of PCR to engineer a product that represented the ligation of the 5′ and 3′ fragments. The ends of this joined product were removed by restriction digestion at sites engineered into the primers. The final fragment was gel purified and ligated to pBluescript II KS(+) (Stratagene). A kanamycin cassette was inserted between the 5′ and 3′ fragments following amplification via PCR from the plasmid pKD4 using primers containing the compatible restriction site located within the overlap between the fragments. pKD4 contains nptII from Tn*5* flanked by FLP recombination target sites (11). These constructs are referred to as the marked plasmids (Table 1).

The construction of the plasmid for creation of unmarked deletion mutants entails cloning of the original overlapping PCR product (lacking the kanamycin cassette) into plasmid pEX18Ap, which contains *sacB*, using the appropriate restriction enzymes (23). This construct is referred to here as the unmarked plasmid (Table 1).

Selection of marked deletion mutants. Marked deletion mutants were created in *B. melitensis* and *B. abortus* via allelic exchange following electroporation of the marked plasmid into either 16M or S2308, respectively. Bacteria were grown as described above and pelleted via centrifugation at 1,700 × *g* for 15 min at 4°C. All subsequent steps were performed on ice or at 4°C. The cell pellet was washed three times with ice-cold sterile water under the same conditions. After the final wash, the cells were resuspended in 1 ml sterile water. The bacterial cell suspension was used in each electroporation with approximately 1 μg DNA in a prechilled 1-mm gap cuvette (Bio-Rad, California) and shocked in a BTX electroporation apparatus set at 2.2 to 2.5 kV and 246 Ω. SOC-B (6% [wt/vol] tryptic soy broth, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) medium was immediately added to the cuvette, transferred to microcentrifuge tubes, and incubated overnight at 37°C with agitation (31). Following incubation, the entire culture was plated onto TSA containing kanamycin. Colonies were replica plated onto TSA containing kanamycin and onto plates containing carbenicillin. Marked deletion mutants from allelic exchange should be kanamycin resistant (Km^r) and carbenicillin sensitive ($Carb^s$). Verification of mutant genotypes was obtained via PCR and Southern blot analysis to ensure that the gene of interest was deleted and the kanamycin cassette was retained.

Selection of unmarked deletion mutants. The unmarked plasmid, containing the *sacB* gene, ligated 5′ and 3′ fragments, and *bla* gene, was used for electroporation into marked deletion strains. Electroporation conditions were identical to those described for the construction of marked mutants. Following electroporation, cells were plated onto TSA containing carbenicillin to select for the first homologous recombination, i.e., a cointegration. Colonies were replica plated onto sucrose plates (TSA without salt, containing 6% [wt/vol] sucrose, without antibiotic) and to TSA containing carbenicillin. Colonies that grew on carbenicillin ($Carb^r$) but not sucrose (Suc^s) were cointegrates with a functional *sacB* gene. Resolution of cointegration occurs spontaneously and was selected for by inoculating 5 ml of sucrose broth (TSB, without salt or antibiotics, and supplemented with 6% [wt/vol] sucrose) and incubating for 24 h with agitation at 37°C, with subsequent plating onto sucrose-containing medium. All knockout candidates were verified via PCR and Southern blot analysis to demonstrate gene deletion as well as loss of the kanamycin cassette.

Clearance of mutants from mice. Survival or persistence of mutants was evaluated following intraperitoneal inoculation of groups of 4- to 6-week-old female BALB/c mice with 1×10^5 to 1×10^6 CFU/ml unmarked deletion mutant or wild-type organisms. Mice were euthanized via carbon dioxide asphyxiation at various times postinfection, depending upon the anticipated clearance rate of the mutant. At each time point, spleens were collected and weighed, homogenized in 1 ml PBS, and serially diluted, and 200-μl aliquots of all dilutions were plated onto TSA. Recovered bacteria were enumerated to evaluate the persistence of each individual organism.

Efficacy studies. The mouse model was used to evaluate efficacies of various unmarked deletion mutants against subsequent virulent infection. Groups of 6 to 10 female 4- to 6-week-old BALB/c mice were vaccinated via intraperitoneal injection of 1×10^6 CFU/ml of unmarked deletion mutant or PBS in naïve controls. Mice were subsequently challenged with 1×10^4 CFU homologous wild-type strain at 12, 16, or 20 weeks postvaccination. One week after the virulent challenge, the mice were euthanized, and spleens were extracted and weighed, homogenized in 1 ml PBS, serially diluted, and plated onto TSA to enumerate recovery of the challenge organism.

To evaluate cross-*Brucella* species protection, groups of five female 4- to 6-week-old BALB/c mice were vaccinated as described above and subsequently challenged with 1×10^4 CFU heterologous wild-type strain 16 weeks postvaccination. One week after the virulent challenge, the mice were euthanized and challenge organisms recovered were enumerated as described above. For both experiments, vaccine efficacy is presented in units of protection (U), representing the bacterial (challenge organism) burden in the spleen as a measure of protective immunity ($\log_{10}$ wild type recovered from unvaccinated mice minus $\log_{10}$ wild type recovered from vaccinates).

Immunological analysis. Correlates of protective immunity were evaluated via splenocyte cytokine memory responses. Splenocytes were pressed through fine mesh into complete RPMI (RPMI 1640 [GIBCO Laboratories, Grand Island, N.Y.] containing L-glutamine, 10% [vol/vol] fetal bovine serum [HyClone Laboratories, Logan, Utah], 1% [vol/vol] sodium pyruvate, 0.1% [vol/vol] β-mercaptoethanol, 1% [vol/vol] penicillin-streptomycin). Cells were pelleted at 1,700 × *g* for 10 min; 10 ml ACK red blood cell lysis buffer (0.15 M NH_4Cl, 10.0 mM $KHCO_3$, 0.1 mM Na_2EDTA) was added to the pellet for 5 min. Splenocytes were pelleted and washed three times with PBS, enumerated using a hemacytometer, and then resuspended in complete RPMI to be seeded in triplicate at a density of 2×10^5 cells/well in 96-well tissue culture plates (Costar, Massachusetts). Samples were stimulated for 3 days with either 1×10^8 CFU heat-killed homologous *Brucella* strain or controls, including unstimulated cells for a baseline level and cells stimulated with 2 μg/ml (wt/vol) concanavalin A (ConA), a positive control mitogen. Supernatants were collected at day 3 and frozen at −80°C. Enzyme-linked immunosorbent assays (ELISAs) were performed on supernatants for gamma interferon (IFN-γ) and interleukin-10 (IL-10) using monoclonal antibody pairs according to the manufacturer's protocol (PeproTech, New Jersey).

