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ENHANCED IMMUNE RESPONSE OF RED DEER (*CERVUS ELAPHUS*) TO LIVE RB51 VACCINE STRAIN USING COMPOSITE MICROSPHERES

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ABSTRACT: Brucellosis is an important zoonotic disease of nearly worldwide distribution. The occurrence of the infection in humans is largely dependent on the prevalence of brucellosis in animal reservoirs, including wildlife. The current vaccine used for cattle *Brucella abortus* strain RB51, has proven ineffective in protecting bison (*Bison bison*) and elk (*Cervus nelsoni*) from infection and abortion. To test possible improvements in vaccine efficacy, a novel approach of immunization was examined from April 2004 to November 2006 using alginate composite microspheres containing a nonimmunogenic, eggshell-precursor protein of the parasite *Fasciola hepatica* (Vitelline protein B, VpB) to deliver live vaccine strain RB51. Red deer (*Cervus elaphus*), used as a model for elk, were vaccinated orally (PO) or subcutaneously (SC) with 1.5×10^{10} viable organisms per animal. Humoral responses postvaccination (immunoglobulin G [IgG] levels), assessed at different time points, indicated that capsules containing live RB51 elicited an anti-*Brucella* specific IgG response. Furthermore, the encapsulated vaccine elicited a cell-mediated response that the nonencapsulated vaccinates failed to produce. Finally, red deer were challenged with *B. abortus* strain 19 by conjunctival exposure. Only animals that received encapsulated RB51 vaccine by either route exhibited a significant reduction in bacterial counts in their spleens. These data suggest that alginate-VpB microspheres provide a method to enhance the RB51 vaccine performance in elk.

Key words: Brucella, elk, microencapsulation, vaccines, Vitelline protein B.

INTRODUCTION

Brucella abortus is a facultative, intracellular, gram-negative, bacterial pathogen and the etiologic agent of brucellosis, an important zoonoses with a nearly worldwide distribution (Boschiroli et al., 2001). Human brucellosis, a debilitating disease characterized by fluctuating fever, is caused mainly by contamination from infected ruminants or consumption of contaminated animal products. Moreover, the disease is a major cause of direct economic losses (Corbel, 1997) and a barrier for international trade of live animals. *Brucella* spp. are also considered a class III pathogen and classified as potential bioterrorist agents.

Brucellosis eradication programs in North America have been successful in controlling the pathogen in domestic livestock but not in wildlife populations (Ragan, 2002). Currently, elk (*Cervus elaphus nelsoni*) and bison (*Bison bison*) are the wildlife reservoirs of *B. abortus* in the Greater Yellowstone area, and transmission from wildlife to cattle has occurred (Thorne, 1980).

Effective vaccines to control brucellosis in wildlife are not currently available. Commercially available vaccine strains used for brucellosis eradication in cattle have been tested in wildlife species (Davis and Elzer, 2002), but results from elk vaccination trials have shown that efficacy is reduced in comparison to cattle. Additional vaccination-related problems include interference with diagnosis (Schurig et al., 2002), resistance to antibiotics, and potential virulence for animals and humans (Berkelman, 2003; Ashford et al., 2004). The *B. abortus* strain 19 (S19) appeared to be safe in adult elk but has been shown to reduce abortion rates only by 30% (Thorne et al., 1981). The S19 vaccine also does not cause morbidity or

mortality in pronghorn antelope (*Antilocapra americana*; Elzer et al., 2002), bison (Davis et al., 1991) or coyotes (*Canis latrans*). Another *Brucella* vaccine, SRB51, has been shown to be safe in a wider range of nontarget species, including ravens (*Corvus corax*), Richardson ground squirrels (*Spermophilus richardsonii*), and deer mice (*Peromyscus maniculatus*; Januszewski et al., 2001); bighorn sheep (*Ovis canadensis*), pronghorn antelope, mule deer (*Odocoileus hemionus*), and moose (*Alces alces shirasi*; Kreeger et al., 2002b); and black bears (*Ursus americanus*; Olsen et al., 2004). However, if administered parenterally, SRB51 did not protect against abortion in elk (Cook et al., 2002; Kreeger et al., 2002a).

The distribution of the disease appears to be correlated with high animal densities associated with winter feeding (Etter and Drew, 2006). Infected and susceptible elk commingling on feed grounds ensure exposure of animals to *B. abortus*, enhancing the probability of transmission. Control of brucellosis should be focused on these sites to prevent or reduce exposure of the pathogen to naïve animals, thus breaking the chain of transmission. Difficulties with integrating *Brucella* vaccination strategies into control efforts have been associated not only with the low efficacy of S19 and RB51 in elk but also with the delivery method used to immunize the animals. Currently, elk vaccination uses a S19 biobullet ballistic approach, and problems arising from this methodology include excessive time and labor, logistics, and high cost.

