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Overexpression of Protective Antigen as a Novel Approach To Enhance Vaccine Efficacy of *Brucella abortus* Strain RB51

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Brucella abortus **strain RB51 is an attenuated rough strain that is currently being used as the official live vaccine for bovine brucellosis in the United States and several other countries. We reasoned that overexpression of a protective antigen(s) of *B. abortus* in strain RB51 should enhance its vaccine efficacy. To test this hypothesis, we overexpressed Cu/Zn superoxide dismutase (SOD) protein of *B. abortus* in strain RB51. This was accomplished by transforming strain RB51 with a broad-host-range plasmid, pBBR1MCS, containing the *sodC* gene along with its promoter. Strain RB51 overexpressing SOD (RB51SOD) was tested in BALB/c mice for its ability to protect against challenge infection with virulent strain 2308. Mice vaccinated with RB51SOD, but not RB51, developed antibodies and cell-mediated immune responses to Cu/Zn SOD. Strain RB51SOD vaccinated mice developed significantly ($P < 0.05$) more resistance to challenge than those vaccinated with strain RB51 alone. The presence of the plasmid alone in strain RB51 did not alter its vaccine efficacy. Also, overexpression of SOD did not alter the attenuation characteristic of strain RB51.**

Intracellular bacteria are responsible for several important infectious diseases of animals and humans. Cell-mediated immune (CMI) responses play a critical role in resistance against intracellular bacterial infections (7). Live bacterial vaccines are considered essential for effectively inducing the appropriate protective CMI responses. Usually, attenuated strains of bacteria are used as live vaccines for intracellular bacterial infections. However, in many cases, even these live vaccines cannot provide high levels of protection. We hypothesized that overexpression of a bacterial protective antigen(s) in its vaccine strain would result in enhancement of the vaccine's efficacy. Our studies with *Brucella abortus* vaccine strain RB51 validate this hypothesis.

Members of the genus *Brucella* are small gram-negative, facultatively intracellular bacteria of zoonotic importance (1). These bacteria are causative agents of brucellosis, a chronic disease of animals and humans. In animals, this disease often results in infertility and abortions leading to severe economic losses to livestock producers (5). Humans acquire the infection by coming in contact with the infected materials or by consuming contaminated meat or dairy products. *B. abortus* is primarily responsible for brucellosis in cattle. *B. abortus* strain RB51, an attenuated rough mutant developed in our laboratory (20), is presently being used in several countries as a live vaccine for the control and eradication of brucellosis in cattle. Similar to most of the intracellular bacterial infections, CMI appears to play a major role in acquired resistance to brucellosis, although antibodies to surface antigens, especially to the O antigen, can confer certain level of protection against a challenge infection in some host species, such as the mouse (2, 5). Studies of mice indicate that protection afforded by strain RB51 vaccination is primarily through induction of specific CMI (6).

Although several immunoreactive antigens of *B. abortus* have been characterized, little is known about the specific proteins necessary for inducing the protective immune responses. Peptides comprising certain epitopes, but not the complete recombinant protein, of Cu/Zn superoxide dismutase (SOD) of *B. abortus* have been shown to induce partial protection against challenge infection with virulent strain 2308 (25). Further, studies involving vaccination of mice with live *Escherichia coli* expressing the *Brucella* Cu/Zn SOD indicated a protective role for this antigen against *Brucella* infections (13). In this paper, we demonstrate that overexpression of *B. abortus* Cu/Zn SOD protein in vaccine strain RB51 significantly increases its protective capabilities in the murine model of brucellosis without altering the attenuation characteristics of the vaccine.

MATERIALS AND METHODS

Bacteria. *B. abortus* virulent strain 2308 and attenuated strain RB51 were from our culture collection. *E. coli* strain DH5α (GibcoBRL, Gaithersburg, Md.) was used for producing the necessary plasmid constructs. Brucellae were grown either in Trypticase soy broth (TSB) or on Trypticase soy agar (TSA) plates. All experiments with live brucellae were performed in a biosafety level 3 facility.