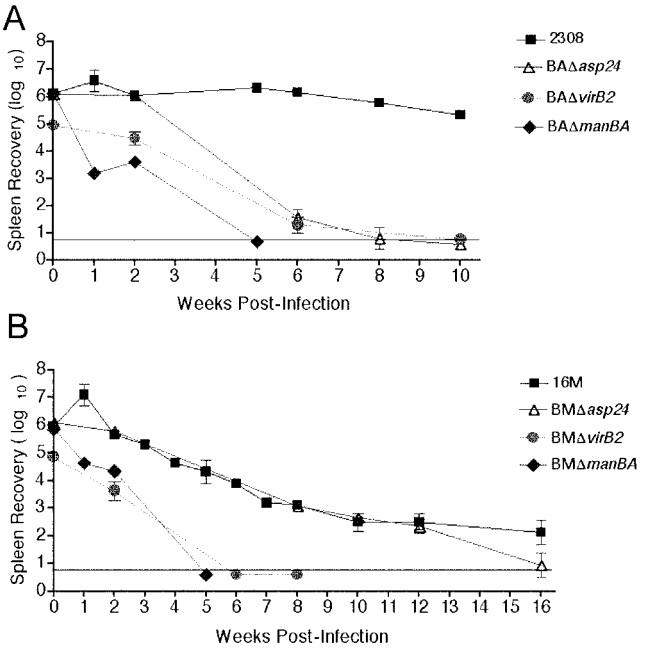

FIG. 1. Kinetics of clearance of unmarked deletion mutants from mice. Four to five female BALB/c mice were infected with 2308, BAΔ*asp24*, BAΔ*virB2*, or BAΔ*manBA* (A) or 16 M, BMΔ*asp24*, BMΔ*virB2*, or BMΔ*manBA* (B) at a dose of 1×10^6 CFU/mouse. Mice were sacrificed at various time points, and remaining *Brucella* organisms persisting in the spleens were enumerated and plotted as the mean CFU/spleen ± the standard error. Statistical significance is based upon an ANOVA comparing the deletion mutant to the wild-type strain and is listed in the text. The solid line at 0.69 logs represents the lower limit of detection, which is ≥5 CFU.

Statistical analysis. Data from survival of mutants as well as in efficacy studies were expressed as mean CFU ± the standard error and were presented graphically as the $\log_{10}$ CFU of *Brucella* recovered per spleen. Culture-negative spleens were assigned a value of 4 CFU, which is below the limit of detection of 5 CFU/spleen.

For survival of unmarked strains in mice, analyses of variance (ANOVAs) were performed to compare individual vaccine groups to the wild-type control group of the same species. Efficacy studies compared vaccinated and subsequently challenged mice to mice receiving PBS as a vaccine control which were challenged with wild-type organisms. ANOVAs were performed on these samples as well, comparing the vaccinated groups to nonvaccinated controls. For all ANOVAs, *P* values less than 0.05 were considered statistically significant.

RESULTS

Safety of attenuated unmarked deletion mutants in the mouse model. Infection of mice with unmarked deletion mutants was used to evaluate clearance of the vaccine strain, modeling field vaccination conditions. Compared to 2308, the unmarked rough *B. abortus manBA* deletion mutant (BAΔ*manBA*) was significantly attenuated by 3.4 logs at 1 week postinfection ($P < 0.0001$), 2.4 logs at 2 weeks postinfection ($P < 0.0001$), and undetected by 5 weeks postinfection ($P < 0.0001$). Between 1 and 2 weeks postinfection, the levels of detectable vaccine strain in the spleens remained unchanged (Fig. 1A). Unmarked rough *B. melitensis* deletion mutants of *manBA* (BMΔ*manBA*) demonstrated a similar clearing trend as BAΔ*manBA*. Compared to wild-type 16M, the mutant was attenuated 2.5 logs at 1 week ($P = 0.01$), 1.3 logs at 2 weeks ($P = 0.001$), and undetected by 5 weeks postinfection ($P = 0.001$) (Fig. 1B). Numbers of bacteria in the spleen remained unchanged between 1 and 2 weeks postinfection, as was observed in the *B. abortus* mutant. From these data we consider the *manBA* unmarked deletion to be highly attenuated.

Mice infected with unmarked *B. abortus virB2* (BAΔ*virB2*) deletion mutants were attenuated 1.6 logs at 2 weeks postinfection ($P = 0.003$), though the attenuation compared to 2308 was not as severe as what was demonstrated with the BAΔ*manBA* mutant at the same time point. BAΔ*virB2* was attenuated 4.7 logs at 6 weeks postinfection ($P < 0.0001$), which was just above the limit of detection, and was undetected at 10 weeks postinfection ($P < 0.0001$) (Fig. 1A). Unmarked *B. melitensis virB2* (BMΔ*virB2*) demonstrated similar survival characteristics to the *B. abortus* mutant. In this case, BMΔ*virB2* was reduced 2.2 logs compared to 16M ($P = 0.001$). By 6 weeks postinfection, the mutant strain was undetected from the mouse spleens ($P < 0.0001$), which held constant at 10 weeks as well ($P = 0.002$) (Fig. 1B). From these data, we consider both BAΔ*virB2* and BMΔ*virB2* to be attenuated at a moderate rate compared to wild-type strains, since they are not cleared as rapidly as *manBA* deletion mutants.

The two *asp24* deletion mutants were the first set of gene deletions that portrayed varied survival phenotypes in the mouse model, depending upon which species of *Brucella* the deletion was created. For the *B. abortus* deletion (BAΔ*asp24*), the mutant was highly attenuated (4.6 log reduction) by 6 weeks postinfection ($P < 0.0001$), barely detectable and 5.0 logs reduced at 8 weeks ($P < 0.0001$), and below the limit of detection at 10 weeks ($P < 0.0001$) (Fig. 1A). In *B. melitensis*, however, BMΔ*asp24* was not reduced in the spleen at a significant level until 16 weeks postinfection, where it persisted at 1.2 logs lower than 16M ($P = 0.04$) (Fig. 1B). One difficulty in assessing the difference between 16M and the knockout arises from the fact that 16M itself clears at a rapid rate compared to 2308 in mice. As such, differences between 16M and knockouts are not as pronounced, especially for mutants that exhibit a delay in attenuation, such as Δ*asp24*.

Efficacy of *B. abortus* mutants against 2308 challenge infection. To evaluate the vaccine potential of the selected unmarked mutants, the level of protection provided against virulent challenge infection was assessed. Degree of efficacy was determined by subtracting the mean CFU/spleen recovered from mice after vaccination for either 12, 16, or 20 weeks plus 1 week challenge with 2308 (corresponding to 13, 17, and 21 weeks postvaccination, respectively) from the mean CFU/spleen recovered from age-matched nonvaccinated but challenged controls. The U value obtained is relative only to the nonvaccinated controls challenged with the wild type at each particular time point.

