

## Construction of Cu-Zn Superoxide Dismutase Deletion Mutants of *Brucella abortus*: Analysis of Survival In Vitro in Epithelial and Phagocytic Cells and In Vivo in Mice

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Cu-Zn superoxide dismutase (SOD) deletion mutants of *Brucella abortus* S2308, a virulent strain, and S19, a vaccine strain, were generated by gene replacement. A deletion plasmid, pBA $\Delta$ sodkn<sup>r</sup>, was constructed by excising the Cu-Zn SOD gene (Cu-Zn sod) from a 2.3-kb *B. abortus* DNA fragment of plasmid pBA20-1527 and inserting a 1.4-kb DNA fragment encoding kanamycin resistance into the Cu-Zn sod excision site. The deletion plasmid was introduced into *B. abortus* by electroporation, and Southern blot analysis confirmed that the antibiotic resistance fragment had replaced Cu-Zn sod in kanamycin-resistant colonies. The survival and growth of Cu-Zn SOD mutant strains were compared with that of the parental strains in HeLa cells and in the mouse macrophagelike cell line J774. The survival and growth of the Cu-Zn SOD mutant strains were similar to those of their respective parental strains in HeLa and J774 cell lines. The kinetics of infection with these strains were examined in BALB/c mice. The splenic levels of the S19 Cu-Zn SOD mutant recovered from intraperitoneally infected BALB/c mice were approximately 10-fold lower than those of the parental strain through 26 days postinfection. Thereafter, infection sharply declined in both groups, and by 105 days postinfection, no organisms were detected. The splenic levels of the S2308 Cu-Zn SOD mutant were lower than those of wild-type S2308-infected mice. The spleen weights of mice infected with the S2308 Cu-Zn SOD mutant were consistently lower than those of wild-type S2308-infected mice. These results suggest that the antioxidant enzyme Cu-Zn SOD plays a role in the survival and pathogenicity of *B. abortus* in vivo.

*Brucella abortus* is a facultative intracellular pathogen capable of survival and replication within host phagocytic cells. Because smooth *B. abortus* strains are significantly more resistant to phagocytic destruction than rough strains, which lack lipopolysaccharide O side chains, it is widely accepted that *B. abortus* lipopolysaccharide serves as a protective barrier to toxic products within macrophages and polymorphonuclear leukocytes (9, 12, 29, 30). Other adaptations contributing to the survival of *B. abortus* within host phagocytic cells have been proposed and include (i) the production of enzymes that defend against oxidative destruction (6), (ii) the production of substances that inhibit phagosome-lysosome fusion in macrophages (18), and (iii) the secretion of adenine and 5'-GMP, which suppress the myeloperoxidase-H<sub>2</sub>O<sub>2</sub> system of neutrophils (7, 13). The work presented here was designed to investigate the first proposed adaptation, specifically, the role of Cu-Zn superoxide dismutase (SOD) of *B. abortus* in its intracellular survival.

SODs form part of the antioxidant defense system that protects cells from the toxic effects of reactive oxygen intermediates by converting superoxide radicals into hydrogen peroxide and oxygen (19, 23). Three forms of SOD have been described. The evolutionarily related manganese and iron SODs are commonly found in prokaryotes (4). Manganese SODs are also found in the matrix of mitochondria. The unrelated Cu-Zn SODs occur primarily in the cytosol of

eukaryotic cells and in chloroplasts (11). The Cu-Zn form has also been found in a few species of bacteria (3, 32).

*B. abortus* possesses two forms of SOD. One has been preliminarily characterized as a manganese SOD (31), and the second has been identified as a Cu-Zn SOD (6). DNA sequence analysis of the *B. abortus* Cu-Zn SOD gene (Cu-Zn sod) revealed the presence of a leader sequence, strongly suggesting an extracytoplasmic location for Cu-Zn SOD (6). This finding may be relevant to understanding how *B. abortus* evades killing within phagocytes. A membrane or periplasmic location for Cu-Zn SOD may be an important adaptation protecting *B. abortus* against the bactericidal oxygen intermediates produced within phagocytic cells. Such a role for SOD has been demonstrated for other facultative intracellular bacteria. The surface-associated and secreted SOD of *Nocardia asteroides* is protective against oxidative killing in vivo during all stages of infection (5). The Fe SOD of *Shigella flexneri* plays a role in the pathogenesis of this organism, even though this protein is located in the cytoplasm (17). Virulent forms of *Mycobacterium tuberculosis* secrete SOD into the medium, and it has been suggested that the enzyme acts as a virulence factor in this organism (1).