Construction of recombinant strain RB51 overexpressing Cu/Zn SOD. Recombinant plasmid pBAII-3, containing the gene for *B. abortus* Cu/Zn SOD (*sodC*) along with its own promoter, was initially obtained from a pUC9 genomic library of *B. abortus* strain 2308 (10). A 1.1-kb fragment containing the *sodC* gene and its promoter sequences was excised from the insert of pBAII-3 with *Cla*I restriction enzyme digestion and subcloned into pBBR1MCS, a broad-host-range plasmid (8); the resulting plasmid was designated pBBSOD. Initially, *E. coli* DH5α was transformed with pBBSOD. A colony of *E. coli* containing pBBSOD was selected on a TSA plate containing chloramphenicol at a concentration of 30 μg/ml. After confirming the expression of Cu/Zn SOD by Western blot analysis, we isolated pBBSOD from *E. coli*. One microgram of pBBSOD or pBBR1MCS was electroporated into *B. abortus* strain RB51 as described elsewhere (12). Several colonies of strain RB51 containing the plasmid were obtained from a TSA plate containing chloramphenicol (30 μg/ml). Strain RB51 containing plasmid pBBSOD and RB51 containing pBBR1MCS were designated RB51SOD and RB51pBB, respectively. Overexpression of Cu/Zn SOD by the recombinant strain RB51 containing the plasmid pBBSOD was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses.

Purification of Cu/Zn SOD. Expression of *Brucella* Cu/Zn SOD by *E. coli* DH5α (pBS/SOD) has been previously described (13). A previously described method was used to extract Cu/Zn SOD from the *E. coli* cells using 10 mM phosphate buffer (pH 7.6) containing 0.1% Triton X-100 (4). The Cu/Zn SOD

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was purified by applying the extracts on an equilibrated anion-exchange column (HiTrapQ; Pharmacia Biotech). All of the proteins except Cu/Zn SOD bound to the resin. Cu/Zn SOD present in the flowthrough was collected, absorbed with polymyxin B beads (Affi-Prep polymyxin support; Bio-Rad Laboratories, Hercules, Calif.) to remove the lipopolysaccharide, and dialyzed extensively against phosphate-buffered saline (PBS). After determining the protein concentration by the Bradford method (3), aliquots of the purified Cu/Zn SOD were stored at −70°C until use for enzyme-linked immunosorbent assay (ELISA) or for in vitro stimulation of splenocytes.

SDS-PAGE. SDS-PAGE was performed using 15% acrylamide gels according to standard procedures (9). The *B. abortus* samples for SDS-PAGE were prepared as follows. *B. abortus* grown on either TSA or TSB was harvested and killed by incubating for 20 min in a 68°C water bath. The killed bacterial cells were washed twice with 10 mM Tris-HCl buffer (pH 8.0), and their cell concentration was adjusted to 10% transmittance at 525 nm. One-milliliter aliquots of such bacterial suspensions were centrifuged, resuspended in 100 μl of 10 mM Tris-HCl buffer, and stored at −20°C. Before use, aliquots were mixed with 100 μl of 2× Laemmli sample buffer (Sigma Chemical Co., St. Louis, Mo.), boiled for 5 min, and used for SDS-PAGE. Gels containing the separated proteins were either stained with Coomassie brilliant blue G (Sigma) or used for Western blot analysis.

Western blotting. Western blotting was performed as previously described (28). Briefly, proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane by using a Trans-blot semidry system (Bio-Rad Laboratories, Hercules, Calif.). The membranes were blocked with 2% bovine serum albumin solution and used for reaction with either goat 48 serum (from a goat hyperimmunized with strain RB51 [18]) or goat anti-*B. abortus* Cu/Zn SOD sera (13). The membranes were developed with appropriate secondary antibody conjugated with horseradish peroxidase (ICN Biochemicals, Inc., Aurora, Ohio).