At 13 weeks postvaccination, mice were protected by BAΔ*asp24* at 4.7 U and S19-vaccinated mice at 4.3 U, both significantly greater than naïve controls ($P < 0.0001$ for both). Mutants that clear more quickly from the host, BAΔ*virB2* and BAΔ*manBA*, protected mice to a lesser degree at 1.5 and 0.9 U, respectively, but significantly better than naïve mice ($P = 0.004$ and $P = 0.001$) (Fig. 2A.). At this time point, BAΔ*asp24* protected mice significantly better than S19, BAΔ*virB2*, and BAΔ*manBA* ($P = 0.01$, $P < 0.004$, and $P < 0.001$, respectively). There was no significant difference between BAΔ*virB2* and

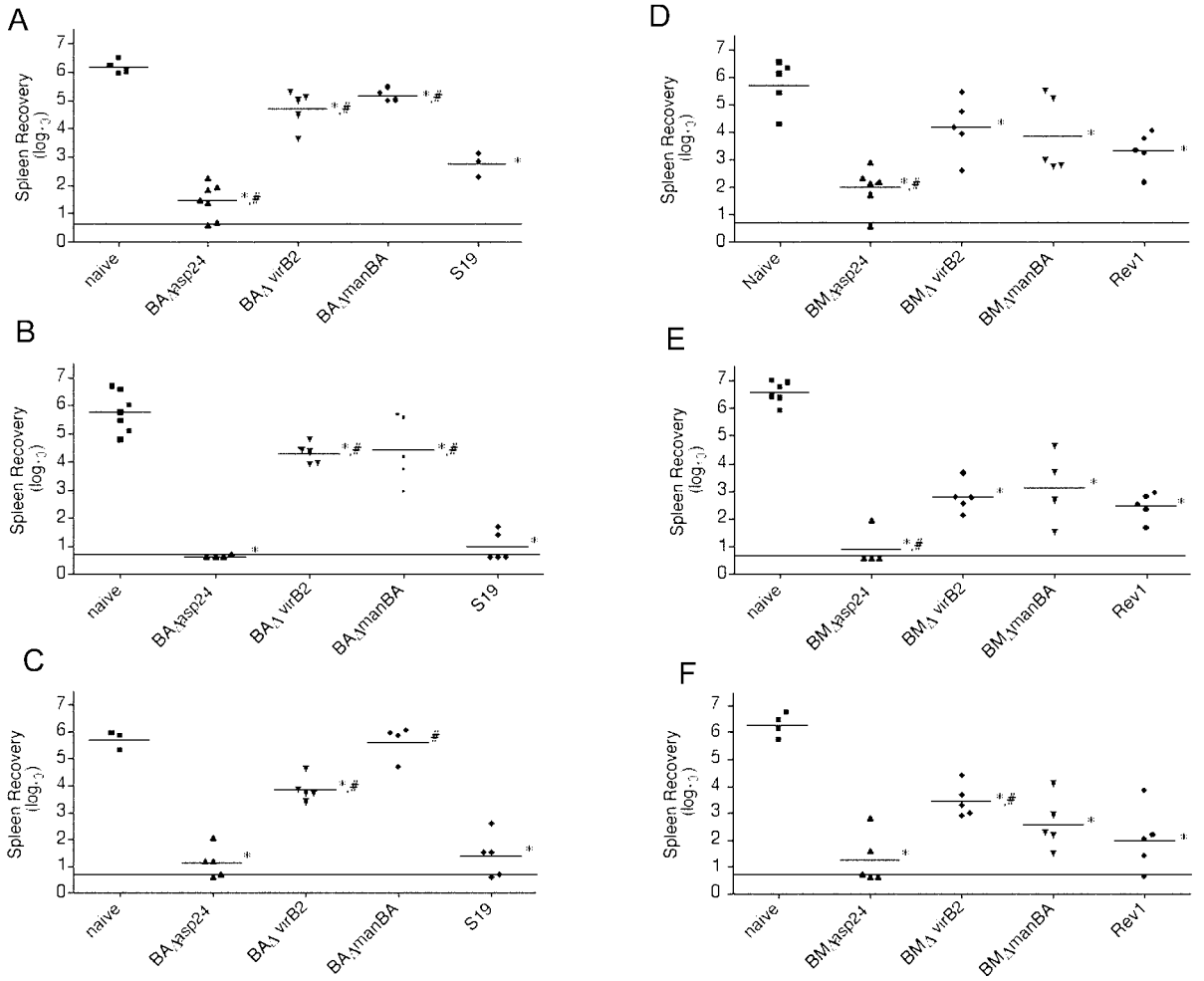

FIG. 2. Homologous efficacy of *B. abortus* and *B. melitensis* vaccine strains. Groups of three to seven female BALB/c mice were vaccinated with 1×10^6 CFU unmarked *B. abortus* vaccine strain, S19, or PBS. Groups were allowed to rest for either 12 weeks (A and D), 16 weeks (B and E), or 20 weeks (C and F) postvaccination. At these time points, mice were subsequently challenged with 1×10^4 CFU 2308 (A to C) or 16M (D to F) and allowed to rest 1 week. After 1 week, mice were euthanized via CO_2 asphyxiation and spleens were collected. Data are reported as the $\log_{10}$ recovery of *Brucella* from spleens, with individual mice in each treatment group represented. The solid line at 0.69 logs represents the lower limit of detection, which is ≥5 CFU. ∗, $P < 0.05$ compared to naïve animals by ANOVA; #, $P < 0.05$ compared to accepted vaccine strain (S19 or Rev 1) by ANOVA.

BAΔ*manBA* vaccinates ($P = 0.22$), and both strains protected mice significantly less than S19 ($P = 0.004$ and $P = 0.0002$, respectively).

At 17 weeks postvaccination, recovery of the challenge organism 2308 from a single mouse vaccinated with BAΔ*asp24* was just above the limit of detection. The challenge organism was not recovered from the three remaining mice, which appeared to exhibit sterile immunity. Overall protection for this group was 4.7 U relative to naïve controls ($P < 0.0001$). S19 protected mice to a similarly high degree at this time point as well, at 4.4 U ($P < 0.0001$). Mice exhibited 0.9 U protection when vaccinated with BAΔ*virB2* ($P = 0.002$) or BAΔ*manBA* ($P = 0.03$), suggesting that persistent yet attenuated mutant strains provide better protection than rapidly cleared mutants (Fig. 2B). At this time point, the protection provided by BAΔ*asp24* against infection was superior to BAΔ*virB2* and BAΔ*manBA* ($P < 0.0001$ and $P = 0.0004$, respectively), though there was no significant difference compared to S19 at this time point ($P = 0.22$). There was no significant difference between BAΔ*virB2* and BAΔ*manBA* groups ($P = 0.84$), and both deletion mutants protected mice significantly less than S19 ($P < 0.0001$ and $P = 0.0003$, respectively).

At 21 weeks postvaccination, BAΔ*asp24* again provided superior protection at 4.5 U ($P < 0.0001$), followed by S19 at 4.3 U ($P = 0.0001$), BAΔ*virB2* at 1.8 U ($P = 0.001$), and BAΔ*manBA* at 0.07 U ($P = 0.86$) relative to naïve controls (Fig. 2C). Similar to earlier time points, BAΔ*asp24* provided significantly higher levels of protection than BAΔ*virB2* and BAΔ*manBA* ($P < 0.0001$ for both strains) but no significant difference relative to S19 ($P = 0.59$). Vaccination with BAΔ*virB2* elicited significantly better protection in mice than BAΔ*manBA* at this time point ($P = 0.002$), although neither protected as well as S19 ($P = 0.0004$ and $P < 0.0001$, respectively).