To investigate the contribution of Cu-Zn SOD to the resistance of *B. abortus* to oxidative killing, we constructed Cu-Zn SOD deletion mutants of S19, a vaccine strain, and S2308, a virulent strain, by gene replacement and compared the mutants with their parental strains for survival in macrophagelike J774 cells and in the nonphagocytic HeLa cell line. In addition, the persistence of Cu-Zn SOD deletion

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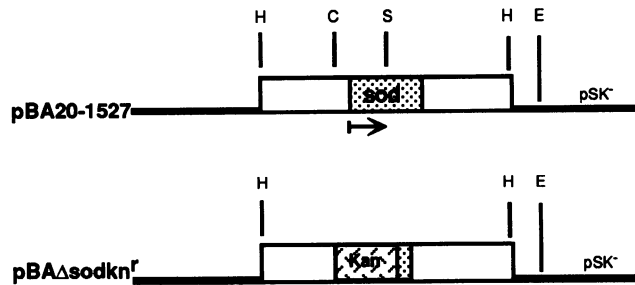


FIG. 1. Diagrammatic restriction map of plasmids pBA20-1527 and pBA $\Delta$ sodkn<sup>r</sup>. H, *Hind*III; C, *Cla*I; S, *Sma*I; E, *Eco*RI.

mutants and their respective parental strains was examined in BALB/c mice.

### MATERIALS AND METHODS

**Materials.** Restriction enzymes, T4 DNA ligase, and the large fragment of DNA polymerase I (the Klenow fragment) were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). Agarose was purchased from FMC Bioproducts, Inc. (Rockville, Md.). Calf alkaline phosphatase and a random hexamer priming kit were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.). Radiolabeled [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol) was obtained from ICN Biomedicals, Inc. (Irvine, Calif.). Modified nylon membranes for Southern blotting and Western immunoblotting were purchased from Schleicher and Schuell, Inc. (Keene, N.H.). Goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate, kanamycin monosulfate, and ampicillin sodium salt were purchased from Sigma Chemical Co. (St. Louis, Mo.).

**Plasmid construction.** Recombinant DNA manipulations were performed by standard procedures as described by Maniatis et al. (25). Plasmid pBA20-1527, encoding Cu-Zn SOD, was described previously (10). This plasmid contains a 2.3-kb *Hind*III *B. abortus* DNA fragment cloned into pUC9. Prior to construction of the deletion vector, the *Sma*I site in the multiple cloning region was removed from pUC9 by digestion with *Xma*I, filling in of the overhangs with deoxynucleoside triphosphates by use of the Klenow fragment of *E. coli* DNA polymerase I, and blunt end ligation with T4 DNA ligase. Construction of the deletion plasmid used for gene replacement in *B. abortus* was done as follows. Restriction endonuclease *Sma*I was used to linearize pBA20-1527 by digestion at a unique *Sma*I site located in the coding region of Cu-Zn *sod* (Fig. 1). Limited exonuclease digestion of pBA20-1527 with *Bal*31 was followed by calf alkaline phosphatase treatment. The deletion plasmids were recircularized with phosphorylated *Sma*I linkers by use of T4 DNA ligase and used to transform CaCl<sub>2</sub>-competent XL1-Blue cells (Stratagene Cloning Systems, LaJolla, Calif.). Plasmids isolated from these clones were digested with restriction enzymes *Sma*I and *Eco*RI. The DNA fragments were excised from low-melting-temperature agarose and ligated into the *Sma*I-*Eco*RI-digested pSK<sup>+</sup> vector (Stratagene Cloning Systems) to generate plasmid pBA $\Delta$ 3'*sod*. Plasmid pBA $\Delta$ 3'*sod* was determined by DNA sequence analysis to contain the translational stop codon and the 10 preceding codons of Cu-Zn *sod*. The 5' end of Cu-Zn *sod*, including promoter sequences identified by primer extension analysis (data not shown), was deleted by digesting pBA20-1527 with restriction enzymes *Sma*I and *Cla*I. The 5' nucleotide over-

hangs were filled in as described above and treated with calf alkaline phosphatase. The remainder of the *B. abortus* fragment contained on the vector was isolated from an agarose gel by electroelution and ligated with T4 DNA ligase and phosphorylated *Sma*I linkers to generate pBA $\Delta$ 5'*sod*. The *Sma*I-*Eco*RI DNA fragment of pBA $\Delta$ 3'*sod* was excised by restriction enzyme digestion, electroeluted from an agarose gel, and ligated into *Sma*I-*Eco*RI-digested pBA $\Delta$ 5'*sod* to generate pBA $\Delta$ sod. A 1.4-kb fragment encoding kanamycin resistance was excised from pBA31-R7Kan7 (20) by *Hind*III digestion. The fragment ends were filled in with deoxynucleoside triphosphates and the Klenow fragment as described above. The kanamycin resistance cassette was blunt end ligated with T4 DNA ligase into the *Sma*I site of pBA $\Delta$ sod to generate plasmid pBA $\Delta$ sodkn<sup>r</sup>. Plasmid pBA $\Delta$ sodkn<sup>r</sup> was the deletion plasmid used for gene replacement in *B. abortus*.

