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Immunologic responses of bison to vaccination with *Brucella abortus* strain RB51: Comparison of parenteral to ballistic delivery via compressed pellets or photopolymerized hydrogels

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Abstract

This study compared responses of bison calves to 10^{10} CFU of *Brucella abortus* strain RB51 (SRB51) delivered by parenteral or ballistic methods. Two types of biobullet payloads were evaluated; compacted SRB51 pellets or SRB51 encapsulated in photopolymerized poly(ethylene glycol) hydrogels. Bison were vaccinated with saline, parenteral SRB51 alone, or in combination with Spirovac™, or ballistically with compressed SRB51 or hydrogel biobullets. Bison parenterally vaccinated with SRB51 had greater ($P < 0.05$) immunologic responses when compared to control bison. Co-administration of Spirovac™ as an adjuvant did not influence immunologic responses. As compared to compressed SRB51 biobullets, ballistic vaccination with hydrogel biobullets increased cellular immune responses at some sampling times. Our data suggest that hydrogel formulations of SRB51 may be a superior alternative to compressed SRB51 tablets for ballistic vaccination of bison. Although preliminary, data suggests that immunologic responses of bison to SRB51 hydrogel bullets are similar to responses after parenteral vaccination with SRB51.

Keywords: Bison; *Brucella abortus*; RB51; Vaccine; Photopolymerized polyethylene glycol

1. Introduction

The Brucellosis Eradication Program in the United States of America is close to its goal of elimination of *Brucella abortus* infections in cattle. The investment in this program has been extensive, with approximately 11 billion dollars spent over the last 70 years and continued spending of approximately $30 million dollars per year for surveillance. The persistence of wildlife reservoirs of *B. abortus* in the United States and the potential for transmission of brucellosis from wildlife to livestock remains a concern for regulatory personnel and producers. Within the last several years, a number of *Brucella*-infected cattle herds have been identified in Wyoming and Idaho [1,2] in which the source of infection was presumed to be free-ranging elk (*Cervus elaphus nelsoni*). The free-ranging bison (*Bison bison*) within Yellowstone National Park have a high seroprevalence for brucellosis [3,4] and management of brucellosis in this reservoir has high public visibility. The current Interagency Bison Management plan for the Greater Yellowstone area requires the development of a safe and effective remotely delivered vaccine for full implementation [5].

Strong cellular immune responses with associated high levels of γ-interferon (γ-IFN) are believed to be important for long-term protection against intracellular pathogens such as *Brucella* [6]. We have previously demonstrated that parenteral vaccination of bison with 10^{10} colony-forming units (CFU) of *B. abortus* strain RB51 (SRB51) is safe and induces antigen-specific proliferative responses and γ-IFN production [7,8]. When evaluated using standard methodology, parenteral vaccination with SRB51 protects bison

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against abortion and fetal or mammary infection after experimental challenge [9]. However, when evaluating a remote delivery system, bison parenterally vaccinated with SRB51 had greater immunologic responses when compared to bison ballistically vaccinated with a bullet containing a compressed SRB51 pellet [10].

In the study reported here, we evaluate the use of a photopolymerized poly(ethylene glycol)-based hydrogels as a payload vehicle for more effective delivery of SRB51 in ballistic bullets [11]. As antigen-specific nitric oxide production has been demonstrated to differ between cattle vaccinated with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and nonvaccinated cattle [12], we evaluated this assay for differentiation of SRB51-vaccinated and nonvaccinated bison. Also a commercially available vaccine that induces strong cellular immune responses in cattle was co-administered with SRB51 to determine if it would enhance protective immune responses to brucellosis vaccination.

2. Materials and methods

2.1. Brucella abortus culture

A master seed stock of SRB51 was obtained from G. Schurig (Virginia Tech, Blacksburg, VA, USA), and after one passage on tryptose agar (Difco Laboratories, Detroit, MI, USA), was designated as the ARS/1 seed stock of SRB51. For experimental use, SRB51 bacteria from the ARS/1 seed stock were grown on tryptose agar for 48 h at 37 °C. For the dot-blot assay, SRB51 suspensions (1.3×10^{12} CFU/mL) were killed by γ-irradiation (1.4×10^{6} rad). Following irradiation, suspensions were washed in 0.15 M sodium chloride (saline) and stored in 1 mL aliquots at −70 °C.