Efficacy of *B. melitensis* mutants against 16M challenge infection. At 13 weeks postvaccination, BMΔ*asp24*-vaccinated

mice exhibited the highest level of protection against virulent *B. melitensis* challenge relative to naïve controls, at 3.7 U ($P < 0.0001$). Rev 1-vaccinated mice were protected at 2.4 U ($P = 0.002$), BMΔ*virB2*-vaccinated mice were protected at 1.9 U, and BMΔ*manBA*-vaccinated mice were protected at 1.5 U ($P = 0.04$ for both strains) compared to naïve controls (Fig. 2D). At this time point, BMΔ*asp24* protected mice significantly better than Rev 1, BMΔ*virB2*, and BMΔ*manBA* ($P = 0.02$, $P = 0.003$, and $P = 0.02$, respectively). There was no significant difference between BMΔ*virB2* or BMΔ*manBA* vaccinates ($P = 0.67$), and there was no significant difference in protection for these strains compared to Rev 1 ($P = 0.16$ and $P = 0.45$, respectively).

At 17 weeks postvaccination, BMΔ*asp24*-vaccinated mice exhibited the highest level of protection at 5.2 U ($P < 0.0001$), Rev 1 at 3.7 U ($P < 0.0001$), BMΔ*virB2* at 3.4 U ($P < 0.0001$), and BMΔ*manBA* at 3.4 U ($P = 0.0002$) against virulent *B. melitensis* compared to naïve controls (Fig. 2E). At this time point, BMΔ*asp24* protected mice significantly greater than Rev 1 and BMΔ*virB2* ($P = 0.006$ and $P = 0.003$, respectively), although there was no significant difference between BMΔ*asp24* and BMΔ*manBA* due to variation between mice ($P = 0.07$). There was no significant difference in protection between BMΔ*virB2* or BMΔ*manBA* vaccinates ($P = 0.92$), and there was no difference for either of these strains compared to Rev 1($P = 0.35$ and $P = 0.72$, respectively).

At 21 weeks postvaccination, BMΔ*asp24* provided the highest level of protection relative to naïve mice at 4.7 U ($P < 0.0001$), followed by Rev 1 at 4.4 U ($P = 0.0002$) (Fig. 2F). The BMΔ*asp24* mutant also protected mice significantly better than mice vaccinated with BMΔ*virB2* ($P = 0.003$). The BMΔ*manBA* mutant protected mice at 4.1 U and BMΔ*virB2* protected at 2.8 U ($P = 0.0001$ and $P < 0.0001$). The protection provided by BMΔ*asp24* vaccination was equivalent to Rev 1 ($P = 0.31$) but was statistically indistinguishable from BMΔ*manBA* ($P = 0.07$) due to variations between mice. There was no significant difference in protection between BMΔ*virB2* and BMΔ*manBA* ($P = 0.14$) or between Rev 1 and BMΔ*manBA* ($P = 0.42$), although Rev 1 vaccination elicited better protection than BMΔ*virB2* ($P = 0.04$).

Efficacy of unmarked mutants against heterologous *Brucella* species. Mice were challenged with a heterologous wild-type strain to determine if the vaccine would be protective against another species of *Brucella*. When challenged with 2308, the BMΔ*asp24* mutant provided the greatest degree of protection compared to naïve control groups, at 4.2 U ($P < 0.0001$) (Fig. 3A). BMΔ*virB2* provided 3.3 U ($P < 0.0001$), and BMΔ*manBA* provided the least protection at 1.6 U ($P = 0.02$). These data indicate that, similar to the homologous efficacy data, an attenuated mutant that persists for a longer period in the host protects better than mutants that clear rapidly. Mice that were vaccinated with BMΔ*asp24* were protected better than BMΔ*virB2*, though this difference was not significant ($P = 0.12$). Both BMΔ*asp24* and BMΔ*virB2* strains elicited significantly better protection than BMΔ*manBA* ($P = 0.003$ and $P = 0.045$, respectively).

Vaccination with BAΔ*asp24* provided the highest level of protection against challenge with 16M at 5.1 U relative to naïve mice ($P < 0.0001$) (Fig. 3B). Mice vaccinated with the BAΔ*virB2* mutant were significantly protected at 2.1 U ($P = 0.0001$), and mice vaccinated with BAΔ*manBA* were protected at 1.4 U ($P = 0.002$) relative to naïve mice. BAΔ*asp24*-vaccinated mice were protected better relative to mice vaccinated with either BAΔ*virB2* ($P = 0.0002$) or BAΔ*manBA* ($P = 0.002$). Although BAΔ*virB2* elicited better protection than BAΔ*manBA*, the difference was not significant between these two groups ($P = 0.95$).