During April 2004 to November 2006, we evaluated the potential for delivering a live RB51 vaccine to elk via a controlled microencapsulated release vehicle. The capsule was made of alginate, a naturally occurring biopolymer that offers the advantages of biocompatibility, low toxicity, and encapsulation conditions that are compatible with live organisms (Wee and Gombotz, 1998). In an attempt to enhance the efficacy of the capsule, we also incorporated a novel protein from the eggshell precursor of the parasite *Fasciola hepatica*, Vitelline protein B (VpB). This recombinant 31-kDa protein possesses an unusual resistance to proteolytic breakdown (Rice-Ficht et al., 1992), which may reduce erosion time and release of the capsule content. To further explore the alternatives of using this method, PO delivery of the microencapsulated vaccine was also investigated, principally because this is the most cost-effective way to deliver a vaccine in wildlife populations.

MATERIALS AND METHODS

Animals

Fifty-four 1–2-yr-old red deer (*Cervus elaphus elaphus*) females from a privately owned tuberculosis-free and brucellosis-free commercial herd were used as an animal model for Rocky Mountain elk (Cervus *elaphus nelsoni*) because of their close genetic relationship. Upon arrival, animals were retested for specific anti-*Brucella* immunoglobulin G (IgG) levels (total IgG) by enzyme-linked immunosorbent assay (ELISA) and were dewormed (moxidectin, Cydectin; Wyeth, Madison, New Jersey, USA). Deer were acclimated for 3 mo before vaccination. All animal care and experimental procedures were performed in compliance with the institutional animal-care protocol.

Bacterial strains

Bacterial strains used in these experiments included the vaccine strains SRB51 and S19. Bacteria were grown on tryptic soy agar (TSA; Difco, BD, Sparks, Maryland, USA) at 37 C with 5% CO_2. Three days postincubation, SRB51 plates were harvested and bacteria were pelleted and standardized for subcutaneous or PO vaccination at a dose of 1.5×10^{10}, whether encapsulated or nonencapsulated. For animal challenge, a dose of 1×10^{9} of vaccine S19 was standardized using a klett meter and plating onto TSA plates retrospectively to confirm the dose.

Preparation of *B. abortus* SRB51 antigen-loaded microspheres

Alginate beads, loaded with 1.5×10^{10} colony-forming units (cfu)/ml of the vaccine SRB51, were prepared as previously described (Abraham et al., 1996) with some modifications. Briefly, enumerated, live SRB51 vaccine

strain (total 1.5×10^{11} for 10 doses) was resuspended in a total of 100 µl of 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (10mM MOPS, 0.85% NaCl, pH 7.4) and mixed with 10 ml of alginate solution (1.5% sodium alginate, 10mM MOPS, 0.85% NaCl, pH 7.3). Spheres (300 µm) were obtained by extruding the suspension through a 200-µm nozzle into a 100 mM calcium chloride solution that was stirred for 15 min using an Inotech encapsulator I-50 (Inotech Biosystems International, Rockville, Maryland, USA). For a permanent cross-linking of the capsule, microspheres were washed three times with 200 ml of MOPS buffer for 10 min and further stirred in a solution containing 0.05% poly-L-lysine (molecular weight = 22,000) for 15 min. Following two successive washes, the beads were stirred in a solution of 0.03% alginate for 5 min to apply a final outer coating. All capsules were stored at 4 C in MOPS buffer until use. To determine the number of bacteria per 1 ml of capsules, spheres were removed from the encapsulator before the permanent cross-linking and were washed three times with 50 ml of MOPS buffer, and particles were dissolved using 10 ml of depolymerization solution (50mM sodium citrate, 0.45% NaCl 10mM MOPS, pH 7.2). Enumeration of bacteria was determined by plating onto TSA plates.

The addition of VpB as a component of the alginate core was achieved by the addition of 1 mg of VpB to the bacteria-alginate suspension described above. Extrusion and capsule formation used the same preparation conditions.