Mice protection and clearance experiments. Three- to four-week-old female BALB/c mice were purchased from Charles River Laboratories, Wilmington, Mass.). Mice were given 1 week of rest before the experiments were started. Ten mice of each group were each vaccinated with ~4×10^8 CFU of RB51SOD, RB51pBB, or RB51. As a negative control, another group of 10 mice was injected with saline alone. Three mice from each group were bled at 3 and 6 weeks postinoculation (p.i.) to obtain sera for ELISA and Western blot analyses. Also at 6 weeks p.i., three mice from each group were sacrificed and their splenocytes were used for in vitro culturing to determine cytokine production. At 7 weeks p.i., seven mice from each group were challenge infected intraperitoneally with 2×10^4 CFU of *B. abortus* strain 2308 i.p. Two weeks after the challenge infection, the mice were killed, bacteria from their spleens were recovered, and numbers of CFU were determined. The protection part of the experiment was repeated two more times with five mice per group; in these experiments, only strains RB51SOD and RB51 were used as vaccines.

In a separate experiment, groups of 15 mice each were inoculated intraperitoneally with ~6×10^8 CFU of strains RB51SOD and RB51. Five mice from each group were sacrificed at 1, 3, and 6 weeks p.i., and the bacterial numbers in their spleens were determined.

ELISA. In the inoculated mice, the presence of serum IgG, IgG1, and IgG2a isotypes with specificity to the Cu/Zn SOD were determined by indirect ELISA. The purified recombinant Cu/Zn SOD was diluted to 5 μg/ml in carbonate buffer (pH 9.6) and used to coat the wells of polystyrene plates (100 μl/well; Nunc-Immuno plate with MaxiSorp surface). After overnight incubation at 4°C, the plates were washed four times in wash buffer (Tris-buffered saline [pH 7.4] with 0.05% Tween 20) and blocked with 2% bovine serum albumin in Tris-buffered saline. After 1 h at room temperature, the blocking solution was discarded, and the serum samples (1:100 dilution in blocking solution) were added to the wells (50 μl/well). Each serum sample was tested in triplicate wells. The plates were incubated for 4 h at room temperature and washed four times, and isotype-specific goat anti-mouse horseradish peroxidase conjugates (Caltag Laboratories, San Francisco, Calif.) were added (50 μl/well) at an appropriate dilution. After 1 h of incubation at room temperature, the plates were washed four times, and 100 μl of substrate solution (TMB Microwell peroxidase substrate; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added to each well. After 20 min of incubation at room temperature, the enzyme reaction was stopped by adding 100 μl of stop solution (0.185 M sulfuric acid), and the absorbance at 450 nm was recorded with a microplate reader (Molecular Devices, Sunnyvale, Calif.).

Cytokine quantitation. Splenocytes from the inoculated mice were obtained as previously described (28) and cultured in the presence of 0.5 to 2.0 μg of Cu/Zn SOD, 10 μg of *B. abortus* RB51 crude extract (28), 0.5 μg of concanavalin A, or no additives (unstimulated control). The cells were cultured for 5 days, and their supernatants were tested for the presence of gamma interferon (IFN-γ) and interleukin-4 (IL-4) by previously described sandwich ELISAs (28) using recombinant mouse IFN-γ or IL-4 (PharMingen, San Diego, Calif.) as standards. In these assays, the lower detection limits were 100 and 10 pg for the IFN-γ and IL-4 assays, respectively. The assays were performed in triplicate.

Statistical analyses. The counts of bacterial CFU in the spleens of mice were analyzed by Student's *t* test. IFN-γ production data were subjected to analysis of variance, and the means were compared using Tukey's honestly significant difference procedure (11).

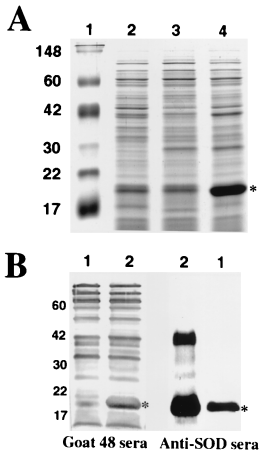

FIG. 1. Demonstration of overexpression of Cu/Zn SOD in strain RB51SOD by SDS-PAGE (A) and Western blot analysis (B). (A) Lane 1, molecular weight marker; lanes 2 to 4, contain antigens of strains RB51, RB51pBB, and RB51SOD, respectively. The gel was stained with Coomassie brilliant blue G. (B) Lanes 1 and 2, the antigens of strains RB51pBB and RB51SOD, respectively. The sera indicated below each blot were used as the primary antibodies for reacting with the antigens. Asterisks indicate the Cu/Zn SOD protein; numbers at the left indicate approximate molecular masses in kilodaltons.

