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Immune Responses of Bison and Efficacy after Booster Vaccination with *Brucella abortus* Strain RB51

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Thirty-one bison heifers were randomly assigned to receive saline or a single vaccination with 10^{10} CFU of *Brucella abortus* strain RB51. Some vaccinated bison were randomly selected for booster vaccination with RB51 at 11 months after the initial vaccination. Mean antibody responses to RB51 were greater ($P < 0.05$) in vaccinated bison after initial and booster vaccination than in nonvaccinated bison. The proliferative responses by peripheral blood mononuclear cells (PBMC) from the vaccinated bison were greater ($P < 0.05$) than those in the nonvaccinated bison at 16 and 24 weeks after the initial vaccination but not after the booster vaccination. The relative gene expression of gamma interferon (IFN-γ) was increased ($P < 0.05$) in the RB51-vaccinated bison at 8, 16, and 24 weeks after the initial vaccination and at 8 weeks after the booster vaccination. The vaccinated bison had greater ($P < 0.05$) *in vitro* production of IFN-γ at all sampling times, greater interleukin-1β (IL-1β) production in various samplings after the initial and booster vaccinations, and greater IL-6 production at one sampling time after the booster vaccination. Between 170 and 180 days of gestation, the bison were intraconjunctivally challenged with approximately 1×10^7 CFU of *B. abortus* strain 2308. The incidences of abortion and infection were greater ($P < 0.05$) in the nonvaccinated bison after experimental challenge than in the bison receiving either vaccination treatment. Booster-vaccinated, but not single-vaccinated bison, had a reduced ($P < 0.05$) incidence of infection in fetal tissues and maternal tissues compared to that in the controls. Compared to the nonvaccinated bison, both vaccination treatments lowered the colonization (measured as the CFU/g of tissue) of *Brucella* organisms in all tissues, except in retropharyngeal and supramammary lymph nodes. Our study suggests that RB51 booster vaccination is an effective vaccination strategy for enhancing herd immunity against brucellosis in bison.

Although *Brucella abortus* can infect other mammalian species, cattle are the preferred host for this species of *Brucella*. As humans are effectively dead-end hosts, in that brucellosis is essentially not transmitted between people, the maintenance of disease in animal hosts is the source of human infection. For that reason, many industrialized countries have invested heavily in brucellosis control programs in livestock due to public health benefits from the reductions in disease prevalence in animal reservoirs. In the United States, it was estimated in 2000 that approximately 11 billion dollars had been spent in efforts to eradicate brucellosis from cattle. This investment was rewarded in 2008 by the announcement from the U.S. Department of Agriculture that for the first time in the United States, all 50 states were simultaneously free of cattle brucellosis. Despite the eradication of *B. abortus* from domestic livestock, the persistence of *B. abortus* infection in free-ranging bison and elk at Yellowstone National Park and the surrounding areas remains a concern for the reintroduction of brucellosis to cattle.

Previous studies have demonstrated that bison are more susceptible to infection with *B. abortus* than are cattle, and a single vaccination with *B. abortus* strain RB51 is effective in reducing the incidence of abortion and infection in bison after experimental challenge (1, 2). In a previous study with a small number of bison, we (3) demonstrated that booster vaccination with RB51 at a 13-month interval increased protection against experimental challenge compared to that provided by a single RB51 vaccination administered during calfhood. In the study reported here, we expand on the previous booster vaccination study with greater experimental units and more extensive bacteriologic and immunologic characterization.

MATERIALS AND METHODS

***B. abortus* cultures.** For the immunologic assays, RB51 suspensions (1×10^{12} CFU/ml) were inactivated by gamma irradiation (1.4×10^6 rads), washed in 0.15 M sodium chloride (saline), and stored at −70°C.

Animals and inoculation. Eight- to 11-month-old bison heifers were obtained from a brucellosis-free herd. After acclimation, the bison were randomly assigned to receive either saline (control; $n = 7$) or a single intramuscular vaccination with RB51 ($n = 24$). Some of the vaccinated bison ($n = 16$) were randomly selected for booster vaccination with RB51 at 11 months after the initial vaccination.

A commercial RB51 vaccine was obtained in lyophilized form (Colorado Serum Company, Denver, CO) and diluted in accordance with the manufacturer's recommendations. All hand inoculations were of 2 ml in volume and administered intramuscularly in the cervical region drained by the superficial cervical lymph node. Following vaccination, the concen-

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trations of viable bacteria within the inocula were determined by standard plate counts.

Serologic evaluation. Blood samples were collected by jugular venipuncture prior to vaccination and at approximately 4-week intervals up to 24 weeks postvaccination. Blood was also obtained after the booster vaccination at approximately 4-week intervals until 16 weeks postbooster. The blood was allowed to clot for 12 h at 4°C and centrifuged. The serum was divided into 1-ml aliquots, frozen, and stored at −70°C.

The serologic antibody responses of the bison after vaccination were determined by a previously described enzyme-linked immunosorbent assay (ELISA) procedure using whole RB51 bacteria as an antigen (1). To determine if RB51 booster vaccination might induce positive serology on surveillance tests, sera obtained after initial or booster vaccination and from nonvaccinated bison were evaluated on the card test and fluorescent polarization assay (FPA) by the National Veterinary Service Laboratories in accordance with standard protocols.