**Electroporation.** Ten micrograms of pBA $\Delta$ sodkn<sup>r</sup> DNA was combined with 50  $\mu$ l (10<sup>10</sup> cells per ml) of thawed *B. abortus* cells in sterile electroporation cuvettes with electrode gaps of 0.2 cm (BioRad Laboratories, Richmond, Calif.). *B. abortus* cells were prepared for electrotransformation by harvesting of cells rapidly grown at 37°C in Trypticase soy broth with vigorous shaking. The cells were chilled on ice for 10 min and centrifuged at 10,000  $\times$  *g* (maximum) for 10 min. The cell pellet was resuspended in an equal volume of sterile cold water and centrifuged as described above. The cells were washed twice more in water and resuspended in a 1/500 volume of 10% glycerol. Aliquots were frozen on dry ice and stored at -70°C. A Gene Pulser transfection apparatus (BioRad Laboratories) at a setting of 25  $\mu$ F and 2.5 kV and a pulser controller set at 200  $\Omega$  were used to transform *B. abortus*. Immediately after electroporation, 1 ml of Trypticase soy broth was added to the cells, and the cells were cultured at 37°C for 6 h before being plated. *B. abortus* transformants were selected on tryptose agar supplemented with 50  $\mu$ g of kanamycin sulfate per ml. Colonies were visible after 4 days at 37°C.

**Isolation of genomic DNA.** Genomic DNA was isolated by the method of Halling and Zehr (21). In brief, the detergent-lysed cells were brought to 4.0 ml with TE, and 4.4 g of cesium chloride and 100  $\mu$ l of 5-mg/ml ethidium bromide were added. The DNA was centrifuged overnight at 5  $\times$  10<sup>4</sup> rpm in a Beckman VTi 65 rotor. Genomic DNA was recovered as described by Maniatis et al. (25).

**Recombinant DNA techniques and Southern hybridization analysis.** Restriction endonucleases and nucleic acid-modifying enzymes were used as described by the manufacturer. DNA restriction fragments were sized on agarose gels (1% with 1 $\times$  TBE (0.09 M Tris, 0.09 M boric acid, 0.002 M EDTA [pH 8.7])). DNA fragments were removed from agarose gels by electroelution of excised agarose bands into 16-mm dialysis tubing (Spectrapor; Thomas Scientific, Philadelphia, Pa.). The eluted DNA was extracted with phenol-chloroform and then ethanol precipitated. *Escherichia coli* strains were transformed with plasmid DNA by the calcium chloride method (25). Radioactively labeled restriction fragment probes were radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP by random oligonucleotide priming. Restriction enzyme-digested genomic DNA (2  $\mu$ g) fragments were separated by gel electrophoresis and transferred to nylon membranes by the procedure suggested by the manufacturer. Hybridization and washing of filters were carried out essentially as described by Maniatis et al. (25). In brief, filters were hybridized overnight at 42°C in 6 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-10  $\mu$ g of sonicated salmon sperm

DNA per ml–50% formamide. The filters were washed once at 37°C in 1× SSC–0.5% sodium dodecyl sulfate (SDS) for 10 min and twice at 65°C in 0.5× SSC–0.5% SDS for 10 min.

**Western blotting.** SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (24). Whole cells were solubilized in 2× Laemmli buffer at 100°C, and cell lysates were electrophoresed on a 4 to 20% SDS-polyacrylamide gel and transferred to nitrocellulose filters as described previously (10). The filters were reacted with rabbit anti-Cu-Zn SOD (diluted 1:200), incubated with a peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (diluted 1:1,000), and stained with 4-chloro-1-naphthol as a chromogen.

**Bacterial cultures.** *B. abortus* S2308, a highly virulent, smooth form of bovine origin, and *B. abortus* S19, an attenuated, smooth vaccine strain, were obtained from the Brucella Culture Collection of the National Animal Disease Center, Ames, Iowa. Isolates of the mutant strains were prepared by streaking on tryptose agar plates containing kanamycin and selecting for single colonies. Working stock cultures of each strain were obtained by spreading single colonies on tryptose agar plates and incubating them at 37°C for 3 days. Cultures were harvested in 2 ml of sterile saline containing 20% glycerol and stored at –70°C. The same stock cultures were used throughout this study.

**Cultivation of bacteria.** *B. abortus* strains were cultured for 48 h at 37°C on tryptose agar plates containing 5% heat-inactivated bovine serum. Cells were harvested by washing of the plate surface with sterile 0.85% NaCl and standardized turbidimetrically to a concentration of 10<sup>9</sup> CFU/ml in saline. This suspension was diluted to 10<sup>7</sup> CFU/ml in Dulbecco's minimal essential medium (MEM) supplemented with 5% fetal calf serum and 2 mM glutamine prior to inoculation of cell lines.