RESULTS

Overexpression of self antigen. As shown in Fig. 1, *B. abortus* strain RB51SOD overexpressed the Cu/Zn SOD protein. Densitometric analysis of the Coomassie blue-stained gel (Fig. 1A) indicated that the expression of Cu/Zn SOD protein in strain RB51SOD was approximately 10 times that of strain RB51 or strain RB51pBB (data not shown). Western blotting with antisera specific to Cu/Zn SOD revealed an additional reactive band of ~40 kDa (Fig. 1B). This band most probably is the dimer of Cu/Zn SOD; the presence of such a dimer that is resistant to SDS treatment has been reported for the purified Cu/Zn SOD of *B. abortus* (4). In addition to Cu/Zn SOD, a protein of ~27 kDa was expressed in significant amounts by strains RB51pBB and RB51SOD (Fig. 1A). Most likely, this protein was chloramphenicol acetyltransferase (26,966 Da in size, based on the deduced amino acid sequence) that was encoded by the antibiotic resistance gene present on plasmid pBBR1MCS (8).

Enhancement in protection conferred by strain RB51SOD. Mice vaccinated with strain RB51SOD had significantly lower bacterial numbers in their spleens compared to those vaccinated with strain RB51 or RB51pBB, indicating enhanced protection against challenge ($P \leq 0.01$ in experiments 1 and 2; $P \leq 0.05$ in experiment 3) (Fig. 2). There was no significant difference in the level of protection between mice vaccinated with strain RB51 and those vaccinated with strain RB51pBB. Mice in these two groups were significantly protected compared to the saline-vaccinated control group ($P \leq 0.001$) (Fig. 2).

To determine if the enhanced protective response could be due to increased survivability of strain RB51SOD in mice, we determined the number of bacteria colonizing the spleens of vaccinated mice over time. At 1, 3, and 6 weeks p.i., no significant difference was observed in the number of bacteria in the spleen of mice inoculated with either strain RB51 or strain RB51SOD, indicating similar attenuation levels. At 1 and 3 weeks p.i., the RB51SOD-inoculated mice had $(4.2 \pm 0.65) \times 10^5$, and $(3.7 \pm 0.55) \times 10^3$ CFU/spleen, respectively, while the RB51-inoculated mice had $(5 \pm 0.3) \times 10^5$ and $(2.82 \pm 0.43) \times 10^3$ CFU/spleen, respectively. At 6 weeks p.i., three mice in both groups contained no detectable number of bacteria (low-