Preparation of peripheral blood mononuclear cells and lymph node cells for lymphocyte proliferation assays. At 4, 8, 12, 16, 20, and 24 weeks after the initial vaccination and 4, 8, 12, and 16 weeks after the booster vaccination, blood was obtained from the jugular vein of each bison, and peripheral blood mononuclear cells (PBMC) were isolated and adjusted to 1×10^7 viable cells, as previously described (4).

Fifty microliters of each cell suspension, each containing 5×10^5 cells, was added to two separate flat-bottom wells of 96-well microtiter plates that contained 100 μl of RPMI 1640 medium only or RPMI 1640 medium containing γ-irradiated RB51 (10^9 to 10^5 bacteria per well). The wells containing 1 μg/ml pokeweed mitogen (Sigma Chemical Company) were used as positive controls for the proliferative responses. The cell cultures were incubated for 7 days at 37°C under 5% CO_2. After 7 days of incubation, the cell cultures were pulsed with 1.0 μCi of [^{3}H]thymidine per well for 18 h. The cells were harvested onto glass filter mats and assessed for radioactivity in a liquid scintillation counter.

Cytokine production. In a similar manner as for the proliferation assays, 50 μl of the PBMC suspension, each containing 5×10^5 cells, was added to flat-bottom wells of 96-well microtiter plates that contained 100 μl of RPMI 1640 medium only or RPMI 1640 medium containing γ-irradiated RB51 (2×10^8 bacteria per well). The cell cultures were incubated at 37°C under 5% CO_2 and the supernatants removed at 48 h after the initiation of culture at 8, 16, and 24 weeks after the initial vaccination and 8 and 16 weeks after the booster vaccination. The supernatants were held at −70°C until assayed in duplicate for gamma interferon (IFN-γ), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) production using a commercial kit (Aushon chemiluminescent array kit; Billerica, MA), as per the manufacturer's directions. Antigen-specific cytokine production was determined for each sample by subtracting the concentrations in the wells without RB51 from the concentrations in the wells with RB51. Any samples below the level of detection of the kit were statistically analyzed as 0 pg/ml.

Cytokine transcription after initial or booster vaccination. At 8, 16, and 24 weeks after the initial vaccination and at 8 and 16 weeks after the booster vaccination, PBMC were isolated from RB51-vaccinated and control bison (6 bison/treatment [trt]/time). Approximately 5×10^5 PBMC were added to each of four separate flat-bottom wells of 96-well microtiter plates that contained RPMI 1640 medium only (control) or RPMI 1640 medium containing γ-irradiated RB51 (10^7 bacteria per well). After 18 h of incubation at 37°C and under 5% CO_2, the cells were pelleted by centrifugation, suspended in 100 μl of RNA*later* (Ambion, Austin, TX), and refrigerated at −20°C until processing for RNA purification.

RNA was purified from the cells using a commercial kit, converted to cDNA, and purified as previously described (3). The samples were analyzed in triplicate on a real-time PCR machine (Rotor-Gene 3000; Corbett Research, Australia), as previously described (3), with the internal normalization of cytokine gene expression carried out using the beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes as the reference genes. The primers for the bovine cytokine sequences were described previously (3, 5).

Experimental challenge. The animals were raised to adulthood and pasture bred at approximately 30 months of age and rectally palpated between 40 and 90 days gestation. At approximately 5 months gestation, the pregnant bison were transferred to a biosafety level 3 containment facility where they were individually housed for the duration of the study. Between 170 and 180 days of gestation, as determined by rectal palpation, the bison were restrained in a squeeze chute and intraconjunctivally challenged with approximately 1×10^7 CFU of *B. abortus* strain 2308, as previously described (1, 2). The concentration of viable bacteria within each challenge inoculum was determined by serial dilution in saline and by standard plate counts.

Blood samples were collected by jugular venipuncture prior to challenge and, in the absence of abortion, at 2, 4, and 8 weeks after challenge. Blood was also obtained at necropsy. The blood was allowed to clot for 12 h at 4°C and was centrifuged. The serum was divided into 1-ml aliquots, frozen, and stored at −70°C. The serologic titers of the bison after challenge were determined using a standard tube agglutination test (6).

Immediately following abortion, or within 72 h of parturition, each cow was euthanized with an intravenous administration of sodium pentobarbital, and samples were collected, as previously described (1, 3). For bacterial culture, the swabs and fluids were plated directly on tryptose agar plates containing 5% bovine serum, whereas the tissues were weighed, triturated in 0.15 M NaCl (saline) using a tissue grinder, and serially diluted prior to plating (1, 7). After 7 days of incubation, *B. abortus* was identified on the basis of colony morphology and growth characteristics (6) and was confirmed by a PCR procedure using primers specific for the identification of the *B. abortus* gene *omp2a* (8). The concentrations of bacteria (CFU/g of tissue) in the tissue samples were determined by performing standard plate counts.

Abortion was defined as the premature birth of a *Brucella*-infected nonviable fetus after 2308 challenge. In addition to a clinical assessment of the viability of live calves, all calves considered viable had milk present within the abomasum at necropsy. Mammary infection was defined as the recovery of the 2308 challenge strain from a supramammary lymph node, milk, or mammary gland tissue. Uterine infection was defined as the recovery of the 2308 challenge strain from the placentome, vaginal swab, or internal iliac lymph node. Fetal infection was defined as the recovery of strain 2308 from any fetal sample.