**Cell cultures.** Mouse monocyte-macrophage J774 cells (J774A.1; ATCC TIB61) and human epithelioid carcinoma cells (HeLa; ATCC CCL2) were purchased from the American Type Culture Collection (Rockville, Md.). J774 cells were routinely grown in Dulbecco's MEM (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 5% fetal calf serum and 2 mM glutamine. HeLa cells were routinely grown in Eagle's MEM (GIBCO Laboratories) supplemented with 5% fetal calf serum and 2 mM glutamine. For monolayer inoculation, 24-well tissue culture plates (Costar, Cambridge, Mass.) were seeded with 1 ml of culture medium containing 1.5 × 10<sup>5</sup> to 2.0 × 10<sup>5</sup> cells. After 24 h of incubation, subconfluent monolayers were washed with phosphate-buffered saline (PBS) at 37°C and further incubated in 1 ml of culture medium without antibiotics.

**Monolayer inoculation.** After overnight incubation, the medium was aspirated from the plates and 1 ml of bacterial suspension was added. The culture plates were centrifuged for 5 min at 800 × g and room temperature and placed in a humidified incubator with an atmosphere of 5% CO<sub>2</sub> at 37°C. After 2 or 4 h, the plates were washed three times with PBS and further incubated with Eagle's MEM or Dulbecco's MEM supplemented with 5% fetal calf serum, 2 mM glutamine, and gentamicin (50 µg/ml) and streptomycin (25 µg/ml) (Pfizer, New York, N.Y.), antibiotic concentrations sufficient to kill extracellular brucellae. The medium was replaced 1 h later and at 24 h. The inoculation period was defined as the period between the exposure of the monolayer to *B. abortus* and the introduction of gentamicin and streptomycin. Experiments were done in duplicate and repeated three times for each experimental treatment. The variation between duplicate assays done on the same days was ±10%.

While the variation between repeat experiments done on different days exceeded ±10%, the patterns of intracellular survival and growth of the mutants relative to the parental strains remained essentially unchanged.

**Enumeration of brucellae.** The numbers of intracellular viable *B. abortus* were determined at 2, 3, 5, 7, 24, 36, and 48 h postinoculation (p.i.) in J774 cells and at 3, 5, 24, and 48 h p.i. in HeLa cells. Except for the 2- and 3-h samples, gentamicin and streptomycin were introduced in all samples 2 h p.i. with J774 cells and 4 h p.i. with HeLa cells. The 2- and 3-h samples were treated with gentamicin and streptomycin for 1 h before bacterial counts were determined. After exposure to antibiotics, inoculated monolayers were washed in PBS and incubated for 20 min in 2 ml of a 0.1% aqueous solution of deoxycholate. This procedure disrupted the host cells without affecting the viability of the brucellae. Samples of the lysate were serially diluted in saline for the quantitation of CFU of *B. abortus* by plating on tryptose agar plates containing 5% heat-inactivated bovine serum. *B. abortus* colonies were identified by colony morphology and growth characteristics.

**Infection of mice and estimation of bacterial numbers.** Two-month-old male BALB/c mice obtained from Charles River Breeding Laboratories Inc. (Wilmington, Mass.) were injected intraperitoneally with approximately 10<sup>4</sup> brucellae in 0.2 ml of PBS. Four groups of 36 mice were infected with *B. abortus* S19, the S19 Cu-Zn SOD mutant, S2308, and the S2308 Cu-Zn SOD mutant, and 6 mice from each group were examined at each sampling period. At 5, 10, 18, 26, 60, and 105 days p.i., mice were killed by cervical dislocation. The spleens were removed, weighed, and cut in half. The tissue used for bacterial estimation was weighed and homogenized in 10 ml of PBS. Tissue homogenates were serially diluted and plated in duplicate. *B. abortus* colonies were counted after 3 days of incubation at 37°C. Viable counts were determined as CFU per milligram of tissue. The remaining tissue was fixed in Formalin for histopathology studies.