2.2. Hand vaccine

For hand vaccination of bison, a commercially prepared SRB51 product (Colorado Serum Company, Denver, CO, USA) derived from the ARS/1 seed stock, was utilized. The lyophilized vaccine was diluted in saline to approximately 10^{10} CFU based upon standard plate counts on other vials with the same lot number. In an effort to booster cell-mediated immune responses to hand vaccination with SRB51, one group of bison received co-administrations of a commercial vaccine (Spirovac™, Pfizer Animal Health, Exton, PA, USA) that induces strong cellular immune responses in cattle against *Leptospira borgpetersenii* serovar hardjo type hardjobovis [13].

2.3. Compressed ballistic bullets

For ballistic vaccination of bison, the lyophilized commercial strain RB51 product was pelleted via compression (Eureka tablet machine, Stokes Pennwalt, Warminster, PA, USA), and incorporated into a 0.25 caliber, hydroxypropyl cellulose Biobullet® (SolidTech Animal Health, Newcastle, OK, USA).

2.4. Hydrogel SRB51 ballistic bullets

Poly(ethylene glycol) macromers (MW ∼ 7000 g/mol) containing photopolymerizable methacrylate endgroups, and PEG macromers containing co-polymerized degradable lactide or glycolide segments (5 mol lactide or glycolide/mol PEG) terminated with methacrylate groups were prepared using a previously published procedure [14]. PEG macromer solutions were dissolved in PBS (pH 7.4) to form a 35% w/w solution. Irgacure 184™ (Ciba Specialty Chemicals, Tarrytown, NY, USA) saturated in ethanol (6 μL) was added to this solution to serve as a photoinitiator. Lyophilized SRB51 (Colorado Serum Company, Denver, CO, USA) was suspended in PEG macromer solutions at a concentration of $\sim 1 \times 10^{11}$ CFU/mL, using the viability provided by the manufacturer. PEG macromer solution containing suspended SRB51 (90 μL) was transferred to hydroxypropyl cellulose biobullet payload compartments, then photopolymerized using a low pressure Hg lamp (Hanovia, 20 mW/cm^2, 90 s). Post-photopolymerization viability was determined after homogenization of gels followed by serial dilution and plating on tryptose agar containing 5% heat inactivated bovine serum.

2.5. Animals and inoculation

Thirty-two, 7- to 9-month-old bison heifers (*Bison bison*) were obtained from brucellosis-free herds. After acclimation for 4 weeks, bison were randomly assigned to treatments: control ($n = 5$), hand SRB51 vaccination ($n = 6$), hand SRB51 combined with Spirovac™ ($n = 6$), compressed SRB51 bullet ($n = 6$), and SRB51 hydrogel bullets (PEG, PEG co-lactide, PEG co-glycolide; $n = 3$/trt). Bison in the hand SRB51 treatment were inoculated subcutaneously (SQ) in the left cervical region with a 2 mL volume of the SRB51 suspension. Bison in the hand SRB51 + Spirovac™ treatment were inoculated with SRB51 in a similar manner and received a co-administration of Spirovac™ (2 mL, SQ) in the left cervical region. Bison in this treatment received an additional Spirovac™ vaccination at 6 weeks after initial vaccination. Bison in the compressed or hydrogel bullet treatments were ballistically inoculated into the left hip region at a distance of approximately 20 m using an air-powered rifle system with a muzzle velocity of approximately 300 m/s (SolidTech Animal Health, Newcastle, OK, USA). The concentration of innoculum in the hand SRB51, compressed bullet, and hydrogel bullet treatments were determined by standard plate counts.

2.6. Serologic evaluation

Blood samples were collected by jugular venipuncture prior to vaccination, and at 4, 8, 12, 16, 20, and 24 weeks post-

innoculation. Blood was allowed to clot for 12 h at 4 °C and centrifuged. Serum was divided into 1 mL aliquots, frozen, and stored at −70 °C.

Antibody titers to *Brucella* were determined by a previously described antibody dot-blot assay in which γ-irradiated SRB51 is used as antigen [15].

2.7. Lymphocyte proliferation assays

At 4, 8, 12, 16, 20, and 24 weeks after vaccination, blood was obtained from the jugular vein of all bison and placed into an acid-citrate dextrose solution. Peripheral blood mononuclear cells (PBMC) were enriched by density centrifugation using a Ficoll-sodium diatrizoate gradient (Sigma Diagnostics, Inc., St. Louis, MO, USA). Peripheral blood mononuclear cells were diluted in RPMI 1640 medium to 1×10^7 viable cells per milliliter as determined by trypan blue dye exclusion.