Immunization of red deer

Fifty-four 1–2-yr-old, female red deer were randomly distributed into six different treatments ($n=9$/group). Three groups were inoculated subcutaneously with a total dose of 1.5×10^{10} cfu of either nonencapsulated SRB51, encapsulated SRB51 with alginate, or encapsulated RB51 with alginate and VpB. Two groups were vaccinated by the PO route by squirting the vaccine into their mouth; one group received 1.5×10^{10} cfu of encapsulated SRB51 with alginate, and the second group received with encapsulated RB51 with alginate and VpB. The control group received a subcutaneous injection of 1 ml of empty capsules (no bacteria entrapped). A single vaccination dose was given to all animals.

Detection of *Brucella*-specific antibody levels.

To determine anti-*Brucella*–specific antibody in serum, blood samples were collected by jugular venipuncture immediately before vaccination and 6, 12, 17, and 28 wk postvaccination. Serum samples were analyzed for anti-*Brucella* IgG (total IgG) determination by ELISA. Heat-killed SRB51 cell antigen was used to coat 96-well plates (Nunc-Immuno plates, high binding protein; Thermo Fisher Scientific, Rochester, New York, USA) at a concentration of 25 µg/well. After overnight incubation at 4 C, plates were washed using phosphate-buffered saline (PBS) containing 0.05% Tween-20, blocked (0.25% w/v bovine serum albumin), and incubated with the diluted deer-serum samples (1:100 in blocking buffer) for 2 hr. Following three more washes, goat anti-deer IgG horseradish peroxidase (KPL Systems, Silver Spring, Maryland, USA) conjugate was added at a dilution of 1:1,000 and incubated at room temperature for 1 hr. After incubation, plates were washed, and *o*-phenylenediamine dihydrochloride peroxidase substrate (Sigma-Aldrich, St. Louis, Missouri, USA) was added following manufacturer's instructions for 20 min. The reaction was stopped by the addition of 50 µl of 0.5M NaOH. The absorbance was measured at 450 nm (A_{450}). All assays were performed in triplicate and repeated at least two times.

Lymphocyte proliferation assay from peripheral blood mononuclear cells

At 12 wk postvaccination, mononuclear cells were isolated from peripheral-blood buffy coats as previously described (Waters et al. 2002) with some modifications. Briefly, 2×10^5 cells/well were seeded in 96-well plates (Falcon, Becton Dickinson, San Jose, California, USA) in RPMI medium containing 10% (v/v) fetal bovine serum, 1mM L-glutamine, and 1mM nonessential amino acids. Cells were stimulated, with *B. abortus* wild-type S2308 lysate at a concentration of 12.5 µg/ml, concanavalin A (5 µg/ml), or medium alone, and were incubated for 6 days at 37 C with 5% CO_2. After this incubation period, 1 µCi of methyl-[^{3}H] thymine was added to each well. Following 18 hr of incubation, cells were harvested onto fiber filters using a 96-well plate cell harvester, and the incorporated radioactivity was measured by liquid-scintillation counting. Lymphocyte proliferation data is represented as mean counts per minute (cpm) ± standard deviation.

Host response to subsequent *B. abortus* S19 exposure

At 7 mo postvaccination, three to four animals from each vaccination group (except RB51/alginate SC; $n=2$) were exposed con-

junctivally, as previously described (Kreeger et al., 2000), using a challenge dose of 1×10^9 cfu/deer of *B. abortus* S19. Dose exposure was confirmed by serial dilutions and plating onto TSA plates. At 2 wk post-challenge, animals were euthanized, and spleens were harvested, weighed and homogenized. For homogenization, 1 ml of peptone saline was added to 1 g of tissue. Each sample was treated for 5–10 min using a stomacher. From each sample, 100 µl was plated onto Farrell's media (Oxoid LTD, Basingstoke, Hampshire, England) in duplicate. At 3–5 days postincubation, bacteria were enumerated. Results are represented as the mean cfu/g of tissue±standard error of the mean (SEM).

Statistical procedures

Anti-*Brucella* IgG levels elicited from vaccination were expressed as the mean absorbance at 450±SD for each group. For lymphocyte proliferation, the cpm from each group were expressed as the mean cpm±SD. Bacterial load from S19 challenge was expressed as mean log cfu±SEM for each group. The significance of differences between groups was determined by analysis of variance (ANOVA); a P value <0.05 was considered statistically significant.

RESULTS

Encapsulation of *B. abortus* SRB51 in alginate microspheres

Two different capsular formulations were prepared using the same alginate base. Variation of the formulation included the addition of VpB within the capsule to modify the degradation kinetics and release of the organism. When capsules were analyzed using light microscopy, all of the capsule formulations appeared spherical and uniform with a mean diameter of 310 µm (Fig. 1). Furthermore, bacterial viability following encapsulation exceeded 95%, as demonstrated by recovery of the organism following dissolution of the capsules (data not shown).