## RESULTS

**Characterization of *B. abortus* Cu-Zn SOD deletion mutants.** Deletion plasmid pBAΔ*sodkn*' was introduced into *B. abortus* S19 and S2308 by electroporation (Fig. 1). We demonstrated previously that plasmids carrying the ColE1 origin do not replicate in *B. abortus* and therefore can be used to construct gene replacement strains of *B. abortus* (20). Gene replacement candidates were isolated by selecting clones on tryptose agar plates containing 50 µg of kanamycin sulfate per ml. Chromosomal DNAs were isolated from eight S19 and six S2308 kanamycin-resistant clones for Southern blot analysis. *Hind*III restriction digests of *B. abortus* DNA were separated on 0.7% agarose gels, transferred to nylon filters, and probed with a radiolabeled 1.4-kb *Hind*III fragment encoding kanamycin resistance. The kanamycin resistance probe hybridized to chromosomal DNA from each kanamycin-resistant mutant but not to DNA from either the *B. abortus* S19 or the *B. abortus* S2308 control (Fig. 2A and C), demonstrating that the antibiotic-resistant clones arose from the integration of plasmid pBAΔ*sodkn*' into the chromosome. A *Hind*III Southern blot of genomic DNAs isolated from the kanamycin-resistant clones was also hybridized with a radiolabeled Cu-Zn *sod* fragment isolated from a *Cla*I-*Sma*I digest of pBA20-1527. Genomic DNAs from seven of the eight S19 kanamycin-resistant clones and five of the six S2308 kanamycin-resistant clones did not hybridize to the Cu-Zn *sod* probe (Fig. 2B and D), indicating that

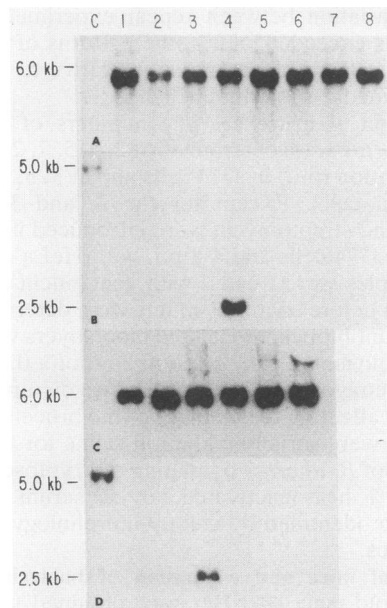


FIG. 2. Southern blots of DNAs from *B. abortus* kanamycin-resistant isolates hybridized with radiolabeled DNA probes. (A) *Hind*III digest of S19 isolates hybridized with a  $^{32}\text{P}$ -radiolabeled kanamycin resistance probe. Lanes: C, DNA from S19 *B. abortus*; 1 to 8, DNAs isolated from S19 kanamycin-resistant clones. (B) Same as panel A, but hybridized with a  $^{32}\text{P}$ -radiolabeled Cu-Zn *sod* probe. (C) *Hind*III digest of S2308 isolates hybridized with a  $^{32}\text{P}$ -radiolabeled kanamycin resistance probe. Lanes: C, DNA from *B. abortus* S2308; 1 to 6, DNAs isolated from S2308 kanamycin-resistant clones. (D) Same as panel C, but hybridized with a  $^{32}\text{P}$ -radiolabeled Cu-Zn *sod* probe.

double crossovers between the deletion plasmid and homologous chromosomal DNA occurred with a high frequency. A single recombinational event between the deletion plasmid and the chromosome gave rise to kanamycin- and ampicillin-resistant *B. abortus* colonies expressing Cu-Zn SOD (data not shown).

Since DNA sequences encoding the carboxy-terminal region of Cu-Zn SOD were present in the suicide plasmid, gene replacement strains used in this work were examined for the expression of Cu-Zn SOD by Western blot analysis. Whole-cell lysates from the S19 and S2308 deletion mutants and control lysates were examined with rabbit antiserum generated against *E. coli*-derived *B. abortus* Cu-Zn SOD (data not shown). As expected, Cu-Zn SOD was immunologically detected in control S2308 and S19 lysates and in S19 and S2308 lysates with *sod*<sup>+</sup> *Kn*<sup>r</sup> *Ap*<sup>r</sup> genotypes. No such product was detected in lysates from isolates with deleted Cu-Zn *sod* sequences.

The generation times and colony morphologies of the two Cu-Zn SOD mutant strains used in this study were compared with those of the parental strains. Generation times were examined *in vitro* by culturing in Trypticase soy broth at 37°C with aeration at 100 rpm. The outer membrane morphology was determined by the acriflavine agglutination assay. Rough strains aggregate when mixed with acriflavine. The smooth morphologies and generation times of the Cu-Zn SOD mutant strains used in this study were indistinguishable from those of their respective parental strains.