Fifty microliters of each cell suspension, containing 5×10^5 cells, was added to each of two separate flat-bottom wells of 96-well microtiter plates that contained 100 μL of RPMI 1640 medium only, or 1640 medium containing γ-irradiated SRB51 (10^5–10^9 bacteria per well). Microtiter plates were prepared prior to initiation of the study and maintained at 70 °C until use. Cell cultures were incubated for 7 days at 37 °C in 5% CO_2. After 7 days incubation, cell cultures were pulsed with 1.0 μCi of [^{3}H]thymidine per well for 18 h. Cells were harvested onto glass filter mats and counted for radioactivity in a liquid scintillation counter.

2.8. γ-Interferon production

In conjunction with proliferation assays, 50 μL of cell suspensions (5×10^5 cells) from each bison at each sampling time were added to separate flat-bottom wells of 96-well microtiter plates that contained 150 μL of RPMI 1640 medium only, or RPMI 1640 medium containing γ-irradiated SRB51 (10^8 or 10^6 bacteria per well). At 72 h after initiation of culture, 100 μL of supernatant was removed from wells containing 10^8 or 10^6 SRB51 or RPMI alone. Data from previous studies demonstrated that bison PBMC produce more γ-IFN at 72 h incubation, as compared to 24 or 48 h incubations. Supernatants were frozen at −70 °C until assayed for γ-IFN using a commercial kit (Bovigam, CSL Limited, Vic., Australia) in accordance with manufacturer's instructions. Standard dilutions of a purified bovine γ-IFN of known quantity (108–0.211 ng/mL) were included on each microtiter plate. Optical density measurements at 450 nm were made using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). Samples with optical densities outside the standard curve were diluted and assayed again. Linear regression analysis was conducted on standards and used to calculate γ-IFN concentrations within individual samples.

2.9. Nitric oxide production

In conjunction with proliferation and γ-IFN assays, 50 μL of cell suspensions (5×10^5 cells) from each bison at each sampling time were added to each of three separate flat-bottom wells of 96-well microtiter plates that contained 150 μL of RPMI 1640 medium only, or RPMI 1640 medium containing γ-irradiated SRB51 (10^8 bacteria per well). At 24, 48, and 72 h after initiation of culture, 100 μL of supernatant was removed from wells containing SRB51 and RPMI alone and placed in separate wells in a microtiter plate. Concentrations of nitrite, the stable oxidation product of nitric oxide, were assayed as described previously [12,16]. Standard dilutions of $NaNO_2$ (2500–39 ng/mL) were included on each plate. After addition of 100 μL of Griess Reageant (Sigma Chemical Company, St. Louis, MO, USA) to each well, the microtiter plate was allowed to incubate for 15 min at room temperature. Following centrifugation (5 min at $400 \times g$) to remove any bubbles in wells, optical densities for each well were determined at 562 nm on an ELISA plate reader. Linear regression analysis was conducted on standards and used to calculate NO concentrations within individual samples.

2.10. Statistical analysis

For analysis of γ-IFN production, data were analyzed as the difference in γ-IFN concentration between paired wells with or without SRB51 antigen. In a similar manner, NO data were analyzed as both NO in wells with or without SRB51 antigen, and as net NO (NO in wells with SRB51–NO in paired wells without antigen). Serologic, proliferation, NO, and γ-IFN data were analyzed as the logarithm of their value. Serologic data were compared over all times using a general linear model procedure (SAS Institute Inc. Cary, NC, USA), whereas proliferation, NO, and γ-IFN production to response to γ-irradiated SRB51 bacteria were analyzed by sampling time. Means for individual treatments were separated by use of a least significant difference procedure ($P < 0.05$).

Data were initially analyzed across all treatments with all three hydrogel formulations (PEG, PEG co-lactide, PEG-co-glycolide) combined as one hydrogel treatment. A second analysis was conducted in which individual hydrogel formulations were compared to responses of nonvaccinated bison.

3. Results

3.1. Vaccine dosages

Standard plate counts indicated that average dosages in hand SRB51, compressed SRB51 biobullet, and hydrogel SRB51 biobullet treatments were 3.2×10^{10}, 1.2×10^{10}, and 4.7×10^{10} CFU of SRB51, respectively.