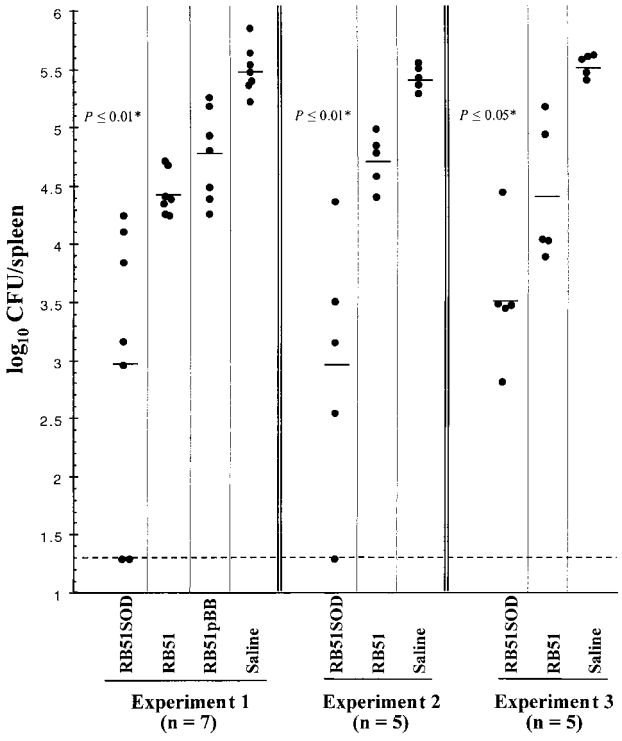

FIG. 2. Resistance to *B. abortus* 2308 challenge infection in mice vaccinated with strains RB51SOD, RB51pBB, and RB51. Two weeks after challenge infection, the number of strain 2308 CFU in the spleen was determined. The mean value in each group is indicated by a solid horizontal line. The horizontal broken line above the *x* axis indicates the lower detection limit (<20 CFU/spleen). The *P* value indicated in the RB51SOD group is in comparison with the RB51 group. In all three experiments, strain RB51-vaccinated mice contained significantly lower numbers of CFUs in comparison with the saline-inoculated group ($P \leq 0.001$). In experiment 1, there was no significant difference in the number of CFU between RB51- and RB51pBB-vaccinated groups ($P > 0.05$).

er detection limit was 20 CFU/spleen); only 80 and 40 CFU could be isolated from the other two mice of the RB51SOD and RB51 groups, respectively.

Immune responses of mice vaccinated with strain RB51SOD. Sera from mice vaccinated with strain RB51SOD, but not those vaccinated with strain RB51 or RB51pBB, contained antibodies to Cu/Zn SOD protein. In ELISA to detect SOD-specific IgG, the absorbance readings of mouse sera collected 3 and 4 weeks after vaccination with RB51SOD were 1.104 ± 0.21 and 1.906 ± 0.16, respectively. Subisotype analysis of these antibodies indicated that they were predominantly of IgG2a (absorbance readings of 0.728 ± 0.18 and 1.682 ± 0.2 for the 3- and 6-week sera, respectively). In both IgG- and IgG2a-specific ELISAs, the absorbance values with sera from other groups of mice were not different from the blank values (<0.01). No IgG1 antibodies specific to the Cu/Zn SOD were detected in sera of any group of mice (data not shown). Also, splenocytes from strain RB51SOD-vaccinated mice secreted IFN-γ upon in vitro stimulation with the recombinant Cu/Zn SOD (Table 1). Splenocytes from all of the vaccinated mice produced similar levels of IFN-γ when stimulated with *B. abortus* RB51 antigen extract (Table 1). However, no IL-4 was detected in any of the culture supernatants of splenocytes stimulated with the specific antigens (data not shown). Splenocytes from all groups of mice, including the saline-inoculated group, produced similar levels of IL-4 and IFN-γ upon stimulation with concanavalin A (data not shown).

TABLE 1. Concentration of IFN-γ in culture supernatants of splenocytes upon in vitro stimulation with recombinant Cu/Zn SOD or RB51 antigen extract for 5 days

Stimulant	Concn of IFN-γ (ng/ml)			
	Naïve mice	RB51-vaccinated mice	RB51SOD-vaccinated mice	RB51pBB-vaccinated mice
Medium	—[a]	—	—	—
RB51 antigen extract	0.42 ± 0.21	16.85 ± 2.72[b]	19.27 ± 2.60[b]	18.2 ± 1.98[b]
Cu/Zn SOD				
0.5 μg	—	—	0.49 ± 0.18	—
1 μg	—	—	1.32 ± 0.34	—
2 μg	—	—	3.09 ± 0.86	—

[a] —, below detection limit (less than 0.1 ng/ml).
[b] No significant difference between the three groups.

DISCUSSION

Overexpression of self proteins has been performed in several bacterial systems to complement the deleted or lost gene expression (14), to study the functional aspects of the gene product (15, 17, 21, 31), or to obtain increased amounts of a protein in its native state (19). However, to the best of our knowledge, this strategy has heretofore not been used to enhance the protective capabilities of live vaccines. The studies presented here clearly indicate that such a strategy indeed results in enhanced protection.

