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Immunization of mice with gamma-irradiated *Brucella neotomae* and its recombinant strains induces protection against virulent *B. abortus, B. melitensis*, and *B. suis* challenge

Dina Moustafa[1], **Virendra K. Garg**[1], **Neeta Jain**[2], **Nammalwar Sriranganathan**[2], and **Ramesh Vemulapalli**[1,*]

[1]Department of Comparative Pathobiology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, USA

[2]Department of Biomedical Sciences and Pathobiology, VA-MD Regional College of Veterinary Medicine, Virginia Tech, VA 24061, USA

Abstract

Human brucellosis, a zoonotic disease of major public health concern in several developing countries, is primarily caused by *Brucella abortus, B. melitensis*, and *B. suis*. No brucellosis vaccine is available for human use. The aim of this study was to determine if *B. neotomae*, a bacterium not known to cause disease in any host, can be used for developing brucellosis vaccines. *B. neotomae* and its recombinant strains overexpressing superoxide dismutase and a 26 kDa periplasmic protein were rendered non-replicative through exposure to gamma-radiation and used as vaccines in a murine brucellosis model. All three vaccines induced antigen-specific antibody and T cell responses. The vaccinated mice showed significant resistance against challenge with virulent *B. abortus* 2308, *B. melitensis* 16M, and *B. suis* 1330. These results demonstrate that the avirulent *B. neotomae* is a promising platform for developing a safe and effective vaccine for human brucellosis.

Keywords

Brucellosis; *B. neotomae*; gamma radiation; vaccine; broad protection

Introduction

Brucellosis is a zoonotic disease caused by members of the genus *Brucella*, which are Gram-negative, facultatively intracellular bacteria [1]. There are six-well recognized (*B. abortus, B. melitensis, B. suis, B. canis, B. ovis*, and *B. neotomae*) and four recently added (*B. ceti, B. pennipedialis, B. microti*, and *B. inopinata*) species in genus *Brucella*. In domestic and wild mammals brucellosis often results in abortions and infertility. In humans, brucellosis manifests itself as a chronic infection with undulant fever and general malaise; other clinical signs vary depending on the affected organ systems [2]. Humans usually acquire the

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*Corresponding author at: Veterinary Pathology Building, 725 Harrison Street, West Lafayette, IN 47907, United States. Tel.: +17654947560; fax: +17654949830. rvemulap@purdue.edu (R. Vemulapalli).

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infection by consuming contaminated dairy or meat products or by coming in contact with the infected animal tissues and secretions [3]. Ingestion, inhalation, and contamination of conjunctiva or broken skin by the infected animal products are the common modes by which humans are infected. In several developing countries, brucellosis is an important public health concern [4]. Of the six well-recognized species of *Brucella, B. melitensis, B. suis* and *B. abortus* are highly virulent to humans [2]. These 3 *Brucella* species are considered potential bioterror agents and they belong to NIAID Category B priority pathogens list. At present there is no vaccine available for human brucellosis. Cell-mediated immunity (CMI) plays a central role in acquired resistance against brucellosis, and antibodies to the O polysaccharide (O antigen) of the lipopolysaccharide (LPS) participate in providing enhanced resistance against infections by *B. abortus, B. melitensis* and *B. suis* [5]. Attenuated, live *Brucella* strains such as *B. abortus* RB51 and 19, and *B. melitensis* Rev1 are being used as vaccines to control brucellosis in domestic animals [6]. However, these vaccines are not suitable for humans since they can cause disease even in individuals with healthy immune system [7-8].

B. neotomae was isolated in 1957 from desert wood rats of the western U.S. [9]. No disease, either in human or other animal species, has ever been attributed to *B. neotomae*. Unlike the virulent *Brucella spp., B. neotomae* does not establish a chronic infection in immuno-competent mouse models of brucellosis (Moustafa and Vemulapalli, unpublished observation). The overall goal of this study was to examine the feasibility of developing a safe and effective vaccine for human brucellosis using *B. neotomae*. We used exposure to a minimum dose of gamma-radiation as a means to increase the vaccine safety by eliminating the bacteria's ability to replicate but retain the metabolic activity. We also tested if overexpression of *Brucella* Cu-Zn superoxide dismutase (SOD) and a 26 kDa periplasmic protein (Bp26) in *B. neotomae* would enhance its vaccine efficacy. Our results demonstrate that mice immunized with gamma-irradiated *B. neotomae* develop significant protective immunity against challenge with virulent *B. abortus* 2308, *B. melitensis* 16M, and *B. suis* 1330.

1. Materials and methods

2.1. Bacteria

B. neotomae strain 5K33 was purchased from the American Type Culture Collection, Manassas, Va. Virulent strains *B. melitensis* 16M, *B. abortus* 2308, and *B. suis* 1330 were from the culture collection at Virginia Tech, Blacksburg, VA. *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA) was used for producing the necessary plasmid constructs and for recombinant protein production. The recombinant *B. neotomae*/Bp26 and *B. neotomae*/SOD were generated by transforming *B. neotomae* with recombinant plasmids pBB4Bp26 and pBB4SOD, respectively. The pBB4Bp26 plasmid was constructed by cloning the gene encoding the 26 kDa periplasmic protein along with its promoter sequences into the *Kpn* I and *Xho* I restriction sites of pBBR1MCS-4 [10]. The gene sequences were first PCR amplified from the genomic DNA of *B. abortus* RB51 using a custom-designed primer-pair (forward primer: 5'-aaggtaccacccgaaagaaagccgggata-3'; reverse primer: 5'-aactcgagcagatcgaaacgcgctctaat-3') and the resulting 1.2 kb DNA fragment was digested with *Kpn* I and *Xho* I enzymes and cloned into the same sites of pBBR1MCS-4. The nucleotide sequence integrity of the cloned fragment was confirmed by sequence analysis. A 1.1 kb fragment containing the *B. abortus sodC* gene and its promoter sequence was excised from pBS/SOD [11] with *Cla*I restriction enzyme digestion and subcloned into pBBR1MCS-4 to generate pBB4SOD. *B. neotomae* was transformed with pBB4Bp26 and pBB4SOD by electroporation following the previously described procedures [12].

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All bacteria were grown in tryptic soy broth (TSB) or tryptic soy agar (TSA) at 37°C. Bacteria harboring the plasmids were grown in the presence of 100 μg/ml concentration of ampicillin. Colony forming units (CFU) of *Brucella* strains were determined by plating 10-fold serial dilutions of the cultures on TSA. All experiments with *B. neotomae* were performed in a Biosafety level (BSL)-2 facility using BSL-3 practices. All experiments with virulent *Brucella* were performed in a BLS-3 facility approved for the select agents work.

2.2. Vaccine preparation

B. neotomae, B. neotomae/Bp26, and *B. neotomae*/SOD were grown in TSB or TSB with ampicillin to mid log phase, and aliquots containing 5×10^{10}- 1×10^{11} CFU/ml were stored at -80°C until use. Two to three weeks before immunization, aliquots of the vaccines were exposed to 350 krads of gamma irradiation using a ^{60}Co source irradiator (Gammacell 220 irraditor). The inability of the irradiated bacteria to replicate was confirmed plating on TSA and incubating for at least 7 days. The irradiated bacteria were stored at 4°C until use for immunization.

2.3. Determination of bacterial metabolic activity

Metabolic activity of the gamma irradiated bacteria was assayed using Alamar blue (BioSource International, Camarillo, CA) and LIVE/DEAD ® BacLight Bacterial viability Kit for microscopy (Molecular Probes, Eugene, OR). Alamar blue is a redox indicator and its color changes from blue to pink in response to chemical reduction. Alamar blue is reduced by FMNH2, FADH2, NADH, and NADPH, which are present in metabolically active cells. Alamar blue is extensively used for monitoring proliferation and viability of various eukaryotic and prokaryotic cells. For the Alamar blue assay, irradiated samples were washed in saline solution and resuspended in TSB to the original concentration. Ninety microliters of the irradiated suspension were mixed with 10 μl of Alamar blue and incubated at 37°C for 1 hour, and the change in color from blue to pink was monitored as described previously [13]. The manufacturer's instructions were followed while using the LIVE/DEAD Baclight kit which utilizes a mixture of the green-fluorescent SYTO® 9 and the red-fluorescent propidium iodide to stain the bacterial nucleic acids. Following staining with the mixture, the live bacteria with intact cell membranes fluoresce green, whereas the dead bacteria with damaged membranes fluoresce red. The stained bacteria were observed using Nikon Eclipse–E400 microscope equipped with a 480/530 and 450/580 bandpass filter sets.