3.2. Serologic evaluation

Mean antibody titers to SRB51 did not differ ($P > 0.05$) between treatments prior to vaccination (Fig. 1). Bison

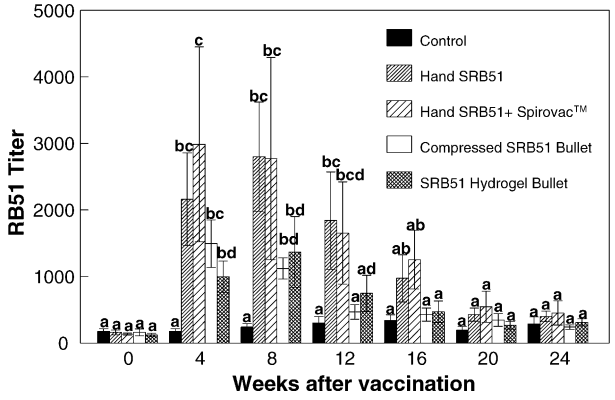

Fig. 1. Serologic responses of control or SRB51-vaccinated bison to γ-irradiated SRB51 in a dot-blot assay. Bison were ballistically vaccinated with 10^{10} CFU of SRB51 in a hydrogel ($n = 9$) or as a compressed pellet ($n = 6$), or parenterally vaccinated with 10^{10} CFU of SRB51 alone, or in combination with Spirovac™ ($n = 6$/trt). Control bison received SQ inoculation with saline ($n = 5$). Bison in the SRB51 + Spriovac™ treatment were booster vaccinated with Spirovac™ at 6 weeks after initial inoculation. Responses are presented as mean antibody titer ± S.E.M. Means with different superscripts are statistically different ($P < 0.05$).

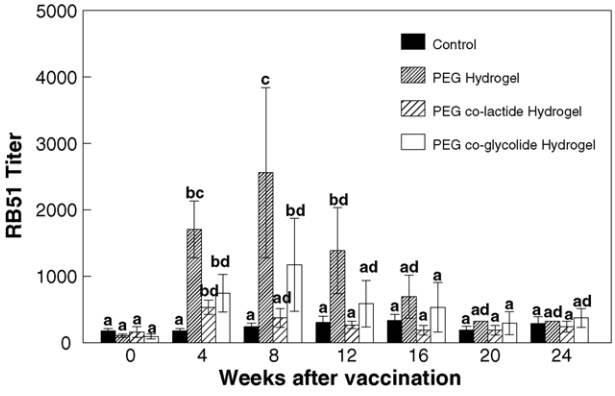

Fig. 2. Serologic responses of control or bison ballistically vaccinated with SRB51 hydrogel bullets to γ-irradiated SRB51 in a dot-blot assay. Bison were SQ inoculated with saline (control; $n = 5$) or were ballistically vaccinated with 10^{10} CFU of SRB51 in three types of hydrogels (PEG; PEG co-glycolide; or PEG co-lactide; $n = 3$/trt). Responses are presented as mean antibody titer ± S.E.M. Means with different superscripts are statistically different ($P < 0.05$).

hand vaccinated with SRB51 alone, or combined with the Spirovac™ vaccine, had greater mean titers ($P < 0.05$) than control bison at 4, 8, and 12 weeks after vaccination. Mean titers of bison ballistically vaccinated with biobullets containing SRB51 as a compressed pellet, or incorporated into hydrogels, were greater ($P < 0.05$) than mean titers of control bison at 4 and 8 weeks after vaccination. By 16 weeks after vaccination, mean titers of all parenteral and ballistic vaccination treatments did not differ ($P > 0.05$) from the control treatment. With the exception of both ballistic treatments at 12 weeks, and the ballistic hydrogel treatment at 4 weeks, mean titers of parenteral vaccination treatments did not differ ($P > 0.05$) from ballistic vaccination treatments throughout the study.

Mean titers of bison vaccinated with the PEG hydrogel bullet were greater than control bison at 4, 8, and 12 weeks after vaccination (Fig. 2). Bison vaccinated with the PEG co-glycolide hydrogel had greater mean titers than control bison at 4 and 8 weeks after vaccination. Whereas mean titers of bison the PEG co-lactide hydrogel treatment were greater than the control treatment only at 4 weeks after vaccination. Although only greater ($P < 0.05$) at 8 weeks after vaccination, there was a tendency for the PEG treatment to induce greater mean titers when compared to the other hydrogel treatments.