Cellular immune response

At 12 wk postvaccination, animals that received the encapsulated vaccine with VpB in the formulation (regardless of the immunization route) were the only individuals that had a statistically significant proliferative response compared with the controls ($P<0.0005$ PO vaccinates, $P<0.005$ SC group; Fig. 2). Interestingly, the cpm counts in animals that received encapsulated RB51 with VpB PO were also higher than in deer that received the same formulation via SC ($P<0.3$). None of the animals that received nonencapsulated vaccine had a significant cellular response compared with naïve nonvaccinated animals.

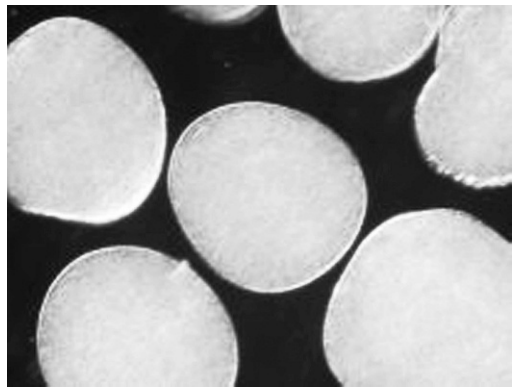

FIGURE 1. Light microscopy image of alginate-vitelline protein B (VpB) microspheres loaded with the vaccine strain SRB51. *Brucella abortus* SRB51 was encapsulated into alginate microspheres.

Anti-*Brucella* IgG response

Immunization with RB51 elicited an anti-*Brucella* IgG response that was clearly detectable by 6 wk postvaccination (Fig. 3). During the initial 17 wk, anti-*Brucella* IgG levels were higher in animals that received the injected vaccine compared with the groups that were immunized PO ($P<0.05$). Between 17 to 28 wk, anti-*Brucella* IgG levels in animals that were PO-vaccinated had an increase in anti-*Brucella* IgG compared with deer SC-vaccinated.