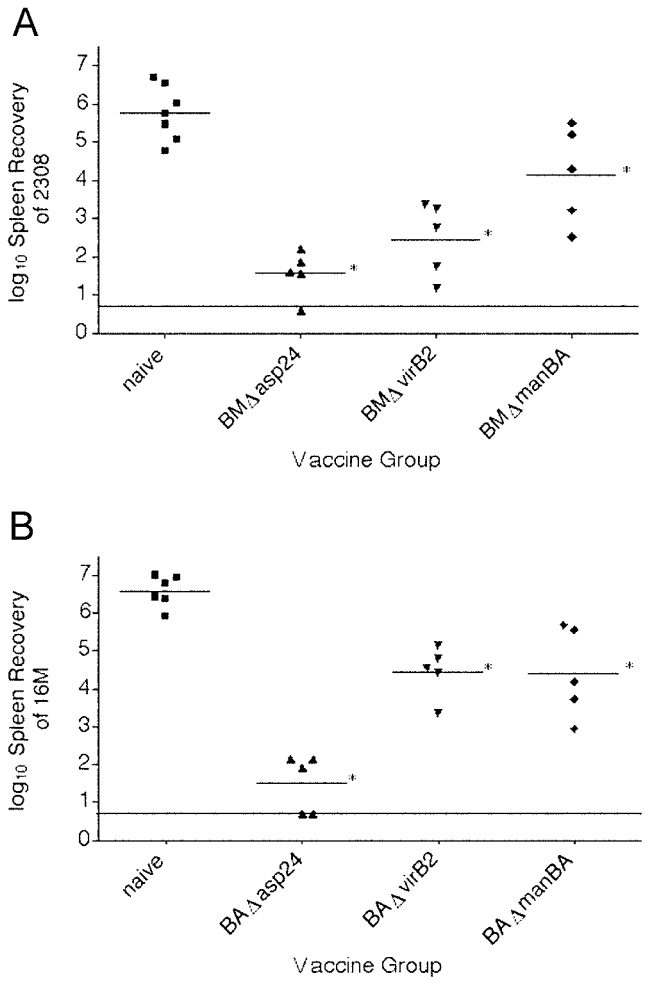

FIG. 3. Heterologous efficacy of *B. abortus* and *B. melitensis* vaccine strains. Groups of 5 to 10 mice were vaccinated with 1×10^6 CFU of unmarked *B. melitensis* vaccine strain or PBS (A) or 1×10^6 CFU of unmarked *B. abortus* vaccine strains or the PBS control (B). Groups were allowed to rest for 16 weeks postvaccination, at which time they were subsequently challenged with 1×10^4 CFU 2308 (A) or 16M (B) and allowed to rest for 1 week. After 1 week, mice were euthanized via CO_2 asphyxiation and spleens were collected. Data are reported as the $\log_{10}$ recovery of *Brucella* from spleens, with individual mice in each treatment group represented. The solid line at 0.69 logs represents the lower limit of detection, which is ≥5 CFU. *, $P < 0.05$ compared to naïve animals by ANOVA.

Production of cytokines from splenocytes of vaccinated mice. Mouse cytokines were evaluated at 21 weeks in an attempt to identify correlates of protective immunity associated with the different vaccine strains. Splenocytes were isolated from mice and stimulated as described above. Splenocytes from mice vaccinated with BMΔ*asp24* produced the highest amount of IFN-γ after stimulation with heat-killed *B. melitensis* ($P <$

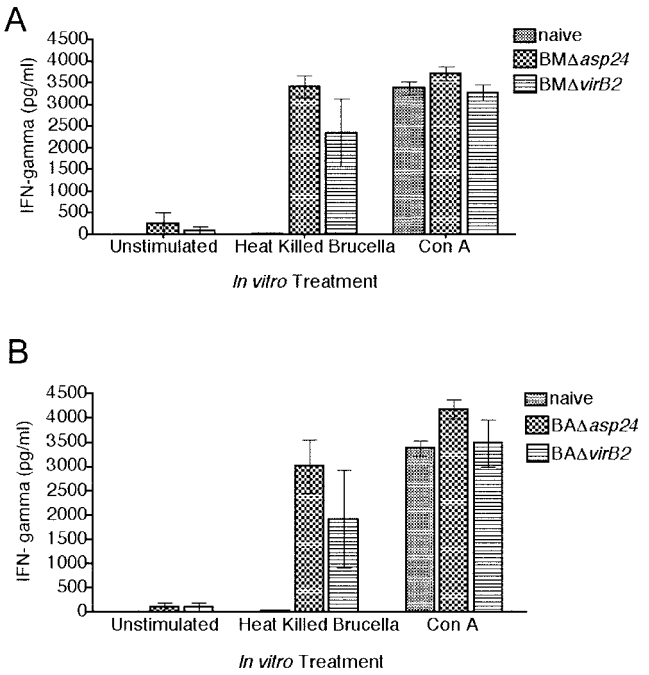

FIG. 4. IFN-γ production from mouse splenocytes. Mice were vaccinated with 1×10^6 CFU of *B. melitensis* deletion mutants (A) or *B. abortus* deletion mutants (B) for 21 weeks, with naïve mice kept as controls. Mice were then euthanized and splenocytes were isolated to measure in vitro IFN-γ responses. Splenocytes were stimulated with either ConA, 1×10^8 CFU heat-killed 16M (A) or 2308 (B) organisms, or unstimulated for a baseline cytokine concentration. IFN-γ production (in pg/ml) was detected after 3 days of growth in the presence of the stimulant and assayed as described in the text, via ELISA.

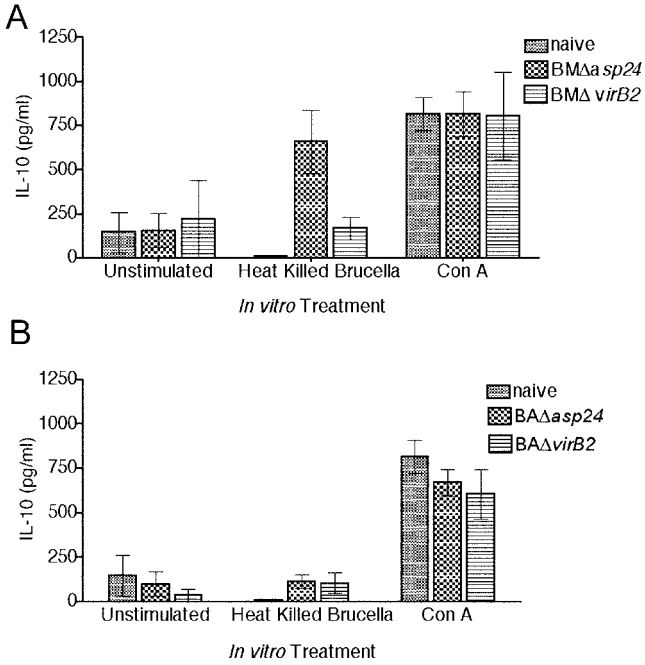

FIG. 5. IL-10 production from mouse splenocytes. Mice were vaccinated with 1×10^6 CFU of *B. melitensis* deletion mutants (A) or *B. abortus* deletion mutants (B) for 21 weeks, with naïve mice kept as controls. Mice were then euthanized, and splenocytes were isolated to measure in vitro IL-10 responses. Splenocytes were stimulated with either ConA, 1×10^8 CFU heat-killed 16M (A) or 2308 (B), or unstimulated for a baseline cytokine concentration. IL-10 production (in pg/ml) was detected after 3 days of growth in the presence of the stimulant and assayed as described in the text, via ELISA.

0.0001) compared to naïve mice stimulated with the same antigen (Fig. 4A). Splenocytes from the BMΔ*virB2* vaccination group also produced a significant amount of IFN-γ compared to naïve mice ($P = 0.02$). There was no significant difference in IFN-γ production between BMΔ*asp24* and BMΔ*virB2* vaccine groups ($P = 0.12$). Splenocytes from mice vaccinated with BMΔ*manBA* did not produce significant amounts of IFN-γ relative to naïve mice (data not shown).

Splenocytes from BAΔ*asp24*-vaccinated mice produced a significant amount of IFN-γ compared to naïve mice when stimulated in the presence of heat-killed *B. abortus* ($P = 0.002$) (Fig. 4B). Production of this cytokine was also greater, though not significantly, than in BAΔ*virB2* vaccinates ($P = 0.37$). Levels of IFN-γ from the BAΔ*virB2* splenocytes, though elevated, were not significant compared to naïve mice ($P = 0.14$).

Relative to naïve controls, IL-10 production from splenocytes of BMΔ*asp24* and BMΔ*virB2* vaccinates was significantly increased ($P = 0.02$ and $P = 0.03$, respectively), and these levels were not significantly different from one another ($P = 0.09$) (Fig. 5A). Levels of IL-10 from the BMΔ*manBA* group were not significantly different from naïve mice (data not shown).