Statistical analysis. Serologic, net IFN-γ, net IL-1, net IL-6, net-TNF-α, and standard tube agglutination titers were analyzed as the logarithms of their values. For any measurements with values of 0, the values were set to 1 for calculation of the logarithm. The cell proliferation results were converted to stimulation indices (counts per minute [cpm] of the wells containing antigen/cpm in the absence of antigen) for statistical comparisons. The serologic data were compared over all sampling times using a two-way analysis of variance model, whereas the differences between the treatments in stimulation indices and the net cytokine data at each sampling time were compared by a general linear model procedure (SAS Institute, Inc., Cary, NC). The means for the individual treatments were separated by the use of a least-significant difference procedure ($P < 0.05$). The data are presented as the mean ± standard error of the mean (SEM).

For the determination of takeoff and amplification values, the threshold on the real-time PCR machine was set at a constant value for all runs. The expression of the cytokine genes was normalized in relationship to the expression of the housekeeping genes (beta-actin and GAPDH genes), and the relative expression differences between the vaccinated bison and controls were determined by an analysis of the takeoff and amplification values on a commercial software program (REST 2009; Qiagen), using previously described mathematical algorithms (9).

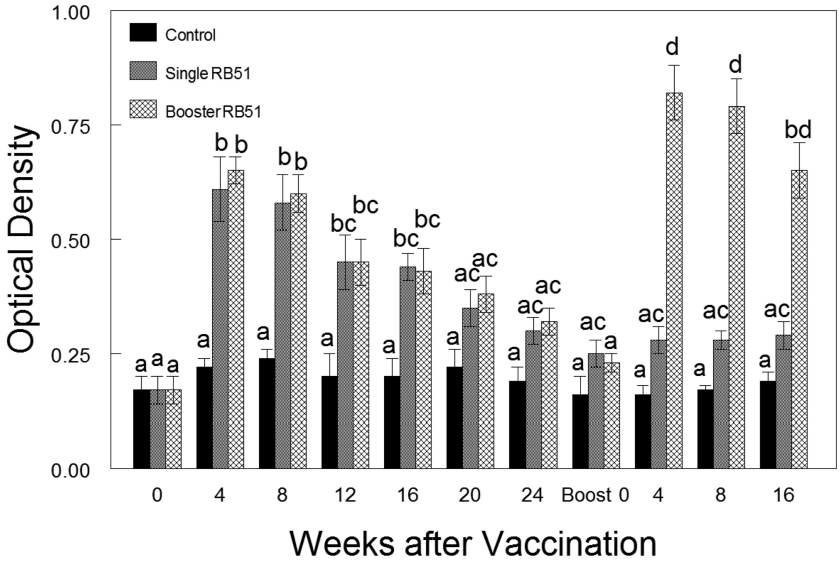

FIG 1 Bison serologic responses to γ-irradiated RB51 in an ELISA in nonvaccinated bison (control), after initial inoculation with 10^{10} CFU RB51, or after booster vaccination 11 months after initial vaccination with a similar dose of RB51. The responses are presented as the mean optical density ± SEM. The mean values with different lowercase letters are significantly different ($P < 0.05$).

RESULTS

Vaccine dosages. The standard plate counts indicated the mean vaccination dosages for the initial and booster RB51 inoculations to be 1.6×10^{10} and 2.8×10^{10} CFU, respectively. The mean ± SEM challenge dose of *B. abortus* strain 2308 was $(8.9 \pm 0.16) \times 10^6$.

Serologic responses after vaccination. The sera obtained from the bison in all treatments were negative on the card and FPA brucellosis serologic tests at all times after the initial or booster vaccination.

With the exception of 20 and 24 weeks after inoculation, the RB51 vaccine recipients had greater ($P < 0.05$) mean ELISA optical densities to RB51 at all sampling times after the initial vaccination than did the nonvaccinated bison (Fig. 1).

At 44 weeks after vaccination, when the booster vaccination was administered, the mean ELISA optical densities to RB51 did not differ ($P > 0.05$) between nonvaccinated bison and those which had received vaccination treatments. However, at 4, 8, and 16 weeks after the booster vaccination, the bison receiving a second inoculation with RB51 had greater ($P < 0.05$) mean responses than did the single-vaccinated and nonvaccinated bison. At these sampling times after the booster vaccination, the mean optical density of the single-vaccinated bison did not differ from the titers of the nonvaccinated bison.

The responses on the card and FPA serologic tests indicated that booster vaccine recipients did not seroconvert on standard surveillance tests and, like the single vaccine recipients, do not develop antibody responses to the immunodominant O side chain on the *Brucella* lipopolysaccharide.

Lymphocyte proliferation responses after vaccination. After the initial vaccination, PBMC from the RB51 vaccine recipients had greater ($P < 0.05$) mean stimulation indices to RB51 at 16 and 24 weeks after vaccination than did the nonvaccinated bison (Fig. 2A). The mean stimulation indices of bison vaccinated with RB51 did not differ ($P > 0.05$) from responses of nonvaccinates at 4, 8, 12, and 20 weeks after initial vaccination.