3.3. Lymphocyte proliferation assays

Proliferative responses to SRB51 of PBMC from bison in all vaccination treatments did not differ ($P > 0.05$) from responses of control bison at 4 weeks after vaccination (representative data in Fig. 3). Peripheral blood mononuclear cells obtained from bison hand vaccinated with SRB51 alone had greater ($P < 0.05$) proliferative responses to γ-irradiated SRB51 at 8, 12, and 20 weeks after vaccination when compared to responses of PBMC from control bison. When Spirovac™ was combined with SRB51 vaccination, proliferative responses by PBMC to SRB51 antigens was greater at 8, 12, 16, 20, and 24 weeks after vaccination. SRB51-specific proliferative responses of PBMC from bison parenterally vaccinated with SRB51 alone, or in combination with Spirovac™, did not differ ($P < 0.05$) at any sampling time. When compared to responses of nonvaccinates, proliferative responses of PBMC from bison ballistically inoculated with a compressed SRB51 bullet did not differ at any sampling time. However, PBMC from bison ballistically inoculated with SRB51 hydrogels had greater ($P < 0.05$) proliferative responses to irradiated SRB51 at

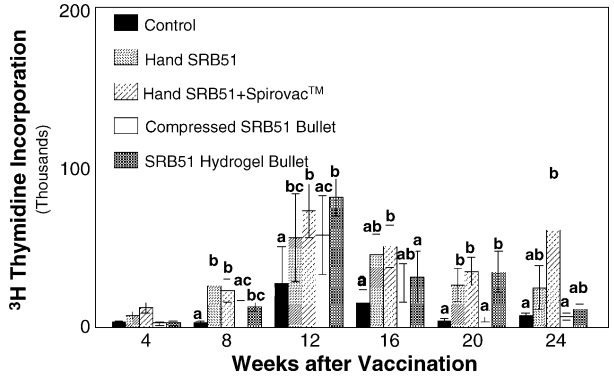

Fig. 3. Proliferative responses of peripheral blood mononuclear cells from control or SRB51-vaccinated bison to 10^8 CFU of γ-irradiated SRB51. Bison were ballistically vaccinated with 10^{10} CFU of SRB51 in a hydrogel ($n = 9$) or as a compressed pellet ($n = 6$), or parenterally vaccinated with 10^{10} CFU of SRB51 alone, or in combination with Spirovac™ ($n = 6$/trt). Control bison received SQ inoculation with saline ($n = 5$). Bison in the SRB51 + Spriovac™ treatment were booster vaccinated with Spirovac™ at 6 weeks after initial inoculation. Cells were incubated at 37 °C in 5% CO_2 for 7 days and pulsed for 18 h with [^{3}H]thymidine. Results are expressed as mean stimulation indexes. Means within a sampling time with different superscripts are statistically different ($P < 0.05$).

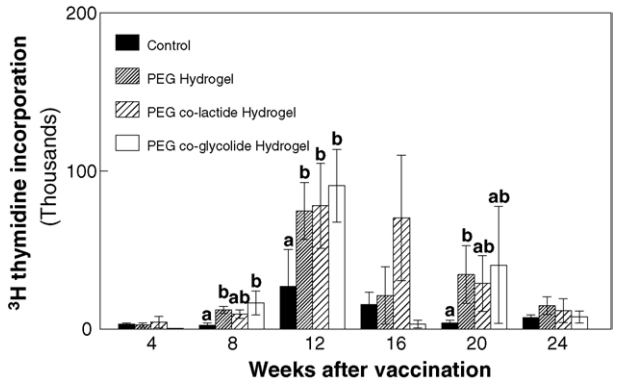

Fig. 4. Proliferative responses to 10^8 CFU of γ-irradiated SRB51 by peripheral blood mononuclear cells from bison inoculated with saline (control; $n = 5$) or ballistically vaccinated with hydrogel bullets. Ballistically vaccinated bison received 10^{10} CFU of SRB51 in three types of hydrogels (PEG; PEG co-lactide; or PEG co-glycolide; $n = 3$/trt) Cells were incubated at 37 °C in 5% CO_2 for 7 days and pulsed for 18 h with [^{3}H]thymidine. Results are expressed as mean stimulation indexes. Means within a sampling time with different superscripts are statistically different ($P < 0.05$).