IL-10 production was significantly increased compared to naïve controls for BAΔ*asp24*-vaccinated mice only ($P = 0.05$) (Fig. 5B). There was no significant increase in levels between any of the other vaccine groups stimulated with heat-killed *Brucella*.

DISCUSSION

Since it is not desirable to release an antibiotic resistance cassette into the field, particularly a transposon, removal of this resistance while retaining attenuation of the mutant is ideal. By utilizing homologous recombination between the bacterial chromosome and a plasmid carrying cloned chromosomal sequences and a selectable marker, unmarked, in-frame deletions can be created via allelic exchange. Mutants generated in this fashion were used in the mouse model to demonstrate a correlation between persistence of the vaccine candidate efficacy.

Genes of interest were chosen based upon differences in survival characteristics of the resulting mutants. *Brucella abortus* mutants deficient in O-antigen exhibit reduced virulence in the host and thus have been considered safe candidate live vaccines (1, 26, 27, 35, 44, 48). Rough mutants are taken up in greater numbers than smooth strains by macrophages and human monocytes, exhibit altered trafficking and host defense responses, and may cause necrotic cell death of the macrophages (27, 28, 42, 44). Due to their defect in O-antigen expression, rough mutants do not interfere with current diagnostic tests but have often proved mildly successful for protection of target livestock and wildlife species (1, 10, 12, 14, 26, 35, 48). A *virB* deletion was chosen because of its inability to persist within macrophages due to altered trafficking, despite the presence of O-antigen (7, 9, 13). The contribution of Asp24 to

intracellular survival is unknown, but the upregulated expression of this gene in macrophages and in response to low pH has suggested a role in the establishment of an intracellular niche leading to chronic infection (33). Since deletions in *asp24* possess both intact O-antigen and the type IV secretion system, it is an ideal candidate to evaluate the importance of both virulence criteria in vaccine efficacy.

The safety of selected vaccine candidates was evaluated based on clearance of unmarked deletion mutants using the mouse model. These experiments mimic utilization of the mutant as a vaccine in the field and may be used to estimate the duration of persistence of the vaccine strain under field conditions. Evaluation of colonization noncompetitively is important, since the presence of the wild-type organism can influence clearance of attenuated mutants from the host and may suggest enhanced rates of clearance (unpublished results). In these experiments, we constructed deletion mutants exhibiting a range of survival characteristics in the mouse model. The rough mutants, BAΔ*manBA* and BMΔ*manBA*, were significantly reduced in number by 1 week postvaccination but required 5 weeks postvaccination to be cleared completely. The *virB2* mutants, BAΔ*virB2* and BMΔ*virB2*, cleared more gradually than the *manBA* deletions, suggesting that their defect affects survival at a later stage of infection. Lastly, BAΔ*asp24* and BMΔ*asp24* cleared from mice at a much slower rate, with BAΔ*asp24* significantly reduced between 8 and 10 weeks postinfection and BMΔ*asp24* significantly reduced after 16 weeks, suggesting that Asp24 may only be required for persistence of the organism. The differences in survival exhibited by these mutants provided an opportunity to evaluate the relationship between protection and persistence of the vaccine strain.

Protection against homologous challenge (i.e., identical vaccine and challenge species) was most successful with the persistent BAΔ*asp24* and BMΔ*asp24* mutants. These mutants protected mice against infection at or near sterile levels at all time points examined. We consider these mutants to be the best candidates for novel attenuated vaccine strains, since they protect mice against infection better than current vaccine strains. In contrast, the rapid clearance of BAΔ*virB2*, BMΔ*virB2*, BAΔ*manBA*, and BMΔ*manBA* may enhance their safety as vaccine strains but provides significantly reduced levels of protection compared to Δ*asp24* mutants or currently available vaccine strains. Due to their enhanced safety, however, their use may be supported if delivered using controlled release, where the duration and presentation to the host can be extended to improve efficacy.

In protection studies against heterologous challenge, both BAΔ*asp24* and BMΔ*asp24* again protected mice significantly against challenge, with very few challenge organisms isolated. These results suggest that survival beyond 8 to 10 weeks does not enhance protection, and since prolonged survival may have side effects, the use of BAΔ*asp24* as a vaccine strain against other species of *Brucella* may be recommended. BAΔ*asp24* may be a protective vaccine strain in the event of a bioterrorist attack or from natural exposure to nonvaccinated, infected animals, providing cross-species protection.

The degree of protection provided by different vaccine candidates was compared with the cytokine profiles elicited from splenocytes in order to identify any correlates of protective immunity that may be used to predict potency or efficacy. The experiments described were designed to evaluate long-term immune response, mimicking field conditions, in which vaccination is expected to protect against challenge exposure occurring at undefined later dates.

IFN-γ, a Th1 cytokine involved in pathogen clearance from activated macrophages, has been well defined as a critical factor in the control of *Brucella abortus* infections in mice via enhanced activation of macrophages and intracellular killing (3, 21, 40). Infection in mice is more severe when the animals are treated with antibody to IFN-γ, and brucellosis becomes a fatal infection in IFN-γ knockout mice, confirming the critical role for IFN-γ in the early stages of infection (3, 36, 37, 40).

Vaccine candidates Δ*asp24* and Δ*virB2* produced significant levels of IFN-γ in response to stimulation with heat-killed *Brucella* relative to naïve mice. However, significant differences in efficacy determined by reduced recovery of the challenge strain were not matched by significant differences in IFN-γ production. As such, there did not seem to be an absolute correlation of protection with IFN-γ in this study, since mice vaccinated with Δ*asp24* mutants demonstrated significantly higher protection than mice vaccinated with Δ*virB2* mutants at all time points tested. Although increased levels of this cytokine may reflect an improved memory response with subsequent protection against challenge, it appears that IFN-γ, although necessary, is not sufficient to provide protection against infection. IFN-γ was not induced significantly in mice vaccinated with a rough mutant, Δ*manBA*. In other studies evaluating cytokine responses from splenocytes from mice vaccinated with rough mutants, a similar result was published (1).

IL-10 is generally considered an antiinflammatory molecule, thought to reduce negative effects caused by proinflammatory cytokines such as IFN-γ (47). Evidence suggests that inhibition of IL-10 activity results in improved clearance of *B. abortus* from the spleens of infected mice (16, 20). Since BALB/c mice are known to mount a very strong inflammatory response early in *Brucella* infection, Th2 cytokines such as IL-10 may be necessary to prevent overwhelming the host and to limit the influx of phagocytic cells and potential spread of the disease (3, 19, 41). Recently, it has been shown that IL-10 develops proinflammatory qualities during active inflammatory responses (47). The fact that IL-10 was generally increased in these experiments compared to naïve controls for BMΔ*asp24*, BAΔ*asp24*, and BMΔ*virB2* may be related to the observed levels of protection, suggesting a proinflammatory role in the mouse model.