**Kinetics of *B. abortus* intracellular growth in HeLa cells.** The numbers of viable brucellae were determined in HeLa

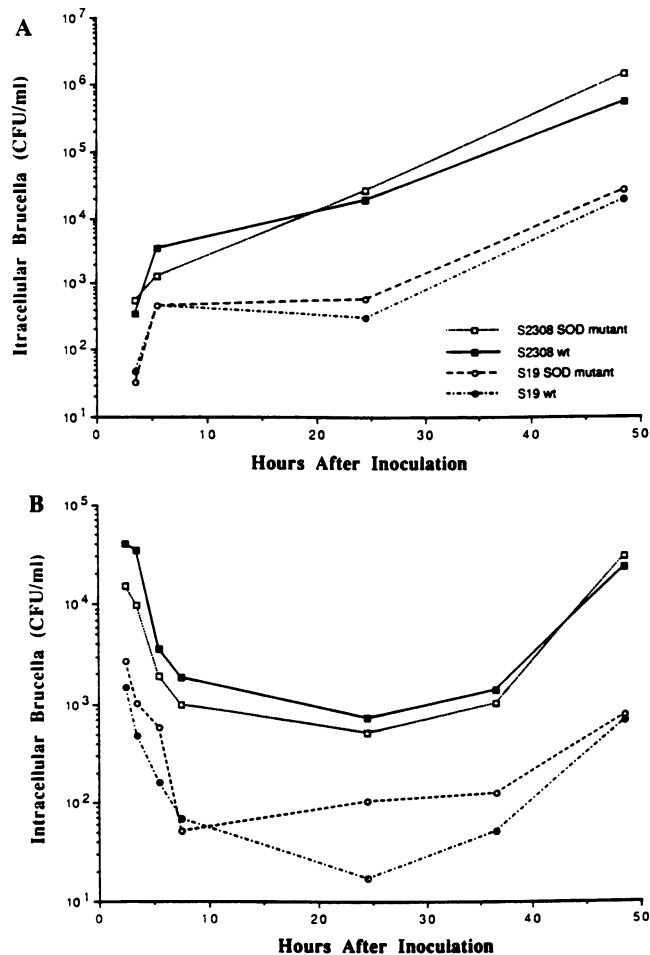


FIG. 3. Survival of wild-type (wt) *B. abortus* S19, the S19 Cu-Zn SOD mutant, wild-type *B. abortus* S2308, and the S2308 Cu-Zn SOD mutant, expressed as the mean  $\log_{10}$  CFU per milliliter in HeLa cells (A) and J774 cells (B). Symbols in panel B are the same as those in panel A. Cells were infected with  $10^7$  *B. abortus* organisms.

cell lysates at 3, 5, 24, and 48 h p.i. (Fig. 3A). Both wild-type bacterial strains showed similar intracellular growth patterns over the course of the experiment. However, at all time points, the number of viable brucellae in the cellular lysates was approximately 1 log larger for S2308 than for S19. The larger number of viable intracellular S2308 recovered from HeLa cells was attributed to greater cellular uptake of S2308 than of S19. Only minor differences in uptake and intracellular growth were seen between the Cu-Zn SOD mutants and their respective parental strains in HeLa cells.

**Kinetics of *B. abortus* intracellular growth in J774 cells.** The numbers of viable brucellae in mouse macrophagelike J774 cells were determined at 2, 3, 5, 7, 24, 36, and 48 h p.i. (Fig. 3B). The patterns of survival and growth of all *B. abortus* strains in J774 cells were clearly different from those in the nonphagocytic HeLa cells. The uptake of S2308 and S19 by J774 cells was much greater than that by HeLa cells. After 3 h p.i., intracellular bacterial numbers were approximately 2 logs larger in J774 cells than in HeLa cells. Unlike in HeLa cells, however, the number of viable brucellae recovered in J774 cells declined steadily over the 24-h p.i. period. The decrease in the number of intracellular brucellae was attributed to the bactericidal activity of these phagocytic cells.