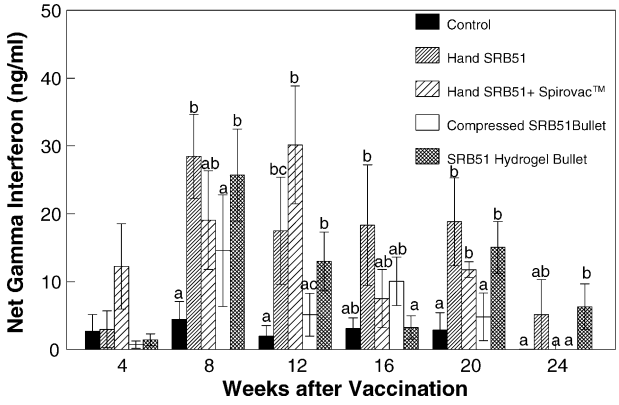

Fig. 5. Production of γ-IFN from peripheral blood mononuclear cells of control or SRB51-vaccinated Bison were ballistically vaccinated with 10^{10} CFU of SRB51 in a hydrogel ($n = 9$) or as a compressed pellet ($n = 6$), or parenterally vaccinated with 10^{10} CFU of SRB51 alone, or in combination with Spirovac™ ($n = 6$/trt). Control bison received SQ inoculation with saline ($n = 5$). Bison in the SRB51 + Spriovac™ treatment were booster vaccinated with Spirovac™ at 6 weeks after initial inoculation. Cells were incubated at 37 °C in 5% CO_2 for 72 in the absence of antigen, or with 10^8 CFU/well of γ-irradiated SRB51. Data is presented as mean net γ-IFN (wells with SRB51 − wells without antigen) ± S.E.M. Means within a sampling time with different superscripts are statistically different ($P < 0.05$).

8, 12, and 20 weeks after vaccination. When both ballistic treatments were compared, PBMC from bison inoculated with SRB51 hydrogel bullets had greater ($P < 0.05$) SRB51-specific proliferative responses to at 12 and 20 weeks after vaccination when compared to proliferative responses of bison inoculated with compressed SRB51 bullets.

Proliferative responses to killed SRB51 by PBMC from bison in the three SRB51 hydrogel treatments did not differ ($P > 0.05$) at any sampling time (representative data in Fig. 4). However, proliferative responses of bison vaccinated with hydrogel treatments did differ ($P > 0.05$) from responses of nonvaccinated bison at various sampling times. In bison ballistically inoculated with bullets containing SRB51 in PEG hydrogels, PBMC had greater ($P < 0.05$) SRB51-specific proliferative responses at 8, 12, and 20 weeks after vaccination when compared to responses of nonvaccinated bison. In a similar manner, PBMC from bison ballistically vaccinated with SRB51 in PEG co-glycolide hydrogels had greater ($P < 0.05$) proliferative responses at 8 and 12 weeks after vaccination as compared to responses of nonvaccinates. Proliferative responses of bison receiving bullets containing SRB51 in PEG co-lactide hydrogels were greater ($P < 0.05$) than responses of control bison only at 12 weeks after vaccination.

3.4. γ-Interferon production

Production of γ-IFN in response to killed SRB51 did not differ ($P > 0.05$) between treatments at 4 weeks after vaccination (Fig. 5). Bison hand vaccinated with SRB51 alone had greater ($P < 0.05$) mean γ-IFN responses to SRB51 at 8, 12, and 20 weeks after vaccination when compared to nonvaccinated bison. In bison vaccinated with both SRB51 and Spirovac™, mean γ-IFN production to SRB51 was greater ($P < 0.05$) than nonvaccinated bison at 12 and 20 weeks after vaccination. In bison vaccinated with hydrogel SRB51 bullets, mean γ-IFN production to SRB51 was greater ($P < 0.05$) than nonvaccinated bison at 8, 12, and 20 weeks after vaccination. Bison ballistically inoculated with compressed SRB51 did not differ ($P > 0.05$) from controls at any sampling time in mean γ-IFN production. In 4 of 5 sampling times between 8 and 24 weeks after vaccination, bison vaccinated with hydrogel SRB51 bullets had greater ($P < 0.05$) mean γ-IFN production to SRB51 when compared to bison vaccinated with compressed SRB51 bullets.

When responses of bison vaccinated with individual hydrogel formulations were compared to responses of nonvaccinated bison, the PEG hydrogels had greater ($P < 0.05$) mean γ-IFN production to SRB51 at 8, 12, and 20 weeks after vaccination (Fig. 6). In comparison, bison ballistically vaccinated with PEG co-lactide hydrogels had greater ($P < 0.05$) mean γ-IFN production at 20 and 24 weeks after vaccination when compared to responses of nonvaccinated bison. Bison vaccinated with the PEG co-glycolide hydrogels had greater ($P < 0.05$) mean γ-IFN production to SRB51 only at 8 weeks after vaccination. Although not statistically different at any sampling time ($P > 0.05$), bison vaccinated with the PEG hydrogels tended to have the greatest mean γ-IFN production when compared to PEG co-glycolide and PEG co-lactide hydrogel formulations.