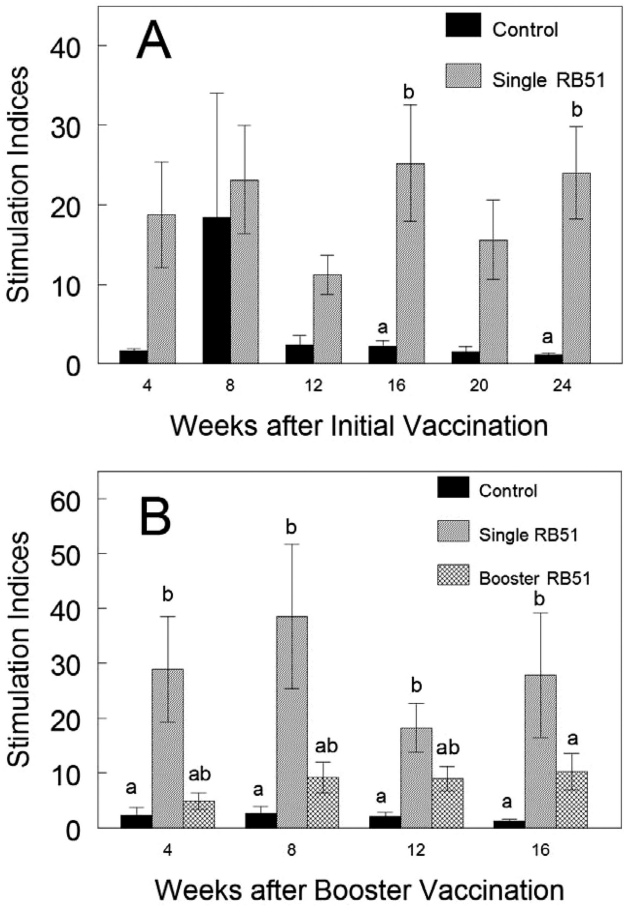

FIG 2 Mean stimulation indices to 10^8 CFU of γ-irradiated RB51 by peripheral blood mononuclear cells from bison vaccinated with saline or once with 10^{10} CFU of RB51 (A) or after booster vaccination 11 months after initial inoculation with an equivalent RB51 dose (B). Cells were incubated at 37°C and 5% CO_2 for 7 days and pulsed for 18 h with [^{3}H]thymidine. Means within a sampling time with different lowercase letters are significantly different ($P < 0.05$).

TABLE 1 Relative expression levels of cytokine gene transcription by PBMC from RB51 vaccinees in comparison to nonvaccinated bison after initial or booster vaccination[a]

	Expression level by vaccination[b]				
	Initial			Booster	
Cytokine	8 wk	16 wk	24 wk	8 wk	16 wk
IFN-γ	14.92*	2.97*	10.71*	6.50*	1.05
IL-1β	0.59	0.92	1.32	0.73	1.88
IL-2	2.84	2.09	1.12	2.47*	1.55
IL-6	0.94	1.95	0.74	1.94	1.36
IL-10	0.72	1.34	0.97	2.34*	1.27
IL-12	2.28	1.80	0.63	1.80	1.40
IL-15	2.81	0.49	0.49	1.24	4.96*
IL-17	0.76	1.07	2.81	3.47*	1.51

[a] PBMC were cultured *in vitro* with 10^7 CFU of γ-irradiated *B. abortus* strain RB51 for 18 h.

[b] The data are expressed as the mean relative expression of genes in the PBMC of vaccinees compared to the expression in PBMC from nonvaccinated bison. An asterisk denotes greater ($P < 0.05$) mean upregulation in vaccinees than that in nonvaccinees. No genes evaluated in the current study demonstrated significant ($P < 0.05$) downregulation in vaccinees compared to that in nonvaccinated bison at any sampling time.

After the booster vaccination, the mean proliferative responses of the PBMC from booster-vaccinated bison did not differ at any sampling time up to 16 weeks compared to the responses of the PBMC from the nonvaccinated bison (Fig. 2B). Compared to the responses of the nonvaccinated bison, PBMC from bison that received only the initial vaccination had greater ($P < 0.05$) mean proliferative responses to the RB51 antigens at all sampling times during this part of the study. The mean proliferative responses of the PBMC from the booster-vaccinated and single-vaccinated bison did not differ ($P > 0.05$) at 4, 8, and 12 weeks after the booster vaccination.

Relative expression of cytokine genes. The expression of IL-1β, IL-6, and IL-12 genes in response to 18 h of incubation of PBMC with RB51 antigens did not differ ($P > 0.05$) between the vaccinated bison and controls at any sampling time after the initial or booster vaccination. Compared to the gene expression in the PBMC from the nonvaccinated bison, the relative expression of IFN-γ was increased ($P < 0.05$) in the RB51 vaccine recipients at 8, 16, and 24 weeks after the initial vaccination and at 8 weeks after the booster vaccination (Table 1). The vaccinated bison also demonstrated greater ($P < 0.05$) antigen-specific expression of IL-2, IL-10, and IL-17 at 8 weeks after the booster vaccination and of IL-15 at 16 weeks after the booster vaccination compared to the expression by PBMC from the control bison.

***In vitro* cytokine production.** At all sampling times evaluated (8, 16, and 24 weeks after the initial vaccination and 8 and 16 weeks after the booster vaccination), PBMC from bison receiving single or booster vaccinations with RB51 had greater ($P < 0.05$) antigen-specific IFN-γ production than those in the nonvaccinated bison (Fig. 3). The PBMC from single- and booster-vaccinated bison did not differ ($P > 0.05$) in their antigen-specific production of IFN-γ at any of the evaluated sampling times.