2.4. Quantification of irradiated bacteria in mice spleens by real-time PCR

The length of persistence of the irradiated *B. neotomae* in spleens of the vaccinated mice was determined by quantifying the bacterial DNA using RT-PCR. Two groups of 12 female BALB/c mice (6 weeks of age) each were inoculated intraperitonially with 10^8 CFU-equivalent of either heat-killed (65°C for 1 hour) or gamma-irradiated *B. neotomae*. Three mice from each group were euthanized at 3 hours, one, three and five days post inoculation, and their spleens were collected aseptically. Total DNA was extracted from the collected spleens using DNeasy® Blood and Tissue Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. The amount of *Brucella* DNA present in the samples was determined using a previously described real-time PCR that amplifies a 178 bp region of *IS*711 element using primers IS421-F (5′-cgctcgcgcggtggat-3') and IS511-R (5'-cttgaagcttgcggacagtcacc-3') and measures the amplified product using a TaqMan probe (Cy5-acgaccaagctgcatgctgttgtcgatg-BHQ2) [14]. DNA extracted from different concentrations of the irradiated *B. neotomae* suspension (10^0 to 10^8 CFU-equivalent/ml) was used to construct the standard curve. After logarithmic conversion, the concentration of each dilution series was plotted against the cycle number at which the fluorescent signal increased above a threshold value (Ct value). The regression equation derived from the standard curve was used to calculate the concentration of *B. neotomae* present in the spleens. The PCR

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reactions were performed in a Stratagene MX3000P thermocycler and the data were analyzed using MxPro QPCR software (Stratagene, La Jolla, CA). All samples and standards were assayed in duplicates.

2.5. Mice immunizations

Female BALB/c mice of 4 to 6 weeks of age were vaccinated by two intraperitoneal inoculations, at day 0 and day 14, with 1×10^8 CFU-equivalent of the irradiated *B. neotomae* and its recombinants *B. neotomae*/Bp26 and *B. neotomae*/SOD. As a negative control, one group of mice was injected with saline alone. Mice were bled by puncturing the retro-orbital plexus under anesthesia at two weeks post inoculation (p.i.) (prior to the booster immunization), and at 6 weeks p.i. (4 weeks after the booster). The serum was separated from the clotted blood and stored at -20°C until use for detection of antigen-specific antibodies by enzyme-linked immunosorbent assay (ELISA). At 6 weeks p.i., all the mice were euthanized by CO_2 asphyxiation followed by cervical dislocation, spleens were collected aseptically and used for determining the antigen-specific T cell immune responses by measuring cytokine production upon in vitro stimulation with specific antigens.

2.6. Preparation of antigens

2.6.1. *B. neotomae* crude extract—Late log phase culture of *B. neotomae* was centrifuged at 6,000 × *g* for 10 min and the pelleted bacteria were washed three times with sterile distilled water and resuspended in 0.5% sodium dodecyl sulfate (SDS). The bacteria were lysed by shaking gently for 2 hours at room temperature and then sonicating for 10 min on ice. The unlysed bacteria were removed by centrifugation at 8,000 × *g* for 10 min. The clear supernatants were collected, and the protein concentration was determined by the Bradford method [15]. Aliquots of the antigen extract were stored at −80°C until use for ELISA.

2.6.2. LPS extraction—Total LPS was extracted from live *B. neotomae* by butanol-water procedure as previously described [16-17]. Briefly, live *B. neotomae* organisms were harvested by centrifugation and 10g of wet pellet was resuspended in 0.85% NaCl at a concentration of 0.25 g wet weight/ml, and thoroughly dispersed by mixing with a magnetic stirrer at 4°C. An equal volume of water saturated butanol was added with constant mixing for 15 min at 4°C. After centrifugation at 35,000 × g for 20 min, the aqueous phase was collected and the insoluble precipitate was further extracted with ½ initial volume of the saline solution. The combined aqueous extracts were centrifuged in order to remove any traces of insoluble materials. LPS was precipitated using 4 volumes of methanol, and the precipitate was dissolved in 0.1M Tris buffer (pH 8) containing 2% SDS and 2% mercaptoethanol. The mixture was heated for 5 min at 100°C followed by 90 min incubation with proteinase K at 60°C. LPS was precipitated by methanol, followed by two washes with cold methanol, and dissolved in water. In order to confirm the presence of any traces of proteins in the extracted LPS, SDS-PAGE gel followed by silver staining was performed to ascertain the quality of the LPS preparation. The extracted LPS was used for ELISA.

2.6.3. Bp26 purification—The Bp26 protein of *B. abortus* was expressed in *E. coli* by using expression vector $pMalC_2$ (New England Biolabs Inc.), and the purification was done according to manufacturer's suggested procedure. Using pMalC2 vector, BP26 protein was expressed as a fusion protein with MBP at the amino terminus so that the recombinant protein can be purified by affinity chromatography with amylose resin. The concentration of the purified protein was determined by the Bradford method. Aliquots of the protein were stored at −80°C until use for ELISA or in vitro stimulation of splenocytes. The purified protein was also used to raise antigen-specific antibodies by hyper-immunizing mice using alum as the adjuvant.

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2.6.4. Purification of SOD—*Brucella* SOD was expressed in *E. coli* DH5α and purified according to the method previously described [18]. Briefly, SOD was extracted from the *E. coli* cells using 10 mM phosphate buffer (pH 7.6) containing 0.1% Triton X-100. Cu/Zn SOD was purified using an equilibrated anion-exchange column (HiTrapQ; Pharmacia Biotech). By applying the extract to the column, all of the proteins except SOD were bound to the resin. SOD present in the flow-through was collected, absorbed with polymyxin B beads (Affi-Prep polymyxin support; Bio-Rad Laboratories, Hercules, CA) to remove the LPS, and dialyzed extensively against phosphate-buffered saline (PBS). The concentration of the purified protein was determined by the Bradford method. Aliquots of the protein were stored at −80°C until use for ELISA or in vitro stimulation of splenocytes.

2.7. SDS-PAGE and Western blotting

To confirm the overexpression of SOD and Bp26 in the recombinant *B. neotomae* strains, SDS-PAGE and Western blot analyses were performed as previously described [19]. As controls, antigen extracts of *B. neotomae*, the purified recombinant MBP-Bp26 fusion protein, and the purified SOD were used. For Western blotting, goat anti-*B. abortus* SOD sera [20], and mouse anti-MBP-Bp26 sera produced in this study were used as the primary antibodies.

2.8. Indirect ELISA

Levels of serum immunoglobulin G (total IgG), as well as IgG1 and IgG2a isotypes with specificity to Bp26, SOD and *B. neotomae* LPS and crude lysate were determined by indirect ELISA [19]. The levels of serum IgG2b, IgG3 and IgM against smooth LPS were also determined. The antigens were diluted in carbonate buffer, pH 9.6, 1 in 10 for *B. neotomae* LPS and to 10 μg/ml of protein concentration for MBP-Bp26, SOD and crude lysate. The wells of polystyrene plates (Nunc-Immunoplate with maxisorp surface) were coated with the diluted antigens (100μl/well). Following overnight incubation at 4°C, plates were washed four times in wash buffer (Tris-buffered saline at pH 7.4, 0.05 % Tween 20) and blocked with 2% bovine serum albumin (BSA) in Tris-buffered saline. After 1hour incubation at 37°C, mouse sera with appropriate dilution in blocking buffer were added to the wells (50 μl/well). Each serum sample was tested in duplicate wells; the plates were incubated for 4 hours at room temperature and washed four times. Horseradish peroxidase–labeled anti-mouse isotype specific conjugates (Southern Biotechnology Associates Inc, Birmingham, Alabama) were added (50 μl/well) at an appropriate dilution. After 1 hour incubation at room temperature, the plates were washed four times. A 100 μl of substrate solution (TMB Microwell peroxidase substrate; Kirkegaard& Perry Laboratories, Gaithersburg, MD) was applied to each well. After 20 min 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 using microplate reader (Molecular devices, Sunnyvale, CA).

2.9. Splenocyte culture and cytokine quantifications

Splenocytes from the vaccinated mice (four mice per group) were obtained as previously described [19], and were cultured in triplicates in 96-well flat-bottomed culture plates (5×10^5 cells /well) in the presence of different stimulants: 10 μg/ml of SOD, 30 μg/ml of MBP-Bp26, and 10^7 CFU-equivalent of gamma-irradiated *B. neotomae*. Cells with plain medium and cells stimulated with 2.5 μg/ml of concanavalin (ConA) were used as controls. The splenocytes were cultured for 5 days, their supernatants were collected and the concentration of selected cytokines was determined using Bio-RAD Bio-Plex Pro™ Mouse cytokine Th1/Th2 Assay according to the manufacturer instructions. The following

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cytokines were tested in the collected supernatants: IL-2, GM-CSF, IFN-γ, TNF-α, IL-4, IL-5, IL-10, IL-12p70.