3.5. Nitric oxide production

Across all times, incubations, and treatments, incubation of PBMC with SRB51 antigen induced greater NO than paired wells containing cells incubated in the absence of antigen (data not shown). However, mean NO concentration in

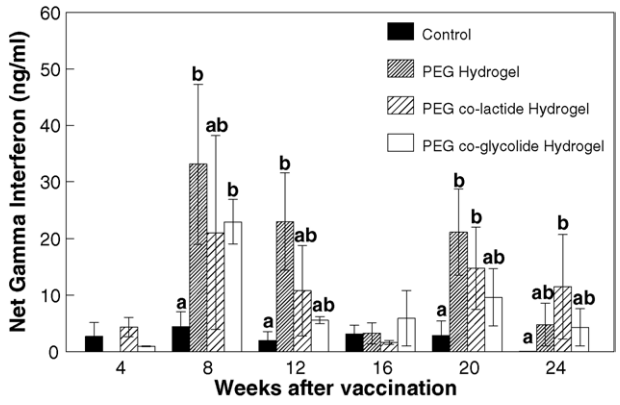

Fig. 6. Production of γ-IFN by peripheral blood mononuclear cells from bison inoculated with saline (control; $n = 5$) or ballistically vaccinated with hydrogel bullets. Ballistically vaccinated bison received 10^{10} CFU of SRB51 in three types of hydrogels (PEG; PEG co-lactide; or PEG co-glycolide; $n = 3$/trt) control or SRB51-vaccinated. Cells were incubated at 37 °C in 5% CO_2 for 72 in the absence of antigen, or with 10^8 CFU/well of γ-irradiated SRB51. Data is presented as mean net γ-IFN (wells with SRB51–wells without antigen) ± S.E.M. Means within a sampling time with different superscripts are statistically different ($P < 0.05$).

wells containing SRB51 antigen did not differ between control and vaccination treatments across various sampling times or incubations. In a similar manner, mean net nitric oxide production by PBMC to γ-irradiated SRB51 did not differ ($P > 0.05$) between control and vaccination treatments for all incubations at all sampling times (data not shown). When the three hydrogel treatments were compared, hydrogel formulation did not influence ($P > 0.05$) NO production by PBMC to SRB51 antigens at any sampling time.

4. Discussion

Previous studies have demonstrated that ballistic vaccination of bison with a bullet containing a compressed SRB51 pellet induces reduced immunologic responses when compared to parenteral delivery [10]. The results reported here suggest that encapsulation of SRB51 in hydrogels for delivery via ballistic bullet may enhance immunologic responses of bison when compared to responses of bison vaccinated with a bullet containing a compressed SRB51 pellet. This could be due to high post processing viability of the *Brucella* within photopolymerized hydrogel formulations, which is typically greater than 90% (data not shown). If increases in antigen-specific γ-IFN production correlate to increased protection against *Brucella*, our data suggest that vaccination of bison with SRB51 hydrogel bullets may producer greater efficacy than currently used bullets containing compressed vaccine pellets. However, unpublished data from our laboratory suggests that even though in vitro assays demonstrated reduced immunologic responses in bison vaccinated with bullets containing a compressed SRB51 pellet, data from paired efficacy experiments suggest that protection against *Brucella* is only slightly reduced when compared to parenteral vaccination (Olsen, unpublished data). Therefore, efficacy studies will have to be conducted before definitive conclusions can be made in regards to protective immunologic responses induced by ballistic vaccination of bison with SRB51 encapsulated within PEG hydrogels.

The PEG hydrogel formulations are designed to swell rapidly and then degrade by hydrolysis to release encapsulated contents by both swelling-controlled and degradation-controlled mechanisms at different rates upon hydration [11]. Previous work studying the release of 1 μm fluorescent beads as models for hydrogel-encapsulated sessile microorganisms shows that PEG-co-glycolide hydrogels release all contents over a period of 9 days, primarily by degradation controlled release, PEG-co-lactide hydrogels release all contents over a period of 45 days by the same mechanism, and PEG hydrogels lacking degradable linkers release less than 1% of encapsulated particles over 45 days, although smaller, soluble compounds are released much more rapidly [11]. These cases contrast differences in degradation-controlled versus swelling-controlled release from the gels upon swelling. While these experiments were performed in buffer alone, they should serve as an approximation of how gels will degrade in vivo in bison tissue sites: these hydrogels in ex vivo and in vivo implant studies in muscle tissue sites behave similarly to buffer studies (Christie, unpublished data). Other factors that could influence the actual release rate in a biobullet formulation in vivo include dissolution of the hydroxpropyl cellulose biobullet casing, and possible gel agitation and break up due to host muscle contractions.