In this study, mice were vaccinated for 20 weeks, and since previous research has never considered such late time points in the mouse, direct comparisons cannot be made to published work. Splenocytes may not be the ideal tissue to evaluate long-term cytokine responses in animals vaccinated for extended periods, and another tissue such as lymph nodes may need to be considered to identify correlates of immunity associated with protection.

Overall, the mouse is a sensitive and popular model for evaluating brucellosis. This model has been invaluable for characterization of genetic components necessary for virulence and is an affordable and consistent method for evaluation of potential vaccine candidates (15, 18, 25). Mutants considered safe and protective in the mouse model are often used as

candidates for vaccines in target animal species. The mouse model cannot be used, however, to gauge the effect of persistence on the colonization of reproductive tissues, thus, the need for other animal models after the initial murine screen. Since the mouse is not an exact model of disease in target livestock and wildlife species, vaccine candidates from the mouse will need to be reevaluated in a suitable animal model. The *asp24* deletion mutants were the most protective in the mouse screen against both homologous and heterologous challenge infections, and perhaps their protective ability in target species may be superior to the other candidates in that model as well.

ACKNOWLEDGMENTS

Melissa Kahl-McDonagh was supported by USDA Food and Agricultural Sciences National Needs Graduate Fellowship Grant 98-38420-5806 (CSREES) and an NIH Training Grant Predoctoral Fellowship Award. Research was funded by USDA/CSREES-NRICGP 99-35204-7550 and NIH 1RO1 AI48496-01.

pEX18Ap was kindly given to the Ficht lab by Herbert Schweizer.

REFERENCES

1. **Adone, R., F. Ciuchini, C. Marianelli, M. Tarantino, C. Pistoia, G. Marcon, P. Petrucci, M. Francia, G. Riccardi, and P. Pasquali.** 2005. Protective properties of rifampin-resistant rough mutants of *Brucella melitensis*. Infect. Immun. **73:**4198–4204.
2. **Allen, C. A., L. G. Adams, and T. A. Ficht.** 1998. Transposon-derived *Brucella abortus* rough mutants are attenuated and exhibit reduced intracellular survival. Infect. Immun. **66:**1008–1016.
3. **Baldwin, C. L., and M. Parent.** 2002. Fundamentals of host immune response against Brucella abortus: what the mouse model has revealed about control of infection. Vet. Microbiol. **90:**367–382.
4. **Bardenstein, S., M. Mandelboim, T. A. Ficht, M. Baum, and M. Banai.** 2002. Identification of the *Brucella melitensis* vaccine strain Rev 1 in animals and humans in Israel by PCR analysis of the *PstI* site polymorphism of its *omp2* gene. J. Clin. Microbiol. **40:**1475–1480.
5. **Boschiroli, M. L., S. Ouahrani-Bettache, V. Foulongne, S. Michaux-Charachon, G. Bourg, A. Allardet-Servent, C. Cazevieille, J. P. Lavigne, J. P. Liautard, M. Ramuz, and D. O'Callaghan.** 2002. Type IV secretion and Brucella virulence. Vet. Microbiol. **90:**341–348.
6. **Boschiroli, M. L., S. Ouahrani-Bettache, V. Foulongne, S. Michaux-Charachon, G. Bourg, A. Allardet-Servent, C. Cazevieille, J. P. Liautard, M. Ramuz, and D. O'Callaghan.** 2002. The Brucella suis virB operon is induced intracellularly in macrophages. Proc. Natl. Acad. Sci. USA **99:**1544–1549.
7. **Celli, J., C. de Chastellier, D. M. Franchini, J. Pizarro-Cerda, E. Moreno, and J. P. Gorvel.** 2003. Brucella evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J. Exp. Med. **198:**545–556.
8. **Celli, J., and J. P. Gorvel.** 2004. Organelle robbery: Brucella interactions with the endoplasmic reticulum. Curr. Opin. Microbiol. **7:**93–97.
9. **Comerci, D. J., M. J. Martinez-Lorenzo, R. Sieira, J. P. Gorvel, and R. A. Ugalde.** 2001. Essential role of the VirB machinery in the maturation of the Brucella abortus-containing vacuole. Cell. Microbiol. **3:**159–168.
10. **Cook, W. E., E. S. Williams, E. T. Thorne, T. J. Kreeger, G. Stout, K. Bardsley, H. Edwards, G. Schurig, L. A. Colby, F. Enright, and P. H. Elzer.** 2002. Brucella abortus strain RB51 vaccination in elk. I. Efficacy of reduced dosage. J. Wildl. Dis. **38:**18–26.
11. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
12. **Davis, D. S., and P. H. Elzer.** 2002. Brucella vaccines in wildlife. Vet. Microbiol. **90:**533–544.
13. **den Hartigh, A. B., Y. H. Sun, D. Sondervan, N. Heuvelmans, M. O. Reinders, T. A. Ficht, and R. M. Tsolis.** 2004. Differential requirements for VirB1 and VirB2 during *Brucella abortus* infection. Infect. Immun. **72:**5143–5149.
14. **Elzer, P. H., J. Smith, T. Roffe, T. Kreeger, J. Edwards, and D. Davis.** 2002. Evaluation of Brucella abortus strain RB51 and strain 19 in pronghorn antelope. Ann. N. Y. Acad. Sci. **969:**102–105.
15. **Endley, S., D. McMurray, and T. A. Ficht.** 2001. Interruption of the *cydB* locus in *Brucella abortus* attenuates intracellular survival and virulence in the mouse model of infection. J. Bacteriol. **183:**2454–2462.
16. **Fernandes, D. M., and C. L. Baldwin.** 1995. Interleukin-10 downregulates protective immunity to *Brucella abortus*. Infect. Immun. **63:**1130–1133.
17. **Ficht, T. A.** 2002. Discovery of Brucella virulence mechanisms using mutational analysis. Vet. Microbiol. **90:**311–315.
18. **Ficht, T. A.** 2003. Intracellular survival of Brucella: defining the link with persistence. Vet. Microbiol. **92:**213–223.
19. **Forestier, C., F. Deleuil, N. Lapaque, E. Moreno, and J. P. Gorvel.** 2000. Brucella abortus lipopolysaccharide in murine peritoneal macrophages acts as a down-regulator of T cell activation. J. Immunol. **165:**5202–5210.
20. **Giambartolomei, G. H., M. V. Delpino, M. E. Cahanovich, J. C. Wallach, P. C. Baldi, C. A. Velikovsky, and C. A. Fossati.** 2002. Diminished production of T helper 1 cytokines correlates with T cell unresponsiveness to Brucella cytoplasmic proteins in chronic human brucellosis. J. Infect. Dis. **186:**252–259.
21. **Golding, B., D. E. Scott, O. Scharf, L. Y. Huang, M. Zaitseva, C. Lapham, N. Eller, and H. Golding.** 2001. Immunity and protection against Brucella abortus. Microbes Infect. **3:**43–48.
22. **Hamdy, M. E., S. M. El-Gibaly, and A. M. Montasser.** 2002. Comparison between immune responses and resistance induced in BALB/c mice vaccinated with RB51 and Rev 1 vaccines and challenged with Brucella melitensis bv. 3. Vet. Microbiol. **88:**85–94.
23. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked Pseudomonas aeruginosa mutants. Gene **212:**77–86.
24. **Hong, P.** 2005. Ph.D. dissertation. Texas A&M University, College Station.
25. **Hong, P. C., R. M. Tsolis, and T. A. Ficht.** 2000. Identification of genes required for chronic persistence of *Brucella abortus* in mice. Infect. Immun. **68:**4102–4107.
26. **Jimenez de Bagues, M. P., P. H. Elzer, S. M. Jones, J. M. Blasco, F. M. Enright, G. G. Schurig, and A. J. Winter.** 1994. Vaccination with *Brucella abortus* rough mutant RB51 protects BALB/c mice against virulent strains of *Brucella abortus*, *Brucella melitensis*, and *Brucella ovis*. Infect. Immun. **62:**4990–4996.
27. **Jimenez de Bagues, M. P., A. Gross, A. Terraza, and J. Dornand.** 2005. Regulation of the mitogen-activated protein kinases by *Brucella* spp. expressing a smooth and rough phenotype: relationship to pathogen invasiveness. Infect. Immun. **73:**3178–3183.
28. **Jimenez de Bagues, M. P., A. Terraza, A. Gross, and J. Dornand.** 2004. Different responses of macrophages to smooth and rough *Brucella* spp.: relationship to virulence. Infect. Immun. **72:**2429–2433.
29. **Ko, J., and G. A. Splitter.** 2003. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. Clin. Microbiol. Rev. **16:**65–78.
30. **Kohler, S., S. Michaux-Charachon, F. Porte, M. Ramuz, and J. P. Liautard.** 2003. What is the nature of the replicative niche of a stealthy bug named Brucella? Trends Microbiol. **11:**215–219.
31. **Lai, F., G. G. Schurig, and S. M. Boyle.** 1990. Electroporation of a suicide plasmid bearing a transposon into Brucella abortus. Microb. Pathog. **9:**363–368.
32. **Lestrate, P., R. M. Delrue, I. Danese, C. Didembourg, B. Taminiau, P. Mertens, X. De Bolle, A. Tibor, C. M. Tang, and J. J. Letesson.** 2000. Identification and characterization of in vivo attenuated mutants of Brucella melitensis. Mol. Microbiol. **38:**543–551.
33. **Lin, J., and T. A. Ficht.** 1995. Protein synthesis in *Brucella abortus* induced during macrophage infection. Infect. Immun. **63:**1409–1414.
34. **Monreal, D., M. J. Grillo, D. Gonzalez, C. M. Marin, M. J. De Miguel, I. Lopez-Goni, J. M. Blasco, A. Cloeckaert, and I. Moriyon.** 2003. Characterization of *Brucella abortus* O-polysaccharide and core lipopolysaccharide mutants and demonstration that a complete core is required for rough vaccines to be efficient against *Brucella abortus* and *Brucella ovis* in the mouse model. Infect. Immun. **71:**3261–3271.
35. **Moriyon, I., M. J. Grillo, D. Monreal, D. Gonzalez, C. Marin, I. Lopez-Goni, R. C. Mainar-Jaime, E. Moreno, and J. M. Blasco.** 2004. Rough vaccines in animal brucellosis: structural and genetic basis and present status. Vet. Res. **35:**1–38.
36. **Murphy, E. A., M. Parent, J. Sathiyaseelan, X. Jiang, and C. L. Baldwin.** 2001. Immune control of Brucella abortus 2308 infections in BALB/c mice. FEMS Immunol. Med. Microbiol. **32:**85–88.
37. **Murphy, E. A., J. Sathiyaseelan, M. A. Parent, B. Zou, and C. L. Baldwin.** 2001. Interferon-gamma is crucial for surviving a Brucella abortus infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunology **103:**511–518.
38. **Murphy, K. C., K. G. Campellone, and A. R. Poteete.** 2000. PCR-mediated gene replacement in Escherichia coli. Gene **246:**321–330.
39. **Oliveira, S. C., N. Soeurt, and G. Splitter.** 2002. Molecular and cellular interactions between Brucella abortus antigens and host immune responses. Vet. Microbiol. **90:**417–424.
40. **Paranavitana, C., E. Zelazowska, M. Izadjoo, and D. Hoover.** 2005. Interferon-gamma associated cytokines and chemokines produced by spleen cells from Brucella-immune mice. Cytokine **30:**86–92.
41. **Pasquali, P., R. Adone, L. C. Gasbarre, C. Pistoia, and F. Ciuchini.** 2001. Mouse cytokine profiles associated with *Brucella abortus* RB51 vaccination or *B. abortus* 2308 infection. Infect. Immun. **69:**6541–6544.