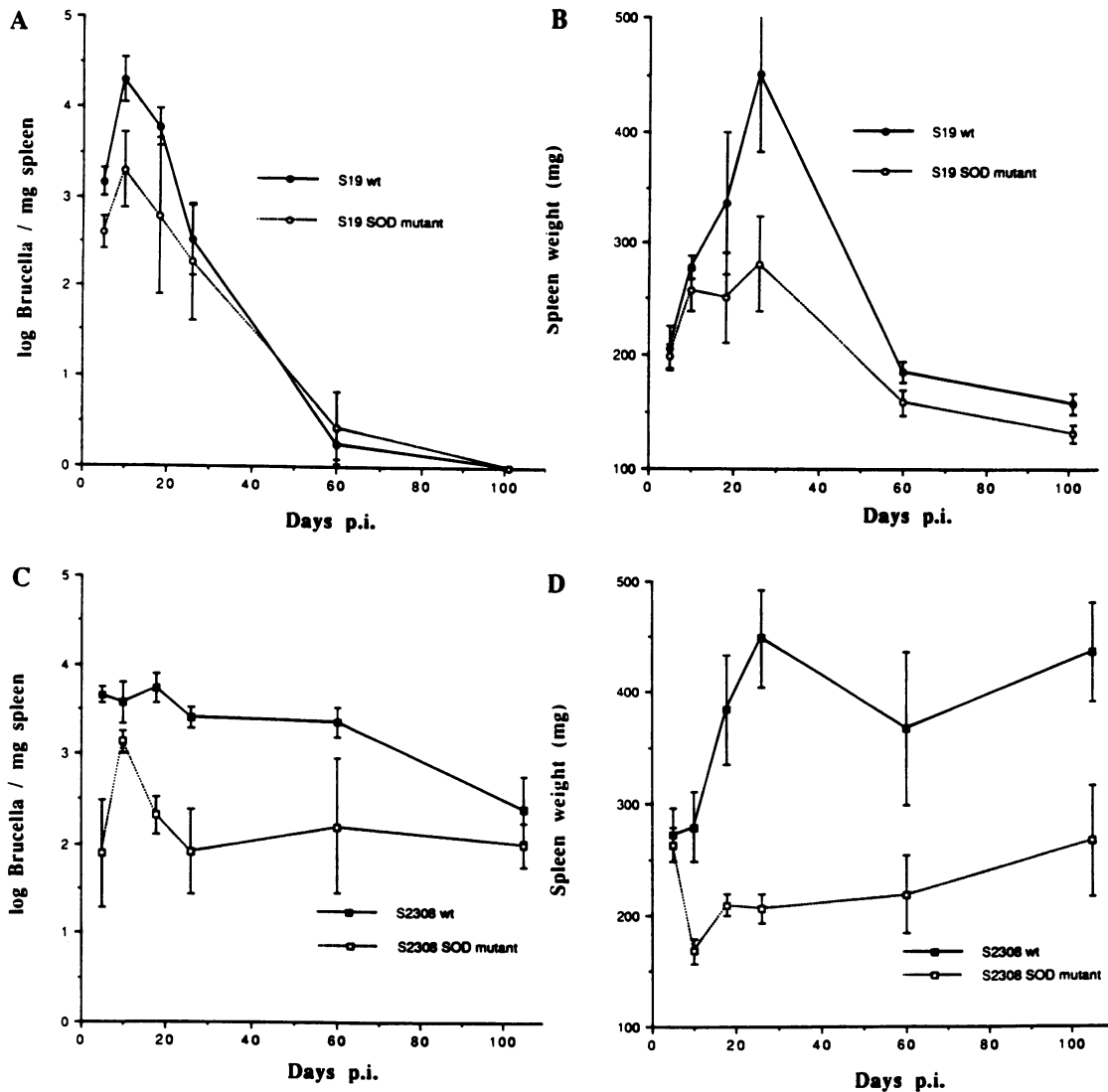


FIG. 4. Persistence of *B. abortus* in spleens of BALB/c mice inoculated i.p. with  $10^4$  organisms and spleen weights of infected mice. (A) Persistence of wild-type (wt) *B. abortus* S19 and the S19 Cu-Zn SOD mutant expressed as the mean  $\log_{10}$  CFU per milligram of spleen. Bars indicate standard errors. (B) Mean spleen weights of BALB/c mice inoculated with wild-type *B. abortus* S19 and the S19 Cu-Zn SOD mutant. Bars indicate standard errors. (C) Same as panel A, but inoculated with wild-type *B. abortus* S2308 and the S2308 Cu-Zn SOD mutant. (D) Same as panel B, but inoculated with wild-type *B. abortus* S2308 and the S2308 Cu-Zn SOD mutant.

After 24 h p.i., intracellular levels of S19 and S2308 increased. Only minor differences were observed between the intracellular growth patterns of the S2308 Cu-Zn SOD mutant and its parental strain (Fig. 3B). The patterns of the S19 Cu-Zn SOD mutant and its parental strain diverged somewhat at 24 h p.i. but were nearly identical by 48 h p.i.

**Course of infection in BALB/c mice.** The levels of wild-type S19 recovered per milligram of spleen in BALB/c mice infected with  $10^4$  bacteria remained approximately 1 log higher than those of the S19 Cu-Zn SOD mutant from 5 through 18 days p.i. (Fig. 4A). At 10 days p.i., the splenic bacterial numbers of both strains peaked, increasing 1 log from day-5 p.i. levels. After 18 days p.i., the splenic levels of the S19 Cu-Zn SOD mutant and wild-type S19 were similar and declined until these strains were cleared from mouse spleens by 105 days p.i.

The loss of Cu-Zn SOD resulted in reduced survival of the S2308 Cu-Zn SOD mutant in mice (Fig. 4C). At day 5 p.i., the splenic levels of the S2308 Cu-Zn SOD mutant were 1.7 logs (>50-fold) lower than those of wild-type S2308, and this difference ( $1.7 \pm 0.6$ ) was statistically significant (*t* test;  $P < 0.05$ ). By day 26 p.i., the levels of the S2308 Cu-Zn SOD mutant were 1.5 logs (>30-fold) lower than those of wild-type S2308. At 60 days p.i., the splenic levels of the S2308 Cu-Zn SOD mutant were 1.2 logs (approximately 16-fold) lower than those of wild-type S2308. Over this 60-day period, the numbers of wild-type S2308 remained relatively unchanged. At 105 days p.i., the splenic counts of the mutant strain and the parental strain appeared to be converging, primarily because of a decrease in wild-type S2308 levels. None of the 36 mice infected with wild-type S2308 were free of bacteria, but 4 of the 36 mice infected

with the S2308 mutant strain were free of bacteria. This difference was statistically significant (chi square, 4.2;  $P < 0.05$ ).