2.10. Flow cytometry analysis for IFN-γ secreting antigen-specific CD8+ and CD4+ T cells

Intracellular staining for IFN-γ was performed as previously described with some modifications [21]. After euthanizing the mice, spleens were collected from the vaccinated mice (4 mice in each group) and single cell suspensions were prepared from spleens. Using ACK solution, erythrocytes were lysed and the splenocytes were cultured in 96 well flat-bottomed plates (10^6 cells /well) with or without specific antigen as described above for splenocyte cultures. After 8 hours of incubation at 37°C in a humid incubator with 5% CO_2, brefeldin A (Golgistop; Pharmingen) was added to the culture medium and incubated for another 8 hours. Cells from each treatment were suspended in PBS containing 1% BSA and 0.2% sodium azide (FACS buffer) and stained for surface markers CD8 and CD4 by incubating for 30 minutes at 4°C with appropriately diluted FITC conjugated anti-mouse CD8 antibody (BD Pharmigen, clone53-6.7) and APC-conjugated anti-mouse CD4 antibody (BD Pharmigen, clone L3T4-RM 4.5). After three washes with FACS buffer to remove unbound antibodies, the cells were subjected to intracellular IFN-γ staining with PE-conjugated rat anti-mouse IFN-γ antibody using the Cytofix/Cytoperm kit (Pharmingen) according to the manufacturer's instructions. Cell stained with PE-conjugated rat IgG1 antibody served as the isotype control. The cells were acquired on BD FACS Canto II™ Flow cytometer (BD Biosciences, CA, USA). The data were analyzed using BD FACSDIVA version 6 software (BD Biosciences, CA, USA) and the proportion of $CD4^+$ and $CD8^+$ T cells that secreted IFN-γ was determined.

2.11. Protection experiments

Groups of 15 mice each were vaccinated by intraperitoneal inoculation with 10^8 CFU-equivalent of irradiated *B. neotomae, B. neotomae*/Bp26 and *B. neotomae*/SOD. A group of mice injected with saline alone served as the control. Two weeks post inoculation the mice were given a booster immunization using the same route and dose. Six weeks after the initial vaccination, 5 mice from each group were challenged by intraperitoneal inoculation with 3×10^4 CFU/mouse of *B. melitensis* 16M, *B. abortus* 2308, or *B.suis* 1330. Two weeks after challenge, the mice were euthanized and the bacterial burden in their spleens was determined as previously described [22].

2.12. Statistical analyses

Absorbance values of ELISA, concentrations of cytokines, and the flow cytometry data of IFN-γ positive T cells were analyzed for differences among the groups by performing analysis of variance with post-hoc Bonferroni for pair-wise comparison using SPSS version 17.0 (SPSS inc, an IBM company, USA). For protection study, one-tailed *t*-test modified for unequal variances between groups was performed to compare the log transformed bacterial loads in spleens of mice from each vaccinated group with the respective saline control group. *P* values of ≤0.05 were considered significant.

2. Results

3.1. Overexpression of SOD and Bp26 in *B. neotomae*

Overexpression of SOD and Bp26 proteins in *B. neotomae*/SOD and *B. neotomae*/Bp26 was detected by SDS-PAGE (Fig. 1A and 1C), which was further confirmed by Western blot analysis using the antigen-specific antibodies (Fig.1B and 1D). Compared to *B. neotomae*, antigen extracts of *B. neotomae*/Bp26 contained an overexpressed protein of 26 kDa in size which was recognized by the mouse anti-MBP-Bp26 serum (Fig. 1A and Fig. 1B).

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Similarly, *B. neotomae*/SOD contained an overexpressed protein of 16 kDa in size which reacted with the goat anti-SOD antibodies (Fig. 1C and 1D). In accordance with the previous reports [23], no detectable level of SOD expression was present in *B. neotomae* (Fig. 1D).

Stable overexpression of both Bp26 and SOD in the respective recombinant *B. neotomae* strains was consistently detected in all subcultures or gamma-irradiated preparations (data not shown).

2.2. Gamma irradiated *B. neotomae*: replication, viability and metabolic activity

Aliquots of *B. neotomae* were exposed to different doses gamma radiation and the CFU present in each aliquot were determined by culturing on TSA plates. As shown in Fig. 2A, gradual decrease in CFU of *B. neotomae* was detected with increased exposure to gamma radiation. A total loss of replicative ability of the bacteria was observed at a minimum dose of 300 kilorads (Fig 2A).

When stained with both green-fluorescent SYTO 9 and red-fluorescent propidium iodide, gamma-irradiated *B. neotomae*, similar to the live bacteria, displayed strong green fluorescence and weak red fluorescence, indicating the undamaged cell membranes (Fig 2B). As expected, the heat-killed bacteria showed strong red fluorescent reflecting the damaged cell membranes (Fig 2B).

Gamma irradiation did not impair the metabolic activity of the bacteria, as indicated by the ability of irradiated and live *B. neotomae* to change the color of Alamar blue dye from blue to pink (data not shown). In contrast, heat-killed *B. neotomae* failed to cause the color change in the Alamar Blue dye (data not shown).

3.3. Persistence of gamma-irradiated *B. neotomae* in mice spleens

Persistence of the irradiated bacteria in spleens of inoculated mice was determined by extracting DNA from the tissues and performing a quantitative real-time PCR specific to *Brucella*. As shown in Table 1, compared to the heat-killed bacteria, gamma-irradiated *B. neotomae* was present in higher numbers in the spleens at 3 hours, 24 hours and 3 days post-inoculation. By day 5 post-inoculation, no bacteria were detected in the spleens of mice inoculated with either of the bacterial preparations.

3.4. Induction of specific antibody responses

Presence of antibodies specific to *B. neotomae* total antigens, Bp26, SOD and LPS in serum of the mice vaccinated with the irradiated vaccines was determined by ELISA. Serum samples collected at 2 and 6 weeks after the initial immunizations were analyzed in comparison with the samples from saline inoculated group. Mice vaccinated with *B. neotomae* and its recombinants *B. neotomae*/Bp26 and *B. neotomae*/SOD developed significantly higher levels of IgG specific to the total antigen of *B. neotomae* at 2 and 6 weeks post vaccination than did mice in the saline inoculated group. Assays with IgG1 and IgG2a specific conjugates revealed that antibodies of both isotypes were present in significantly higher levels than in saline inoculated mice (data not shown).

At 2-weeks post vaccination, only mice vaccinated with *B. neotomae*/Bp26, developed significantly higher levels of Bp26-specific IgG than saline inoculated mice (Fig. 3). However, by week 6, all vaccinated groups showed production of significant levels of Bp26-specific antibodies. Both IgG1 and IgG2a isotypes specific to Bp26 were detected in the vaccinated mice. As expected, the level of Bp26-specific antibodies in mice vaccinated with *B. neotomae*/Bp26 was higher compared to that detected in mice vaccinated with *B. neotomae* or *B. neotomae*/SOD (Fig. 3).

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Significant levels of IgG specific to SOD were detected in serum of all vaccinated mice (Fig. 3). The induced antigen-specific antibodies were of both IgG1 and IgG2a isotypes. No significant differences were detected in the levels of SOD-specific antibodies among the different vaccine groups (Fig. 3).

Serum of all vaccinated mice contained significant levels of IgG and IgM to *B. neotomae* LPS (Fig. 4). The IgG and IgM levels were higher at 6 weeks than at 2 weeks. The produced LPS-specific antibodies included IgG1, IgG2a, IgG2b, and IgG3 isotypes (Fig. 4). There was no difference in the lgG1 levels between the 2- and 6-week serum samples. In contrast, the levels of IgG2a, IgG2b, and IgG3 were higher in the 6-week samples (Fig. 4).

Antibody levels were also measured in ELISA titers by endpoint titration of the pooled serum samples of each vaccinated group (Supplementary Tables 1-3). Differences in antibody titers between the time-points or groups mirrored that of the antibody levels based on ELISA absorbance values presented in Figs. 3 and 4.

3.5 Antigen-specific cellular immune responses

Specific CMI responses of the vaccinated mice at 6 weeks post initial immunization were determined by quantification of a panel of Th1/Th2 cytokines secreted by the splenocytes and the number of IFN-γ secreting $CD4^+$ and $CD8^+$ T cells upon in vitro stimulation with irradiated *B. neotomae*, MBP-Bp26 and SOD.

When stimulated with gamma-irradiated *B. neotomae*, splenocytes from all vaccinated mice, but not the saline inoculated ones, secreted significantly higher levels of IFN-γ, IL-12p70, IL-5 and IL-10 compared to the unstimulated controls (Fig 5). The concentrations of IL-4 in all culture supernatants were low (< 10 pg/ml) and were not significantly different from the corresponding unstimulated controls (Fig. 5).

Stimulation with MBP-Bp26 and SOD resulted in the secretion of significantly higher amounts of IL-12p70 and IL-10 by splenocytes from all vaccinated mice compared to the unstimulated controls; the concentrations of IFN-γ and IL-5 were low and variable among the different vaccine groups (Fig. 5).