42. **Pei, J., and T. A. Ficht.** 2004. *Brucella abortus* rough mutants are cytopathic for macrophages in culture. Infect. Immun. **72:**440–450.
43. **Porte, F., A. Naroeni, S. Ouahrani-Bettache, and J. P. Liautard.** 2003. Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome fusion in murine macrophages. Infect. Immun. **71:**1481–1490.
44. **Rittig, M. G., A. Kaufmann, A. Robins, B. Shaw, H. Sprenger, D. Gemsa, V. Foulongne, B. Rouot, and J. Dornand.** 2003. Smooth and rough lipopolysaccharide phenotypes of Brucella induce different intracellular trafficking and cytokine/chemokine release in human monocytes. J. Leukoc. Biol. **74:**1045–1055.
45. **Schurig, G. G., R. M. Roop II, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan.** 1991. Biological properties of RB51; a stable rough strain of Brucella abortus. Vet. Microbiol. **28:**171–188.
46. **Schurig, G. G., N. Sriranganathan, and M. J. Corbel.** 2002. Brucellosis vaccines: past, present and future. Vet. Microbiol. **90:**479–496.
47. **Sharif, M. N., I. Tassiulas, Y. Hu, I. Mecklenbrauker, A. Tarakhovsky, and L. B. Ivashkiv.** 2004. IFN-alpha priming results in a gain of proinflammatory function by IL-10: implications for systemic lupus erythematosus pathogenesis. J. Immunol. **172:**6476–6481.
48. **Ugalde, J. E., D. J. Comerci, M. S. Leguizamon, and R. A. Ugalde.** 2003. Evaluation of *Brucella abortus* phosphoglucomutase (*pgm*) mutant as a new live rough-phenotype vaccine. Infect. Immun. **71:**6264–6269.
49. **Ugalde, J. E., C. Czibener, M. F. Feldman, and R. A. Ugalde.** 2000. Identification and characterization of the *Brucella abortus* phosphoglucomutase gene: role of lipopolysaccharide in virulence and intracellular multiplication. Infect. Immun. **68:**5716–5723.

Editor: V. J. DiRita