At 8 weeks after the initial vaccination, PBMC from single- and booster-vaccinated bison had greater ($P < 0.05$) antigen-specific production of IL-1β than did PBMC from the nonvaccinated bison (Fig. 4), whereas there was no difference ($P > 0.05$) at 16 and 24 weeks. At 8 and 16 weeks after the booster vaccination, PBMC from

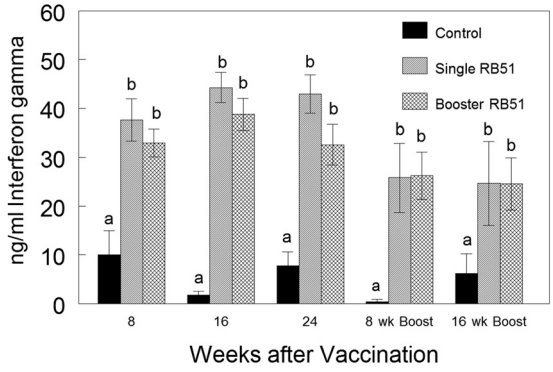

FIG 3 Mean production of γ-interferon bison PBMC in response to incubation for 48 h with 10^8 CFU of γ-irradiated RB51. The bison were vaccinated with saline (control), once with 10^{10} CFU of RB51 (single RB51), or booster vaccinated with 10^{10} CFU of RB51 at 11 months after the initial vaccination (booster RB51). The mean values within a sampling time with different lowercase letters are significantly different ($P < 0.05$). wk, week.

bison vaccinated twice with RB51 had greater ($P < 0.05$) antigen-specific production of IL-1β than did PBMC from nonvaccinated bison. However, at these sampling times, the mean IL-1β production by PBMC of bison vaccinated once with RB51 was intermediate between the production by PBMC from booster-vaccinated and control bison and did not differ ($P > 0.05$) from the means of either treatment.

The antigen-specific production of IL-6 by PBMC did not differ ($P > 0.05$) between treatments prior to 16 weeks after the booster vaccination. At 16 weeks after the booster vaccination, PBMC from the booster vaccine recipients had a greater mean IL-6 production to RB51 antigens than did the PBMC from the nonvaccinated bison (Fig. 5). At that sampling time, the mean IL-6 production by PBMC of single-vaccinated bison did not differ ($P > 0.05$) from the mean production of PBMC from the control or booster-vaccinated bison.

The antigen-specific production of TNF-α by PBMC did not

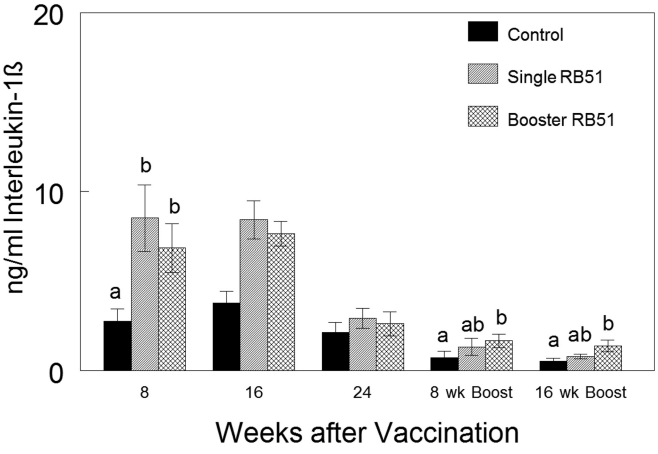

FIG 4 Mean production of interleukin-1β by bison PBMC in response to incubation for 48 h with 10^8 CFU of γ-irradiated RB51. The bison were vaccinated with saline (control), once with 10^{10} CFU of RB51 (single RB51), or booster vaccinated with 10^{10} CFU of RB51 at 11 months after the initial vaccination (booster RB51). The mean values within a sampling time with different lowercase letters are significantly different ($P < 0.05$). wk, week.

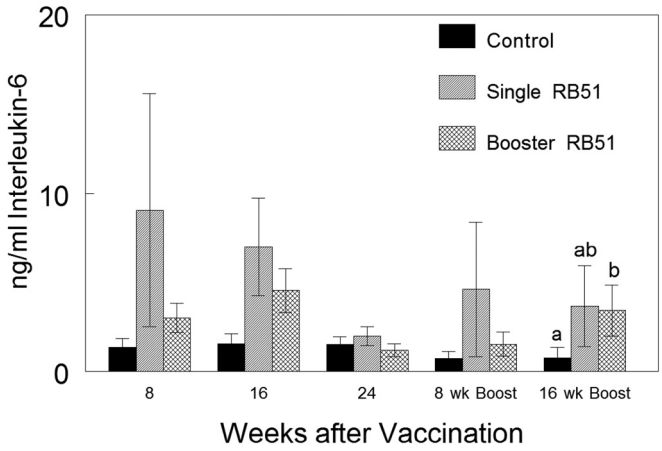

FIG 5 Mean production of interleukin-6 by bison PBMC in response to incubation for 48 h with 10^8 CFU of γ-irradiated RB51. The bison were vaccinated with saline (control), once with 10^{10} CFU of RB51 (single RB51), or booster vaccinated with 10^{10} CFU of RB51 at 11 months after the initial vaccination (booster RB51). The mean values within a sampling time with different lowercase letters are significantly different ($P < 0.05$). wk, week.

differ ($P > 0.05$) between the treatment groups at any sampling time evaluated (data not shown).