Splenocytes from all vaccinated, but not saline inoculated, mice secreted significantly higher amounts of TNF-α upon stimulation with irradiated *B. neotomae* (Fig. 5).

Stimulation with irradiated *B. neotomae*, MBP-Bp26 and SOD induced secretion of similar high levels of GM-CSF from splenocytes of all vaccinated, but not saline inoculated, mice (data not shown). In contrast, splenocytes from all groups secreted similar low levels of IL-2 upon in vitro stimulation with all the antigens (data not shown). Mitogen stimulation with conA resulted in the secretion of similarly high levels of all the cytokines from splenocytes of all groups (data not shown).

Using flow cytometry analysis of splenocytes stimulated in vitro with irradiated *B. neotomae*, significantly higher proportions of IFN-γ-secreting CD4+ (1.5-2.3%) and CD8+ (0.9-1.8%) T cells were detected in all vaccinated mice compared to the saline inoculated ones (Fig. 6). In vitro stimulation with MBP-Bp26 and SOD resulted in detection of low proportion of IFN-γ-secreting CD4+ and CD8+ T cells in splenocytes from vaccinated mice.

3.6. Protection against challenge with virulent *Brucella spp*

Compared to the saline-inoculated controls, vaccination of mice with 2 doses of irradiated *B. neotomae, B. neotomae*/Bp26, or *B. neotomae*/SOD prior to challenge with virulent *B. abortus* 2308, *B. suis* 1330 or *B. melitensis* 16M significantly reduced the number of virulent

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brucellae in the spleens 2 weeks after challenge (Table 2). The vaccinated mice contained approximately 2-3 logs, 3.5 logs, and 1.3-2.3 logs lower *B. abortus* 2308, *B. suis* 1330, and *B. melitensis* 16M, respectively (Table 2). For each challenge strain, there were no statistically significant differences in the spleen bacterial loads among the different vaccine groups.

4. Discussion

In this study, we demonstrated that vaccination of mice with gamma-irradiated *B. neotomae* results in the development of protective immunity against virulent *B. abortus, B. suis*, and *B. melitensis*. The vaccine potential of *B. neotomae* was first suggested in 1963 by Stoenner for controlling swine brucellosis [24]. However, to the best of authors' knowledge, there are no published reports of examining the usefulness of *B. neotomae* as a brucellosis vaccine. Though *B. neotomae* is not known to be a pathogen, safety concerns may preclude its use as a live vaccine, especially in humans. Therefore, we used exposure to a minimum dose of gamma radiation as a means to abolish the ability of *B. neotomae* to replicate. The use of ionizing radiation has been used in the development of vaccines for preventing infectious diseases of animals and humans that are caused by different viruses, bacteria, and parasites [13,25-29]. However, a recently developed strategy specifically uses exposure to a minimum dose of radiation, which is sufficient to abolish replication of the organism, for developing safer vaccines for diseases caused by intracellular pathogens [13,28-29]. Similar to the previously reported findings for *B. abortus* RB51 and *B. melitensis* Rev1 [13,29], *B. neotomae* exposed to a minimum of 300-350 krads of radiation lost its ability to replicate on nutrient rich TSA medium. The irradiated *B. neotomae*, however, remained metabolically active as demonstrated by the bacteria's ability to reduce Alamar blue dye and prevent the penetration of propidium iodide through the cell membranes (Fig. 2). By retaining the metabolic activity, gamma irradiated bacteria can mimic the actual host cell infection of the live bacteria [28-29]. In addition, the bacteria exposed to a minimum dose of radiation retain their de novo protein synthesis capabilities [13,29]. This feature in case of intracellular bacterial pathogens can lead to elicitation of CMI responses to antigens that are expressed when the bacteria are inside the host cells. Our PCR analysis suggests that the irradiated bacteria persisted at higher numbers and for longer time than the heat-killed bacteria in the spleens of the inoculated mice. Though we have not compared the immune responses induced by the irradiated and heat-killed *B. neotomae*, similar previous studies with *B. abortus* RB51 and *B. melitensis* showed that the irradiated bacteria are better at inducing antigen-specific and protective immune responses [13,29].

All three irradiated *B. neotomae* vaccines used in this study conferred similar levels of protection against each of the virulent *Brucella spp* (Table 1). Overexpression of a protective antigen in the live vaccine strain was previously used as a strategy to induce enhanced protective immunity against brucellosis and tuberculosis in mouse models [18,30-31]. The selection of SOD and Bp26 for overexpression in *B. neotomae* was based on the previous findings that these are protective antigens of *B. abortus* and *B. melitensis*, respectively [20,32]. Moreover, when used as a live vaccine, overexpression of SOD in *B. abortus* RB51 (strain RB51SOD) led to induction of increased SOD-specific immune responses and enhancement of its protective efficacy against *B. abortus* challenge in BALB/c mice [18]. Unexpectedly, in this study, we did not detect significantly enhanced SOD-specific antibody and T-cell immune responses in mice vaccinated with the irradiated *B. neotomae*/SOD. Though the irradiated *B. neotomae*/SOD vaccine, unlike the live RB51SOD vaccine, cannot replicate, we expected that there would be a significantly enhanced SOD-specific immune responses following the booster immunization. This lack of enhanced SOD-specific immune responses suggests that the amount of SOD present in the irradiated vaccine dose was not sufficiently high enough or that the other immunodominant antigens of

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B. neotomae affected the immunogenic potential of SOD. It is also possible that the overexpression of SOD did not occur under in vivo conditions. In case of Bp26, mice vaccinated with the irradiated *B. neotomae*/Bp26 developed increased levels of antibodies and more numbers of IFN-γ-secreting CD8+ T cells specific to Bp26. Nevertheless, these increased Bp26-specific immune responses did not translate to enhanced protection against the challenge with any of the 3 virulent *Brucella spp*. (Table 1). If overexpression of some other protective proteins can lead to enhancement of the vaccine efficacy remains to be tested.

In general, a Th1 type of immune responses is considered desirable for protection against intracellular bacterial infections, such as brucellosis. However, some published reports document that the acquired resistance against brucellosis can occur even in presence of a mix of Th1 and Th2 immunity [33-34]. The presence of antigen-specific IgG1 and IgG2a antibodies in serum of the vaccinated mice suggest that the irradiated *B. neotomae* vaccines induced a mix of Th1 and Th2 immune responses. However, based on cytokine secretion by the antigen-specific splenocytes, it appears that the induced Th1 response was more prominent, because of the significantly higher concentration of IFN-γ in supernatants of cultures stimulated with irradiated *B. neotomae*; the concentrations of Th2 cytokines IL-4 and IL-5 were marginally higher, compared to the spontaneous release cell controls (Fig. 5). The presence of increased concentration of IL-12p70 and TNF-α in the antigen-stimulated culture supernatants also suggests the development of Th1 type effector cells. Although T cells are not a major source for IL-12, a key facilitator of Th1differentiation during an immune response, antigen-specific Th1 cells can positively affect IL-12 secretion by antigen-presenting cells through contact-dependent manner [35].

All three *B. neotomae* vaccines induced the development of antigen-specific IL-10-secreting lymphocytes (Fig. 5). IL-10 is usually considered to be an anti-inflammatory cytokine which participates in reducing the adverse effects resulting from the excessive production of proinflammatory cytokines such as IFN-γ [36]. Previous research demonstrated that inhibition of IL-10 activity leads to increased clearance of *B. abortus* from infected mice [37]. However, there appears to be no correlation between IL-10 secretion by the vaccine-induced antigen-specific T cells and resistance to virulent *Brucella* challenge. For example, studies with some experimental *Brucella* vaccines, such as certain deletion mutants of *B. abortus* and *B. melitensis*, and plasmid DNA and recombinant protein of Omp31, induction of IL-10 secreting antigen-specific lymphocytes had no negative effect on protection to challenge infection [38-39]. Recently, it has been shown that IL-10 can also promote inflammation during active inflammatory responses [36]. Therefore, it is possible that vaccine-induced IL-10 secretion may play a beneficial role in mediating protection against virulent *Brucella* challenge.

Both T cells and anti-smooth LPS antibodies play a role in mediating protection against brucellosis [40-41]. Antigen-specific T cells that secrete IFN-γ are primarily responsible for the acquired CMI against virulent *Brucella* infection [41]. Passive transfer experiments showed that antibodies to the O-antigen can mediate protection [42-47]. Anti-O antibodies of isotypes IgM, IgG2a, IgGb and IgG3 are effective at affording protection against brucellosis in mice [45-47]. All three irradiated *B. neotomae* vaccines induced LPS-specific antibodies of these isotypes, which increased upon booster immunization (Fig. 4). Usually, IgM is mainly produced during the primary immune response by short-lived plasma cells, thereafter, following the isotype switching in germinal centers, long-lived plasma cells develop which produce antibodies of other isotypes and the production of IgM decreases. Therefore, it is unclear why the levels of IgM remained elevated even after 4 weeks after the booster immunization. Whether this feature is specific to *B. neotomae* remains to be determined.