After the first sampling period at 5 days p.i., mice infected with the S2308 Cu-Zn SOD mutant had consistently lower spleen weights than did those infected with wild-type S2308 (Fig. 4D). Mice infected with the S19 Cu-Zn SOD mutant also had consistently lower spleen weights than did those infected with the respective parental strain, but the differences were pronounced only on days 18 and 26 p.i. (Fig. 4B).

## DISCUSSION

The loss of Cu-Zn SOD reduced the levels of *B. abortus* S2308 and S19 in mouse spleens during the initial phase of infection, albeit our findings indicated that Cu-Zn SOD had a greater effect on the survival of S2308 than of S19 in mice. The reduced effect on survival due to mutation of Cu-Zn SOD in S19 may be attributable to attenuation of the parent. Mice infected with S19 rapidly resolve the infection, whereas S2308 produces a chronic infection (8, 26, 28). The patterns of infection by S19 and S2308 in mice also correspond to the relative virulence of these strains in cattle (33). Attenuated S19 is used as a bovine vaccine, and S2308 is used as a challenge strain in cattle. There is no correlation between Cu-Zn SOD and the relative virulence of wild-type S19 and S2308, however, since both strains possess comparable levels of enzyme activity (31). Collectively, these data suggest that Cu-Zn SOD influences the initial level of infection but that the enzyme is not necessary for the persistence of *B. abortus* in mice.

The deletion mutation resulting in the loss of Cu-Zn SOD is not likely to have caused a polar mutation affecting other *B. abortus* genes. Northern (RNA) blot analysis revealed that the Cu-Zn *sod* transcript is <1.0 kb long (data not shown). Since the distance from the transcriptional start site to the stop codon of Cu-Zn *sod* is approximately 700 bases, these data indicate that Cu-Zn *sod* is transcribed as a unique message.

The elimination of *B. abortus* from mice and cattle requires cell-mediated immunity, and macrophages activated by T-cell lymphokines are thought to be central to this process (2, 27). However, passive transfer experiments indicate that humoral immunity also plays a role in controlling *B. abortus* infections (2) and antibodies that opsonize *B. abortus* have been shown to stimulate oxidative metabolism and increase bactericidal activity in both neutrophils and macrophages (12, 22).

Although we have not directly demonstrated impaired survival within macrophages, the sharp reduction in the splenic levels of the S2308 Cu-Zn SOD mutant over 10 to 26 days p.i. suggests that Cu-Zn SOD contributes to the increased survival of *B. abortus* within murine macrophages during the early stages of infection. Consistent with this idea are the findings that murine macrophages possess maximum brucellacidal activity approximately 14 days after infection with *B. abortus* (14) and that virulent strains of *B. abortus* are better able to survive within macrophages than nonvirulent strains (27). It is clear from the persistence in mice that the loss of Cu-Zn SOD did not render the S2308 Cu-Zn SOD mutant avirulent. A possible basis for the persistence of *B. abortus* has been suggested to involve proliferation of the organism in monocytes and nonactivated macrophages (14, 16).

The contribution of Cu-Zn SOD to the pathogenicity of *B.*

*abortus* S2308 was indicated by consistently lower mean spleen weights in mice infected with the S2308 Cu-Zn SOD mutant than with wild-type S2308. In addition, fewer splenic lesions occurred in mice infected with the S2308 Cu-Zn SOD mutant than with wild-type S2308 (data not shown).

The loss of Cu-Zn SOD did not affect the survival of S19 or S2308 in the mouse macrophagelike J774 cell line. These results may indicate that J774 cells do not possess sufficient oxidative bactericidal activity to elicit different intracellular survival rates for the Cu-Zn SOD mutants and their respective parental strains. Alternatively, activation of bactericidal activity by T-cell lymphokines may be necessary to achieve reduced intracellular survival rates for Cu-Zn SOD mutants within macrophages.

The kinetics of survival and growth of both the Cu-Zn SOD mutant strains and their parental strains were also very similar in the nonphagocytic HeLa cell line. We attributed these results to the limited ability of HeLa cells to kill *B. abortus*. Patterns of intracellular survival and growth similar to those found in HeLa cells were also reported for S19 and S2308 in the nonphagocytic Vero cell line (15).

In summary, the deletion of Cu-Zn *sod* from *B. abortus* resulted in decreased survival during the initial phase of infection and reduced spleen weights in mice. Together, these results indicate that Cu-Zn SOD may play a role in the pathogenesis of *B. abortus*.

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