Postchallenge results. Prior to challenge, the mean titers on the standard tube agglutination test did not differ ($P > 0.05$) between treatments (data not shown). All treatments had greater ($P < 0.05$) mean titers at 4 weeks after challenge than the mean titers prior to challenge. The mean titers of the vaccination and control treatments did not differ ($P > 0.05$) at any sampling time after experimental challenge (data not shown).

In the control treatment, 6 of 6 (100%) bison aborted compared to 1 of 5 (20%) in the single-vaccination group and 1 of 14 (7%) in the booster vaccination group (Table 2). In addition to abortion being greater ($P < 0.05$) in the control group, the nonvaccinated bison had a greater ($P < 0.05$) incidence of uterine and mammary infection than did those in either vaccination treatment group. The booster vaccine recipients, but not the single-vaccine recipients, had a reduced ($P < 0.05$) incidence of infection in fetal tissues or other maternal tissues not associated with uterine or mammary systems.

Compared to the samples obtained from nonvaccinated bison at necropsy after experimental challenge, both vaccination treatments had lower colonization (CFU/g of tissue) in all tissues, except in retropharyngeal and supramammary lymph nodes (Table 3). Of particular interest was the 7.5-log reduction in the mean colonization in the placentome/cotyledon in booster vaccine recipients compared to that in the nonvaccinated bison and a comparative 5.6-log reduction in the placentomes of the single-vaccine recipients. All tissue samples obtained at necropsy from the booster vaccine recipients demonstrated a ≥1-log reduction in mean colonization compared to the colonization in the samples from the nonvaccinated bison. The reduction in colonization in the tissues from single-vaccine recipients tended to be less than that in the booster vaccine recipients, but colonization did not statistically differ ($P > 0.05$) between the single- and booster-vaccinated bison in any tissue.

In regards to the ability to recover the challenge strain from the samples obtained from individual bison, both single- and booster vaccine recipients had reductions in the incidences of recovery from tissue, fluid, and swab samples. With the exception of bronchial, hepatic, and retropharyngeal lymph nodes, the incidence of recovery of the challenge strain from milk, blood, vaginal swab, and individual tissues was lower ($P < 0.05$) in the booster-vaccinated bison than that in the samples from the controls. In a comparison of single-vaccine recipients to the nonvaccinated bison, the incidence of recovery was reduced ($P < 0.05$) in milk and in some tissues, but the incidence of recovery did not differ ($P > 0.05$) in the spleen, mammary glands, and placentome, as well as in the iliac, mandibular, parotid, prescapular, and retropharyngeal lymph node samples.

DISCUSSION

Vaccination is a cost-effective technique that facilitates the control of brucellosis in reservoir hosts but by itself will not eradicate the disease. Any vaccination-based program for the control of brucellosis in free-ranging wildlife will likely require a long-term commitment of substantial financial and human resources. For that reason, it is imperative that any vaccine and/or vaccination protocol be optimized to its highest possible efficacy to ensure the greatest impact on reducing brucellosis prevalence in the targeted wildlife population.

The results of the current study demonstrate that booster vaccination of bison at approximately 1 year after the initial vaccination is more efficacious in protecting against abortion and colonization after experimental challenge than is a single vaccination with 10^{10} CFU of *B. abortus* strain RB51 administered prior to 12 months of age. The data from the current study are similar and expand on the results from a previous study (3), which evaluated the efficacy of a similar booster vaccination protocol in only 5

TABLE 2 Efficacies of *B. abortus* strain RB51 vaccination strategies in protecting against experimental challenge at midgestation with 10^7 CFU of *B. abortus* strain 2308

Vaccination group	Rate of abortion or infection (% [no. aborted or infected/total])[e]				
	Abortion	Uterine infection[a]	Mammary infection[b]	Fetal infection[c]	Remaining maternal tissues[d]
Single RB51	20 (1/5)*	40 (2/5)*	40 (2/5)*	60 (3/5)	80 (4/5)
Booster RB51	7 (1/14)*	21 (3/14)*	21 (3/14)*	42 (6/14)*	71 (10/14)*
Control	100 (6/6)	100 (6/6)	100 (6/6)	83 (5/6)	100 (6/6)

[a] Placentome, vaginal swab, and/or internal iliac lymph node.
[b] Mammary tissues (4 quarters), milk, and/or supramammary lymph node.
[c] Fetal lung, liver, spleen, gastric contents, bronchial lymph node, or rectal swab.
[d] All maternal tissues not included in Uterine infection or Mammary infection column.
[e] An asterisk indicates a result that is significantly different ($P < 0.05$) from the control treatment.