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Immunization of mice with any of the three gamma-irradiated *B. neotomae* vaccines conferred a significant level of protection against virulent *B. abortus, B. suis*, and *B. melitensis* challenges. Based on the units of protection afforded by the three vaccines, the level of resistance against *B. abortus* and *B. suis* appeared to be better than that against *B. melitensis* challenge (Table 2). One contributing factor for this difference could be the resistance of *B. melitensis* to complement-mediated lysis. Both smooth and rough *B. melitensis* are more resistant to complement-mediated killing than *B. abortus* with similar phenotypes [48-49]. Another factor could be the variation in epitope dominance in the O antigen of *Brucella* smooth LPS between *B. melitensis* and *B. abortus* or *B. suis*. Two epitopes, designated C (for common among all *Brucella* smooth LPS) and C/Y (for common between smooth LPS of *Brucella* and *Yersinia enterocolitica* O:9), are present in smooth LPS from all *Brucella* species [50]. Two additional epitopes, designated A (for *abortus*) and M (for *melitensis*), are identified in the O-antigen portion of the smooth LPS [51-52]. Different smooth *Brucella* strains possess varying proportions of A and M epitopes. The O-antigen of *Brucella* smooth LPS is a homopolymer of 4.6-dideoxy-4-formamido-α-D-mannopyranosyl units. These units are linked in α-1,2 in A-dominant smooth *Brucella* strains, but every fifth residue is linked in α-1,3 in M-dominant strains. Antibodies to the dominant epitope of the O-antigen are more effective in mediating protection [50]. *B. neotomae, B. abortus* 2308, and *B. suis* 1330 are A-dominant strains, while *B. melitensis* 16M is a M-dominant strain [50]. Although we did not identify the epitope specificity of the anti-smooth LPS antibodies produced by the vaccinated mice, it is highly likely that the irradiated *B. neotomae* vaccines induced more antibodies to specific to A than M epitope. In addition, antigen specificity of the T cell responses could also have affected the level of protection conferred by the vaccines against *B. melitensis* challenge. Enhancement of T cell responses through strategies such as microencapsulation might increase the protective efficacy of the irradiated *B. neotomae* vaccines [53].

In the previous studies with the irradiated *B. abortus* RB51 and *B. melitensis* Rev1, a single dose of 10^9 CFU equivalent per mouse was used for immunization [13,29]. However, in our preliminary experiments, we observed that the mice inoculated with 10^9 CFU equivalent of irradiated *B. neotomae* exhibited clinical signs consistent with the vaccine-induced sickness during the first 3-5 days after immunization. On the contrary, mice inoculated with 10^8 CFU equivalent of irradiated *B. neotomae* did not show any signs of distress. Therefore, an empirical regimen of 2 doses of 10^8 CFU equivalent of the irradiated bacteria each at 2-weeks apart was used for the studies. Further studies are warranted to determine the minimum effective dose of the vaccine and the duration of the protective immune status in the vaccinated animals. In addition, examining the potential of the irradiated *B. neotomae* as an oral vaccine would be very pertinent for human application.

In conclusion, the results suggest that gamma-irradiated *B. neotomae* can be used for developing an effective vaccine against brucellosis caused by *B. abortus, B. suis* and *B. melitensis*. The non-pathogenic feature of *B. neotomae* along with the inability of the irradiated bacteria to replicate in the host makes it a safer alternative to the other live attenuated vaccine candidates for human brucellosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Corbel MJ. Brucellosis: an overview. Emerg Infect Dis. 1997; 3(2):213–21. [PubMed: 9204307]
2. Franco MP, Mulder M, Gilman RH, Smits HL. Human brucellosis. Lancet Infect Dis. 2007; 7(12): 775–86. [PubMed: 18045560]
3. Cutler SJ, Whatmore AM, Commander NJ. Brucellosis - new aspects of an old disease. J Appl Microbiol. 2005; 98(6):1270–81. [PubMed: 15916641]
4. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. Lancet Infect Dis. 2006; 6(2):91–9. [PubMed: 16439329]
5. Ficht TA, Kahl-McDonagh MM, Arenas-Gamboa AM, Rice-Ficht AC. Brucellosis: The case for live, attenuated vaccines. Vaccine. 2009; 27:D40–D3. [PubMed: 19837284]
6. Schurig GG, Sriranganathan N, Corbel MJ. Brucellosis vaccines: past, present and future. Vet Microbiol. 2002; 90(1-4):479–96. [PubMed: 12414166]
7. Perkins SD, Smither SJ, Atkins HS. Towards a *Brucella* vaccine for humans. FEMS Microbiol Rev. 2010; 34(3):379–394.
8. Blasco JM, Díaz R. *Brucella melitensis* Rev-1 vaccine as a cause of human brucellosis. The Lancet. 1993; 342(8874):805.
9. Stoenner HG, Lackman DB. A new species of *Brucella* isolated from the desert wood rat, *Neotoma lepida* Thomas. Am J Vet Res. 1957; 18(69):947–51. [PubMed: 13470254]
10. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM 2nd, et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 166(1):175–6. [PubMed: 8529885]
11. Vemulapalli R, He Y, Boyle SM, Sriranganathan N, Schurig GG. *Brucella abortus* strain RB51 as a vector for heterologous protein expression and induction of specific Th1 type immune responses. Infect Immun. 2000; 68(6):3290–6. [PubMed: 10816476]
12. McQuiston JR, Schurig GG, Sriranganathan N, Boyle SM. Transformation of *Brucella* species with suicide and broad host-range plasmids. Methods Mol Biol. 1995; 47:143–8. [PubMed: 7550728]
13. Sanakkayala N, Sokolovska A, Gulani J, HogenEsch H, Sriranganathan N, Boyle SM, et al. Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant *Brucella abortus* RB51. Clin Diagn Lab Immunol. 2005; 12(12):1429–36. [PubMed: 16339067]
14. Bounaadja L, Albert D, Chenais B, Henault S, Zygmunt MS, Poliak S, et al. Real-time PCR for identification of *Brucella spp*.: a comparative study of IS*711, bcsp31* and *per* target genes. Vet Microbiol. 2009; 137(1-2):156–64. [PubMed: 19200666]
15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–54. [PubMed: 942051]
16. Morrison DC, Leive L. Fractions of Lipopolysaccharide from *Escherichia coli* O111-B4 prepared by two extraction procedures. J Biol Chem. 1975; 250(8):2911–9. [PubMed: 804483]
17. Phillips M, Pugh GW, Deyoe BL. Chemical and protective properties of *Brucella* lipopolysaccharide obtained by butanol extraction. Am J Vet Res. 1989; 50(3):311–7. [PubMed: 2494911]
18. Vemulapalli R, He Y, Cravero S, Sriranganathan N, Boyle SM, Schurig GG. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. Infect Immun. 2000; 68(6):3286–9. [PubMed: 10816475]
19. Vemulapalli R, Duncan AJ, Boyle SM, Sriranganathan N, Toth TE, Schurig GG. Cloning and sequencing of *yajC* and *secD* homologs of *Brucella abortus* and demonstration of immune responses to YajC in mice vaccinated with *B. abortus* RB51. Infect Immun. 1998; 66(12):5684–91. [PubMed: 9826342]
20. Onate AA, Vemulapalli R, Andrews E, Schurig GG, Boyle S, Folch H. Vaccination with live *Escherichia coli e*xpressing *Brucella abortus* Cu/Zn superoxide dismutase protects mice against virulent *B. abortus*. Infect Immun. 1999; 67(2):986–8. [PubMed: 9916121]
21. Murali-Krishna K, Altman JD, Suresh M, Sourdive DJD, Zajac AJ, Miller J, Ahmed R. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity. 1998; 8(2):177–87. [PubMed: 9491999]