Data obtained in this study indicate little correlation to immune responses based on the PEG hydrogel formulation used. It is possible that the high concentration of SRB51 (approximately 10^{11} CFU/bullet) used within PEG hydrogels for these experiments alters the controlled release behavior observed for 1 μm fluorescent particles (approximately 10^9 particles/bullet)[11]. Figs. 1, 3, and 5 represent all combination of all hydrogel formulations as one data point ($n = 9$). Nondegradable PEG biobullets showed comparable(or slightly higer) immune responses, suggesting factors other than hydrogel hydrolysis alone may contribute to release of SRB51 in vivo. Biobullet placement in the host may produce effects on hydrogel degradation and release SRB51 in vivo not readily duplicated in vitro. In addition, as the SRB51 vaccine contains live bacteria, sufficient release of *Brucella* may occur to allow tissue colonization and in vivo replication to stimulate detectable immunologic responses. It cannot be excluded that full release of the 10^{10} CFU of bacteria entrapped in the hydrogel was not required to induce significant immunologic responses.

Hydrogel formulations offer several advantages for preparation of ballistic bullets as compared to the previously used compressed SRB51 tablets. When lyophilized SRB51 is compressed into a pellet, we have noted viability losses of approximately 80% (Olsen, unpublished data). Current data suggest that loss of viability during preparation of SRB51 hydrogels are far less (<10%) as compared to preparation

of compressed pellets. An additional benefit of hydrogels is that the process is less labor intensive and appears to be more readily scalable for production of larger numbers of bullets. One disadvantage of these PEG hydrogel-based biobullets is their tendancy to soften the hydroxypropryl cellulose bullet casing over time, reducing the structural integrity and firing reliability of the biobullet. We are currently investigating the use of lyophilized PEG hydrogels as vaccine carriers for biobullets that can easily be prepared and stored for extended lengths of time.

We had expected a beneficial effect by co-administration of Spirovac™ with parenteral SRB51. If Spirovac™ induces cytokines associated with Th-1 immune responses [17], it was hypothesized that co-administration might also enhance protective Th-1 immune responses to SRB51. However, in general, our data suggests that co-administration of Spirovac had no effect on *Brucella*-specific immunologic responses. One explanation may be that brucellosis vaccination provides maximal stimulation of cell-mediated immune responses in bison. Others have found that heat killed *B. abortus* induces strong Th-1 responses in human monocytes and mice [18–20]. As we did not see a reduction in immune responses to SRB51 by co-administration of Spirovac™, the hypothesis of immune overload does not seem to be applicable. However, others have noted that combining foot and mouth disease and vesicular stomatitis live virus vaccines [21], or rinderpest and contagious bovine pleuropneumonia vaccines to cattle [22] reduced immune responses to a least one vaccine when compared to responses in cattle receiving only one vaccine. In the study using rinderpest and contagious bovine pleuropneumonia vaccines [22], serologic responses of cattle receiving both vaccines simultaneously did not differ from responses of cattle receiving single vaccinations. However, protection against bovine pleuropneumonia was reduced after experimental challenge in cattle vaccinated simulataneously with both vaccines as compared to cattle vaccinated with only the bovine pleuropneumonia vaccine.

Our data suggest that bison PBMC incubated with SRB51 induce greater NO production than paired wells incubated without antigen. This observation concurs with others who noted significant NO production when J774-A1 macrophage cells were infected with rough, but not smooth strains of *Brucella suis* or *Brucella melitensis* [23]. However, NO production in response to SRB51 appears to be an innate, rather than adaptive immune response, as it did not differ between SRB51-vaccinated and control bison. Although NO production may have diagnostic implications in regards to *M. bovis* infection or vaccination [12], our data suggests this assay has no diagnostic value for detecting SRB51-vaccinated bison.

In summary, our data suggests that hydrogel formulations of SRB51 may be a superior alternative to compressed SRB51 tablets for ballistic vaccination of bison. Although preliminary, data suggests that immunologic responses to SRB51 hydrogel bullets are similar to responses after parenteral vaccination with SRB51. At the present time, immunologic correlates of protective immunity against *Brucella* are not characterized in bison, and the limited data on induction of γ-IFN production by RB51 vaccination of bison has not been correlated to protection against experimental challenge with *B. abortus*. Therefore, additional studies evaluating efficacy will be required before the influence of SRB51 hydrogel biobullets on protective immunity against brucellosis can be definitely determined.

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

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