TABLE 3 Colonization and incidence of recovery of *B. abortus* in tissues at necropsy after experimental strain 2308 challenge in bison that had received saline (control) or a single or booster vaccination with 10^{10} CFU of *B. abortus* strain RB51

	Mean ± SD CFU/g of tissue (no. recovered/total no.)[a]		
		RB51 vaccinees	
Sample type	Control	Single	Booster
Lung	1.2 ± 0.4 (4/6) A	0.4 ± 0.4 (1/5) B	0 ± 0 (0/14)* B
Liver	1.7 ± 0.4 (5/6) A	0 ± 0 (0/5)* B	0.1 ± 0.1 (1/14)* B
Spleen	1.7 ± 0.4 (5/6) A	0.6 ± 0.4 (2/5) B	0.2 ± 0.2 (1/14)* B
Bronchial LN[b]	2.1 ± 0.2 (6/6) A	0.2 ± 0.2 (1/5)* B	0.5 ± 0.2 (6/14) B
Hepatic LN	1.5 ± 0.5 (5/6) A	0 ± 0 (0/5)* B	0.2 ± 0.1 (2/14) B
Iliac LN	1.6 ± 0.6 (4/6) A	0.8 ± 0.5 (2/5) B	0.1 ± 0.1 (1/14)* B
Mandibular	2.2 ± 0.4 (5/6) A	0.7 ± 0.4 (2/5) B	0.2 ± 0.2 (2/14)* B
Mesenteric	2.0 ± 0.4 (6/6) A	0.3 ± 0.3 (1/5)* B	0.1 ± 0.1 (1/14)* B
Retropharyngeal LN	1.6 ± 0.7 (4/6)	0.4 ± 0.4 (1/5)	0.4 ± 0.3 (2/14)
Mammary gland	1.0 ± 0.3 (3/6) A	0.3 ± 0.3 (1/5) B	0 ± 0 (0/14)* B
Parotid LN	2.4 ± 0.1 (6/6) A	0.9 ± 0.3 (4/5) B	0.6 ± 0.3 (5/14)* B
Prescapular LN	1.4 ± 0.5 (4/6) A	0.4 ± 0.4 (1/5) B	0.2 ± 0.2 (1/14)* B
Supramammary LN	2.0 ± 0.3 (6/6)	1.1 ± 0.7 (2/5)*	0.6 ± 0.5 (1/14)*
Placentome/cotyledon	9.1 ± 0.6 (5/6) A	3.5 ± 1.7 (2/5) B	1.6 ± 0.8 (3/14)* B
Milk	(6/6)	(1/5)*	(2/14)*
Blood	(5/6)	(1/5)	(1/14)*
Vaginal swab	(4/6)	(1/5)	(2/15)*
Conjunctival swab	(1/6)	(0/5)	(1/15)

[a] Means denoted with different uppercase letters statistically differ ($P < 0.05$). Asterisks indicate incidences of infection that are significantly different ($P < 0.05$) from the control treatment.
[b] LN, lymph node.

bison. The efficacy data in the current study are similar to unpublished cumulative data from 11 studies conducted in our laboratory, in which 31% of the RB51-vaccinated bison ($n = 73$) and 86% of the control bison ($n = 58$) aborted after experimental challenge that was conducted in a manner similar to that in the current study. As abortion events are the predominant mechanism for the lateral transmission of brucellosis within a reservoir population, extrapolating the current data from booster-vaccinated bison would suggest that the use of a similar inoculation protocol under field conditions might dramatically reduce abortions, the shedding of *Brucella*, and the transmission of disease within the population. Even with the high efficacy of booster vaccination with RB51, the impact on disease prevalence in bison under field conditions would be dependent on the proportion of the population that is successfully vaccinated, receiving either a single inoculation or those in which two inoculations were successfully administered. As experimental studies ensure that all animals are pregnant and receive an infectious dose of a virulent strain during midgestation, when they are most susceptible (10), it is possible that vaccine efficacy under field conditions might be greater than the results obtained in this study under experimental conditions. However, the data from the field studies of brucellosis vaccines would suggest that numerous variables, known and unidentified, can influence the prevalence of disease and vaccine protection under field conditions. Regardless, the promising results in the current study support the initiation of a field study evaluating booster vaccination in an infected bison herd.

In the current study, we further extended the characterization of cellular immunity to include an evaluation of both the transcription and protein expression of cytokines by PBMC. The inability to detect significant proliferative responses in the peripheral blood after booster inoculation, as observed in the current study, was also noted in our previous study (3). Since the booster-vaccinated bison in the current study had greater protection against experimental challenge than did the single-vaccine recipients, the lack of responsiveness in PBMC suggests that booster vaccination may impact cell trafficking in the blood for ≥24 weeks after the second inoculation. It was not surprising that increased transcription and expression of IFN-γ were the most common antigen-specific cytokine responses observed after single or booster vaccination, as this cytokine (i.e., IFN-γ) has been frequently correlated with protection in other studies evaluating vaccine efficacy against intracellular bacteria, including *Brucella* species (11–14). Although our data appear to suggest that the mean expression of IFN-γ was greater after the initial vaccination, the samples obtained after the initial vaccination were run separately from the samples obtained after the booster vaccination. For that reason, we believe it is not appropriate to statistically compare IFN-γ expression after the initial and booster vaccinations.

The increase in the expression of IL-1β at various sampling times in the current study may reflect its role as a central mediator in innate immunity. This cytokine contributes to bacterial clearance by promoting phagolysosomal maturation (15), and the downregulation of IL-1β appears to be an important mechanism used by many pathogenic intracellular bacteria, including *Mycobacterium tuberculosis* (16). Adaptive immunity may also include increases in the expression of IL-1β, as cattle infected with *Mycobacterium bovis* demonstrate increased expression of IL-1β in whole-blood cultures in response to incubation with *M. bovis* antigens (17). In the present study, it was surprising that protein expression and gene transcription of IL-1β did not correlate in some sampling times, whereas IFN-γ demonstrated consistent increases in both gene transcription and protein expression after the initial and booster vaccinations. This may partly be explained by the observation that the inactive precursor murine IL-1β (pmIL-1β) requires cleavage by caspase-2 to become active and secreted.