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22. Schurig GG, Roop RM, Bagchi T, Boyle S, Buhrman D, Sriranganathan N. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol. 199; 28(2):171–88. [PubMed: 1908158]
23. Bricker BJ, Tabatabai LB, Judge BA, Deyoe BL, Mayfield JE. Cloning, expression, and occurrence of the *Brucella* Cu-Zn superoxide dismutase. Infect Immun. 1990; 58(9):2935–9. [PubMed: 2201639]
24. Stoenner HG. The behavior of *Brucella neotomae* and *Brucella suis* in reciprocal superinfection experiments in mice and guinea pigs. Am J Vet Res. 1963; 24:376–80. [PubMed: 13984251]
25. Hubbert WT, Miller JN. Studies on immunity in experimental leptospirosis: the immunogenicity of *Leptospira icterohemorrhagiae* attenuated by gamma-irradiation. J Immunol. 1965; 95(4):759–64. [PubMed: 5841058]
26. Jarrett WF, Jennings FW, Mc IW, Mulligan W, Urquhart GM. Irradiated helminth larvae in vaccination. Proc R Soc Med. 1958; 51(9):743–4. [PubMed: 13591294]
27. Martin SS, Bakken RR, Lind CM, Garcia P, Jenkins E, Glass PJ, et al. Comparison of the immunological responses and efficacy of gamma-irradiated V3526 vaccine formulations against subcutaneous and aerosol challenge with Venezuelan equine encephalitis virus subtype IAB. Vaccine. 2010; 28(4):1031–40. [PubMed: 19914193]
28. Datta SK, Okamoto S, Hayashi T, Shin SS, Mihajlov I, Fermin A, et al. Vaccination with irradiated *Listeria* induces protective T cell immunity. Immunity. 2006; 25(1):143–52. [PubMed: 16860763]
29. Magnani DM, Harms JS, Durward MA, Splitter GA. Nondividing but metabolically active gamma-irradiated *Brucella melitensis* is protective against virulent *B. melitensis* challenge in mice. Infect Immun. 2009; 77(11):5181–9. [PubMed: 19703982]
30. Dhar N, Rao V, Tyagi AK. Recombinant BCG approach for development of vaccines: cloning and expression of immunodominant antigens of *M. tuberculosis*. Fems Microbiol Lett. 2000; 190(2): 309–16. [PubMed: 11034297]
31. Rao V, Dhar N, Tyagi AK. Modulation of host immune responses by overexpression of immunodominant antigens of *Mycobacterium tuberculosis* in bacille Calmette-Guérin. Scand J Immunol. 2003; 58(4):449–61. [PubMed: 14507310]
32. Yang X, Hudson M, Walters N, Bargatze RF, Pascual DW. Selection of protective epitopes of *Brucella melitensis* by DNA vaccination. Infect Immun. 2005; 73(11):7297–303. [PubMed: 16239526]
33. Velikovsky CA, Goldbaum FA, Cassataro J, Estein S, Bowden RA, Bruno L, et al. *Brucella* lumazine synthase elicits a mixed Th1-Th2 immune response and reduces infection in mice challenged with *Brucella abortus* 544 independently of the adjuvant formulation used. Infect Immun. 2003; 71(10):5750–5. [PubMed: 14500496]
34. Delpino MV, Estein SM, Fossati CA, Baldi PC, Cassataro. Vaccination with *Brucella* recombinant DnaK and SurA proteins induces protection against *Brucella abortus* infection in BALB/c mice. Vaccine. 2007; 25(37-38):6721–9. [PubMed: 17686554]
35. Ria F, Penna G, Adorini L. Th1 cells induce and Th2 inhibit antigen-dependent IL-12 secretion by dendritic cells. Eur J Immunol. 1998; 28(6):2003–16. [PubMed: 9645382]
36. Sharif MN, Tassiulas I, Hu Y, Mecklenbrauker I, Tarakhovsky A, Ivashkiv LB. IFN-alpha priming results in a gain of proinflammatory function by IL-10: implications for systemic lupus erythematosus pathogenesis. J Immunol. 2004; 172(10):6476–81. [PubMed: 15128840]
37. Fernandes DM, Baldwin CL. Interleukin-10 downregulates protective immunity to *Brucella abortus*. Infect Immun. 1995; 63(3):1130–3. [PubMed: 7868238]
38. Cassataro J, Velikovsky CA, Bruno L, Estein SM, de la Barrera S, Bowden R, et al. Improved immunogenicity of a vaccination regimen combining a DNA vaccine encoding *Brucella melitensis* outer membrane protein 31 (Omp31) and recombinant Omp31 boosting. Clin Vaccine Immunol. 2007; 14(7):869–74. [PubMed: 17428946]
39. Kahl-McDonagh MM, Ficht TA. Evaluation of protection afforded by *Brucella abortus* and *Brucella melitensis* unmarked deletion mutants exhibiting different rates of clearance in BALB/c mice. Infect Immun. 2006; 74(7):4048–57. [PubMed: 16790778]

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40. Gonzalez D, Grillo MJ, De Miguel MJ, Ali T, Arce-Gorvel V, Delrue RM, et al. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysacharide rough mutants defective in core and O-polysaccharide synthesis and export. PLoS One. 2008; 3(7):e2760. [PubMed: 18648644]
41. Yingst S, Hoover DL. T cell immunity to brucellosis. Crit Rev Microbiol. 2003; 29(4):313–31. [PubMed: 14636042]
42. Limet J, Plommet AM, Dubray G, Plommet M. Immunity conferred upon mice by anti-LPS monoclonal antibodies in murine brucellosis. Ann Inst Pasteur Immunol. 1987; 138(3):417–24.
43. Araya LN, Elzer PH, Rowe GE, Enright FM, Winter AJ. Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. J Immunol. 1989; 143(10):3330–3337. [PubMed: 2509555]
44. Montaraz JA, Winter AJ, Hunter DM, Sowa BA, Wu AM, Adams LG. Protection against *Brucella abortus* in mice with O-polysaccharide-specific monoclonal antibodies. Infect Immun. 1986; 51(3):961–3. [PubMed: 3005173]
45. Elzer PH, Jacobson RH, Jones SM, Nielsen KH, Douglas JT, Winter AJ. Antibody-mediated protection against *Brucella abortus* in BALB/c mice at successive periods after infection: variation between virulent strain 2308 and attenuated vaccine strain 19. Immunology. 1994; 82(4):651–8. [PubMed: 7835931]
46. Elzer PH, Jacobson RH, Nielsen KH, Douglas JT, Winter AJ. BALB/c mice infected with *Brucella abortus* express protracted polyclonal responses of both IgG2a and IgG3 isotypes. Immunol Lett. 1994; 42(3):145–50. [PubMed: 7890314]
47. Cloeckaert A, Jacques I, de Wergifosse P, Dubray G, Limet JN. Protection against *Brucella melitensis* or *Brucella abortus* in mice with immunoglobulin G (IgG), IgA, and IgM monoclonal antibodies specific for a common epitope shared by the *Brucella* A and M smooth lipopolysaccharides. Infect Immun. 1992; 60(1):312–5. [PubMed: 1370276]
48. Fernandez-Prada CM, Nikolich M, Vemulapalli R, Sriranganathan N, Boyle SM, Schurig GG, et al. Deletion of wboA enhances activation of the lectin pathway of complement in *Brucella abortus* and *Brucella melitensis*. Infect Immun. 2001; 69(7):4407–16. [PubMed: 11401980]
49. Fernandez-Prada CM, Zelazowska EB, Nikolich M, Hadfield TL, Roop RM 2, Robertson GL, et al. Interaction between Brucella melitensis and human phagocytes: bacterial surface O-polysaccharide inhibits phagocytosis, bacterial killing, and subsequent host cell apoptosis. Infect Immun. 2003; 71(4):2110–9. [PubMed: 12654833]
50. Cloeckaert A, Weynants V, Godfroid J, Verger JM, Grayon M, Zygmunt MS. O-Polysaccharide epitopic heterogeneity at the surface of *Brucella spp*. studied by enzyme-linked immunosorbent assay and flow cytometry. Clin Diagn Lab Immunol. 1998; 5(6):862–70. [PubMed: 9801349]
51. Caroff M, Bundle DR, Perry MB, Cherwonogrodzky JW, Duncan JR. Antigenic S-type lipopolysaccharide of *Brucella abortus* 1119-3. Infect Immun. 1984; 46(2):384–8. [PubMed: 6437981]
52. Bundle DR, Cherwonogrodzky JW, Perry MB. Structural elucidation of the *Brucella melitensis* M antigen by high-resolution NMR at 500 MHz. Biochemistry. 1987; 26(26):8717–26. [PubMed: 3442684]
53. Arenas-Gamboa AM, Ficht TA, Kahl-McDonagh MM, Rice-Ficht AC. Immunization with a single dose of a microencapsulated *Brucella melitensis* mutant enhances protection against wild-type challenge. Infect Immun. 2008; 76(6):2448–55. [PubMed: 18362129]

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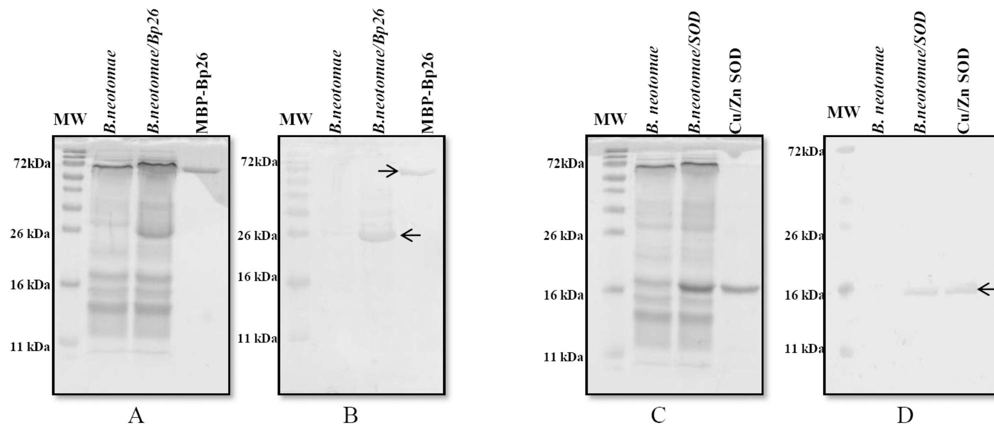

Figure 1.