There is sufficient evidence that the complete protein or certain epitopes of *Brucella* Cu/Zn SOD can induce protective immunity in mice (13, 25). However, the contribution of *B. abortus* Cu/Zn SOD protein to the protective immunity conferred by a live vaccine strain is not known. The absence of detectable antibody and selected CMI responses against Cu/Zn SOD in mice vaccinated with strain RB51 suggest that there is no significant role for this protein in the protection conferred by this attenuated vaccine strain. As demonstrated in this study, mice vaccinated with RB51SOD, but not RB51, developed Cu/Zn SOD-specific immune responses. This suggests that a quantity of Cu/Zn SOD that is higher than that produced by strain RB51 is required to induce an immune response in mice. The amount of Cu/Zn SOD produced by strain RB51 from the chromosomal gene, which is detectable by Western blotting (Fig. 1B), may not be sufficient to induce an immune response. Also, Rafie-Kolpin et al. (16) reported that the expression of Cu/Zn SOD in *B. abortus* virulent strain 2308 is decreased during intracellular growth in bovine macrophages. Studies in our laboratory indicate that the activity of the *sodC* gene promoter in strain RB51 is decreased upon intracellular localization in J774A.1 cells, a murine macrophage-like cell line (29). Taken together, the available data indicate that the decreased expression is a probable reason for the lack of an immune response against *Brucella* Cu/Zn SOD in strain RB51-vaccinated animals.

Mice vaccinated with strain RB51SOD developed a Th1-type of immune response to the Cu/Zn SOD, as indicated by the specific induction of serum IgG2a, but not IgG1, antibodies and by the secretion of IFN-γ, but not IL-4, by the Cu/Zn SOD-stimulated splenocytes (24). The enhanced protective immunity conferred by strain RB51SOD could be attributed to the specific cell-mediated responses, especially IFN-γ secretion (32). It is also possible that the overexpression of Cu/Zn SOD in strain RB51 alters the processing and presentation of other protective antigens by a yet unidentified mechanism and that the observed immune response to Cu/Zn SOD does not play a crucial role in the enhanced protection. Further studies

aimed at unraveling the basis for the observed enhanced protection are under way in our laboratory. Such studies may also provide clues as to the reasons for the observed highly variable degree of protection afforded by strain RB51SOD, especially in experiments 1 and 2 (Fig. 2). Although the matter is still controversial (10, 22, 26), the Cu/Zn SOD is considered a virulence factor of *B. abortus* by some researchers (26). Overexpression of a virulence factor may enhance the persistence or virulence characteristic of an attenuated pathogen. However, this appears not to be the case in our study, since the patterns of clearance or persistence of strains RB51 and RB51SOD in the vaccinated mice were similar (Fig. 3). It remains to be tested if the induction of a measurable immune response to Cu/Zn SOD and the enhanced protective response seen in RB51SOD-vaccinated mice can also be achieved in other animal species such as cattle. Cattle vaccinated with strain RB51 also do not develop antibodies or a lymphocyte proliferation response to Cu/Zn SOD (23). If cattle were to respond to strain RB51SOD immunization as did mice, in addition to increasing the efficacy of the vaccine, it could also be the basis for development of a serological assay for the detection of strain RB51SOD-vaccinated animals.

Overexpression of a nonprotective 18-kDa outer membrane lipoprotein of *B. abortus* (27) in strain RB51 did not enhance its protective ability although it increased IFN-γ production (unpublished data). This indicates that selection of an appropriate protein for the overexpression is important to achieve the enhanced protectivity of the vaccine. It is our opinion that overexpression of some other *Brucella* protective antigen(s) in strain RB51 could also result in enhanced protective capabilities. Moreover, this strategy can also be used for other live bacterial vaccines as long as the overexpressed antigen does not affect the attenuation characteristic of the vaccine strain. Because of the enhanced vaccine efficacy, lower doses of the vaccine may be used to obtain the same level of protection as provided by the original vaccine.

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