Signaling by IL-1β and its role in innate immunity have also been proposed to be important in the pathogenesis of brucellosis. This is supported by the observation that IL-1 receptor knockout mice are more susceptible to infection with virulent *B. abortus* (18) and the induction of expression in macrophages after inflammasome formation. Inflammasomes are multiprotein complexes formed after Nod-like receptors recognize pathogen-associated molecular patterns (PAMPS) associated with bacterial products inserted into the host cell cytoplasm by bacterial type II or type IV secretion systems, the recognition of bacterial nucleic acids, or mitochondrial reactive oxygen species produced by live bacteria (18, 19). The expression of caspase 1, IL-1β, and IL-18 by murine macrophages after inflammasome formation plays a role in susceptibility to Gram-negative bacteria, including *Brucella* species (18). Data using bovine cell lines suggest that IL-1β and innate immunity play important roles in the protection against infectious agents, including Gram-negative bacteria. Bovine monocytes primed with IFN-γ release IL-1β after stimulation with calcium-binding calgranulins (20), and bovine macrophages infected with *Mycobacterium avium* subsp. *paratuberculosis* have increased gene transcription and protein expression of IL-1β that are dependent on the influx of extracellular calcium and phagosome acidification (21). *Brucella* spp. may have evolved a mechanism to subvert innate immunity and IL-1 during infection. Recently, there has been an increased interest in the Toll intracellular domain/IL-1 receptor-containing protein TcpB of *Brucella* due to its possible interference with Toll–interleukin-1 receptor domain-containing adapter protein (TIRAP)/Mal and MyD88 signaling through Toll-like and interleukin-1 receptors (22–24).

It was of interest that the gene transcription of IL-6 did not differ between vaccinated and nonvaccinated bison, but increased protein expression was detected at one time point after booster vaccination. Interleukin-6 is a proinflammatory cytokine associated with innate immune responses and T cells. In human cells, the induction of IL-6 by *Brucella* spp. has been proposed as a mechanism to elude or evade immune recognition by the downmodulation of major histocompatibility complex class II (MHC-II) molecule expression and the subsequent presentation of soluble antigens to MHC-II-restricted T cells (25). Others have suggested that differences in innate immunity, specifically increased interleukin-6 protein expression, may explain the differences between cattle breeds in resistance to infection with *M. bovis*. In that study, the more susceptible breed (Holstein) had greater interferon responses and lower IL-6 expression than those of the more resistant Sahiwal zebu breed (26). We previously observed that bison are more susceptible to *Brucella* infection than are cattle, and the peak expression of IFN-γ requires longer *in vitro* incubation times for bison PBMC than those for cattle.

In the current study, there were single observations of increased expression of IL-2, IL-10, IL-15, and IL-17 genes in bison vaccinated with an RB51 booster. Interleukin-2 is a cytokine associated with TH1 responses, regulatory cells, and the stimulation of cellular immunity, whereas IL-10 is generally associated with TH2 responses and the stimulation of nonprotective humoral responses. The increased production of IL-2 and decreased production of IL-10 have been associated with beneficial effects on *Brucella* infections in mice (27). The proinflammatory cytokine IL-15 is a cell surface-associated cytokine that functions to upregulate lectin expression in inflamed tissue, maintain memory CD8 T cells, and induce the differentiation of natural killer cells (28, 29). Interleukin-17 has been proposed to function as a bridge between innate and adaptive immunity and has been demonstrated to be important for protective immunity against extracellular bacteria and some intracellular bacteria. Increased IL-17 expression has been demonstrated to correlate with reduced *Brucella* infection in several cytokine knockout mouse strains (27, 30). However, the importance of IL-17 in protection against some intracellular bacterial pathogens, such as *M. tuberculosis*, remains controversial (31). The lack of consistent increases in the expression of IL-2, IL-10, IL-15, and IL-17 genes in the current study did not allow changes in the expression of these cytokines to be directly correlated to the greater protection against *Brucella* observed in booster vaccine recipients.

In summary, our data demonstrate a phenotypic improvement in protection against brucellosis in bison receiving booster RB51 vaccination at approximately 1 year after initial vaccination compared to single-vaccinated or nonvaccinated bison. Although the humoral responses were increased, other assays failed to demonstrate robust anamnestic responses after booster vaccination or an immune mechanism that explained the observed phenotypic differences in protection. Gamma interferon demonstrated the most consistent increases in gene transcription and gene expression after vaccination, but transcription and expression did not increase after booster vaccination. Regardless, single and booster RB51 vaccination significantly reduced abortion and tissue colonization after experimental *Brucella* challenge compared to those in nonvaccinated bison, with the reductions being greater in the booster vaccine recipients. Our study suggests that a protocol in which bison receive an initial RB51 vaccination of 10^{10} CFU, followed by booster vaccination approximately 1 year later with an equivalent dose, is currently the most effective vaccination strategy for enhancing herd immunity against brucellosis.

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Names are necessary to report factually on the available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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