Detection of expression of Bp26 and SOD proteins in *B. neotomae*/Bp26 and *B. neotomae*/SOD by SDS-PAGE and Western blot analyses. The whole antigen of *B. neotomae* and *B. neotomae*/Bp26 and *B. neotomae*/SOD were separated by 12.5 % SDS-PAGE and either stained with Commassie brilliant blue (A and C) or analyzed by Western blotting with mono-specific serum to MBP-Bp26 (B) or SOD (D). MBP-Bp26k and SOD are proteins purified from overexpressing *E. coli*. The arrow heads indicate the reacting protein. Lane MW, molecular weight marker in kilodaltons (kDa).

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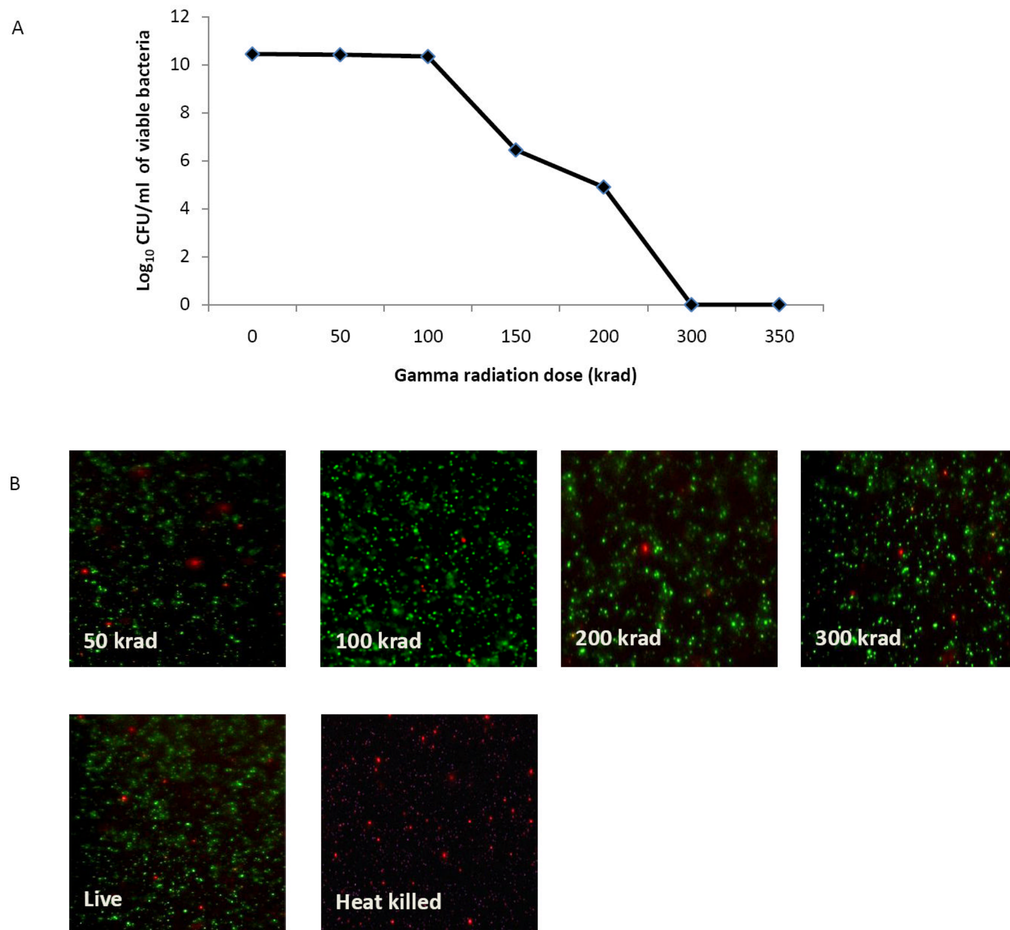

Figure 2.

Effect of gamma-irradiation on the viability of *B. neotomae*. Aliquots of *B. neotomae* were exposed to different doses of gamma radiation, the viable bacteria in each aliquots were determined by plating 10-fold dilutions on TSA plates (A), and staining with live/dead BacLight kit for microscopy (B). As a control, an aliquot each of live and heat-killed bacteria was included for microscopy.

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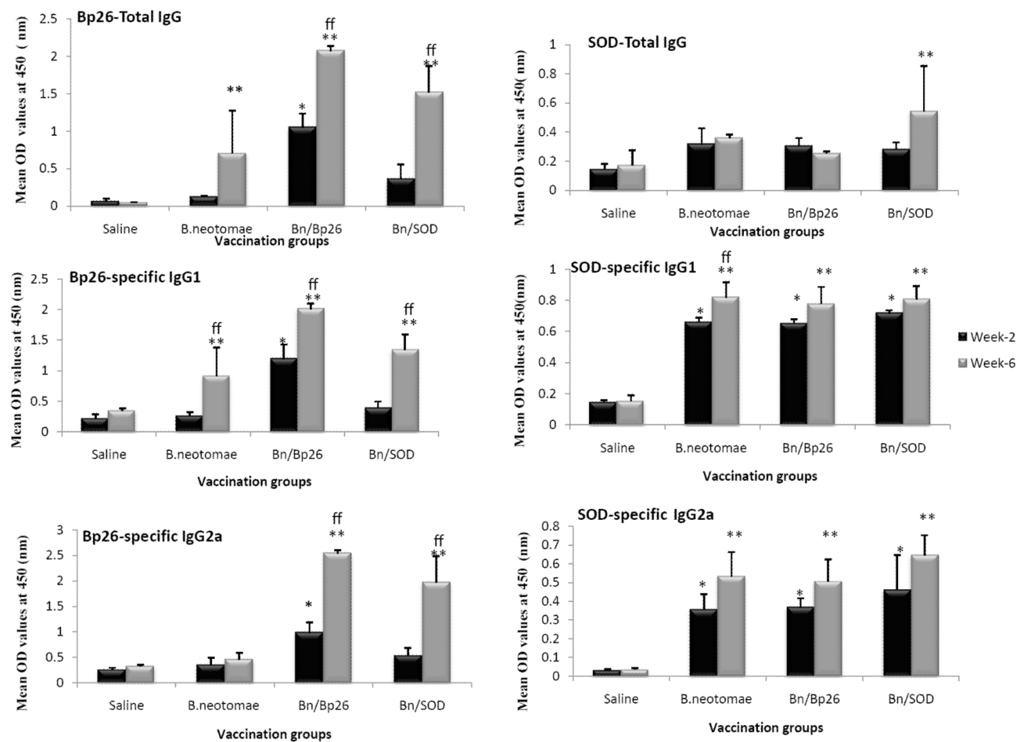

Figure 3.

ELISA detection of Bp26- and SOD-specific IgG, IgG1, and IgG2a antibodies in serum of mice vaccinated with gamma irradiated *B. neotomae, B. neotomae*/Bp26, and *B. neotomae*/SOD, or inoculated with saline. Serum samples were collected at 2 and 6 weeks after initial vaccination, and were diluted 1 in 200 and assayed for the presence of total antigen specific antibodies. Results are shown as mean ± standard deviation (n=4) of absorbance at 450 nm of the color developed.

* Significantly different from the corresponding saline group at week 2 ($P<0.05$).

** Significantly different from the corresponding saline group at week 6 ($P<0.05$).

^{ff} Week 6 is significantly different from week 2 within each vaccine group ($P<0.05$).

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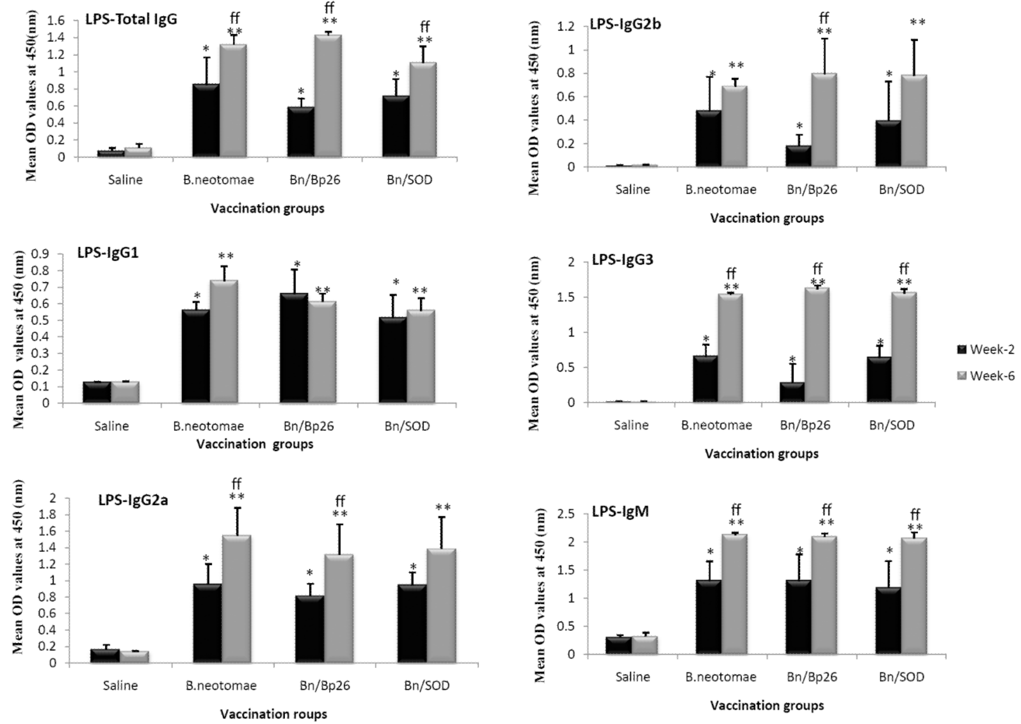

Figure 4.

ELISA detection of *B. neotomae* LPS specific antibodies IgG, IgG1, and IgG2a antibodies in serum of mice vaccinated with gamma irradiated *B. neotomae, B. neotomae*/Bp26, and *B. neotomae*/SOD, or inoculated with saline. Serum samples were collected at 2 and 6 weeks after initial vaccination, and were diluted 1 in 200 and assayed for the presence of total antigen specific antibodies. Results are shown as mean ± standard deviation (n=4) of absorbance at 450 nm of the color developed.

* Significantly different from the corresponding saline group at week 2 (P<0.05).

** Significantly different from the corresponding saline group at week 6 (P<0.05).

ff Week 6 is significantly different from week 2 within each vaccine group (P<0.05).

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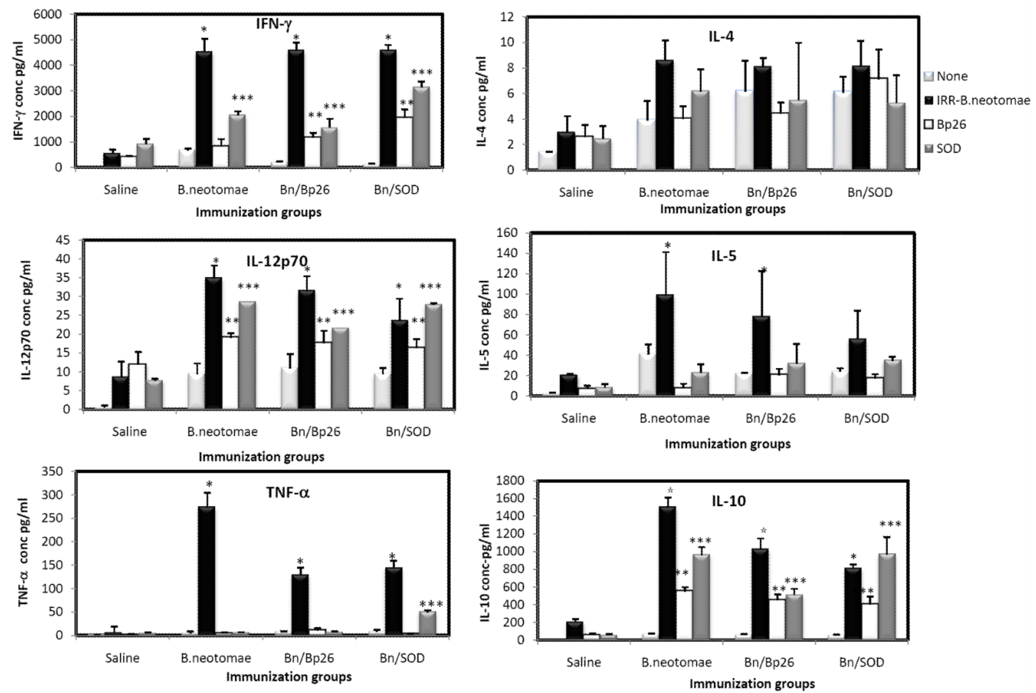

Figure 5.

Production of specific cytokines by splenocytes of BALB/c mice vaccinated with gamma irradiated *B. neotomae* and its recombinants after in vitro stimulation with irradiated *B. neotomae* (IRR-B.neotomae), MBP-Bp26 (Bp26), or SOD. Values are means ± standard deviation (n=4).

* Significantly different from the corresponding unstimulated control with *B. neotomae* stimulation ($P<0.05$)

** Significantly different from the corresponding unstimulated control with MBP-Bp26 stimulation ($P<0.05$)

*** Significantly different from the corresponding unstimulated control with SOD stimulation ($P<0.05$)

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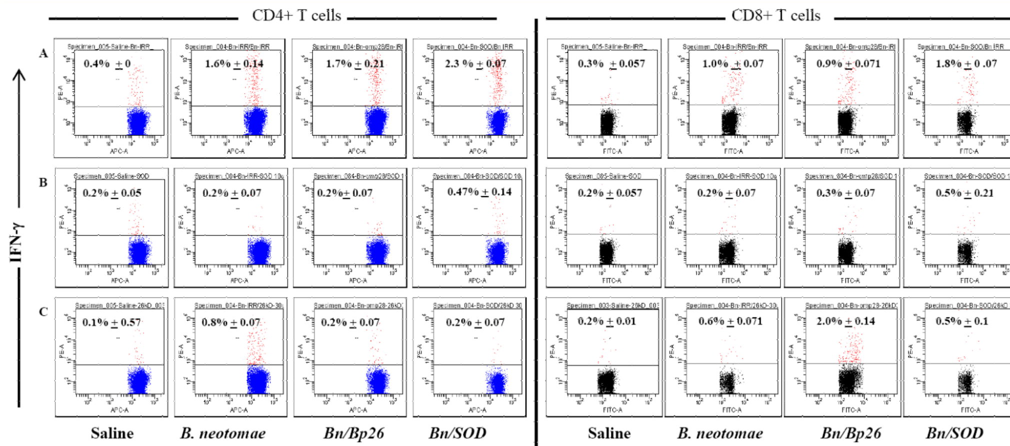

Figure 6.

Flow cytometric analysis showing the percentage of interferon-γ secreting CD4+ and CD8+ T cells in the spleens of BALB/c mice immunized with gamma irradiated *B. neotomae* and its recombinants following in vitro stimulation with: (A) gamma irradiated *B. neotomae*, (B) SOD,(C) MBP-Bp26.

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Table 1

Persistence of gamma-irradiated and heat-killed *B. neotomae* in mouse spleens as detected by the real-time quantitative PCR.

Post-inoculation time	log $_{10}$ CFU equivalent/spleen (mean ± stdev)	
	Irradiated *B. neotomae*	Heat-killed *B. neotomae*
3 hours	5.04±0.01	4.76±0.13
24 hours	4.19±0.02	1.8±0.07
3 days	3.34±0.02	0.21±0.15
5 days	-	-

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Table 2

Protection against challenge with 3 virulent *Brucella* species in mice vaccinated with 2 doses of gamma-irradiated *B. neotomae* and its recombinant strains.

Challenge strain	Vaccine	Bacterial load in spleen $\log_{10}$ CFU (mean ± stdev)	Units of protection[a]	*P* value vs corresponding control group
B.abortus 2308	None (saline)	5.27 ± 0.09	-	-
	B. neotomae	3.32 ± 0.84	2.95	0.0015
	B. neotomae/Bp26	2.29 ± 0.32	2.98	0.00006
	B. neotomae/SOD	3.24 ± 0.19	2.03	0.00005
B. suis 1330	None (saline)	5.71 ± 0.29	-	-
	B. neotomae	2.13 ± 1.16	3.58	0.003
	B. neotomae/Bp26	2.15 ± 0.81	3.56	0.0028
	B. neotomae/SOD	2.31 ± 0.99	3.4	0.0005
B. melitensis 16M	None (saline)	5.66 ± 0.11	-	-
	B. neotomae	3.62 ± 1.4	2.04	0.025
	B. neotomae/Bp26	4.32 ± 0.43	1.34	0.003
	B. neotomae/SOD	3.33 ± 1.73	2.33	0.045

[a]Units of protection were calculated by subtracting the mean $\log_{10}$ CFU for a vaccinated group from the mean $\log_{10}$ CFU of the corresponding saline control group.