Host response against challenge by conjunctival S19 exposure

The host protective response was determined by subtracting the mean cfu of

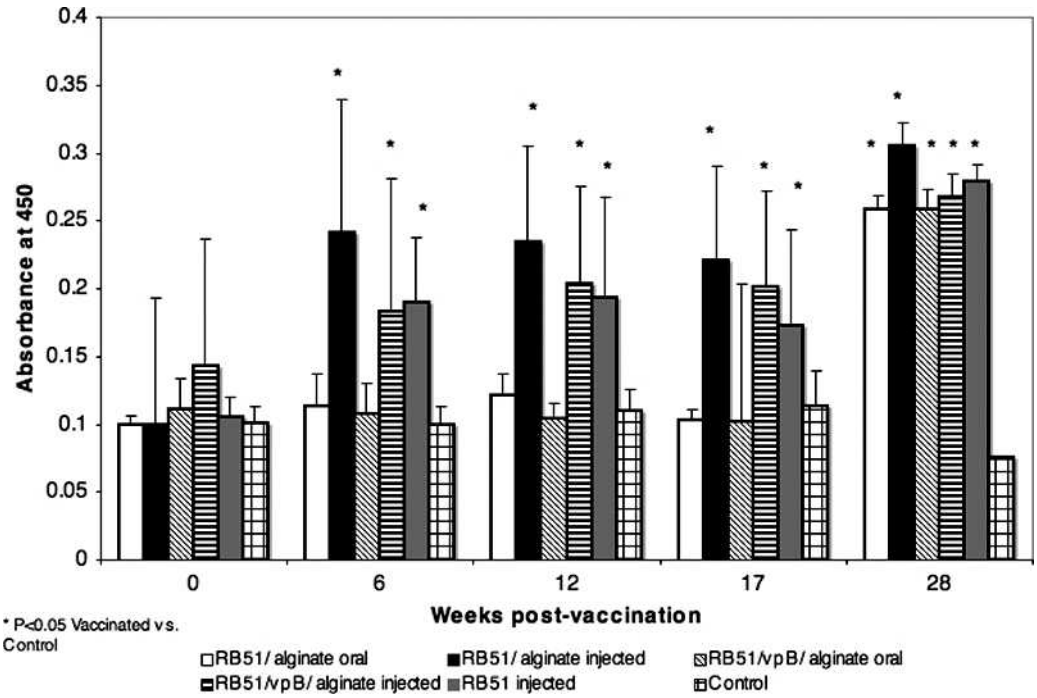

FIGURE 2. Immunoglobulin G (IgG) anti-*Brucella* antibodies in serum from deer vaccinated with SRB51. Red deer (n=9/group) were inoculated subcutaneously (SC) with 1.5×10^{10} colony-forming units (cfu) of either nonencapsulated SRB51, encapsulated SRB51, or encapsulated SRB51 with vitelline protein B (VpB). Control group received empty capsules. Two groups received encapsulated oral (PO) vaccine (SRB51 with alginate or SRB51 with VpB) at the same dose. At 0, 6, 12, 17, and 28 wk postvaccination, serum samples were collected and analyzed for anti-*Brucella* IgG determination by enzyme-linked immunosorbent assay (ELISA). Results are shown as the means±standard deviations of absorbance at 450 nm.

S19 recovered per gram of spleen from deer vaccinated with the nonencapsulated or encapsulated vaccine from the mean cfu per gram recovered from naïve nonvaccinated but infected deer. At 2 wk postchallenge, only animals that received encapsulated SRB51 with VpB had a significant decrease in bacterial load in the spleen (Fig. 4). Red deer that received the vaccine PO were the only group that was statistically significant compared with the nonencapsulated, injected SRB51 ($P<0.04$). Animals that were PO-immunized with the VpB capsules had a 1.27 log reduction in spleen counts compared with animals vaccinated with nonencapsulated SRB51 and a 1.68 log reduction compared with naïve, nonvaccinated, but S19 exposed, animals. *Brucella abortus* strain 19 spleen counts in deer that received the VpB capsules via SC were also diminished by 1.21 log compared with the nonencapsulated RB51 and by 1.62 log compared with non-SRB51 vaccinated controls ($P<0.2$).

DISCUSSION

The ultimate goals of vaccination are to control disease and reduce or eliminate transmission from reservoir species. To accomplish these goals in elk using current *Brucella* vaccines, the development of more efficacious vaccination mechanisms are needed to enhance vaccine efficacy.

Recent data indicate that the manner in which antigen reaches the lymph organs and how it is delivered to the antigen-presenting cells are fundamental in the induction of an optimal immune response. There is experimental evidence to support the observation that microencapsulation

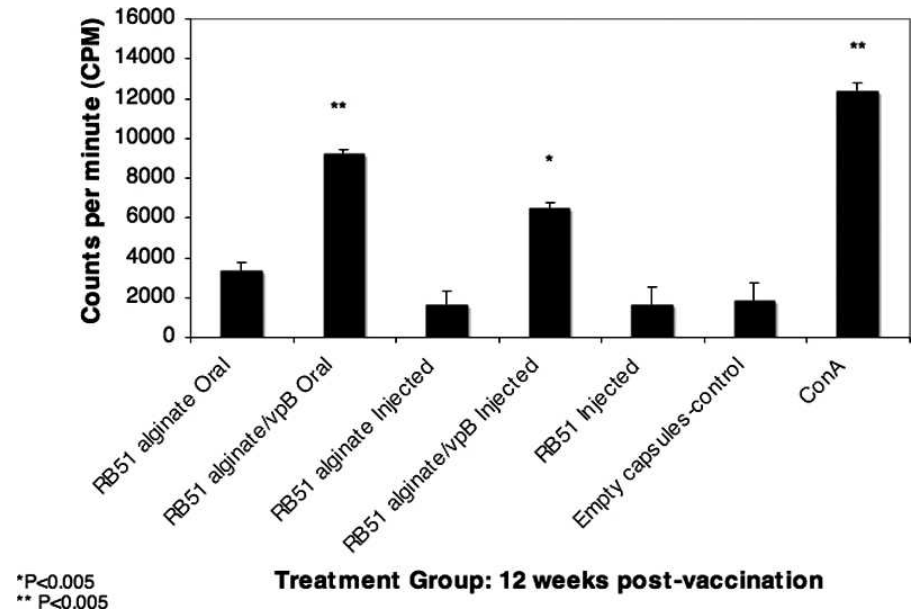

FIGURE 3. Lymphocyte-proliferative responses of peripheral blood mononuclear cells from deer immunized with RB51. Red deer ($n=9$/group) were vaccinated subcutaneously (SC) with 1.5×10^{10} colony-forming units (cfu) of either nonencapsulated SRB51, encapsulated SRB51 (SRB51 with alginate), or encapsulated SRB51 with vitelline protein B (VpB). Two groups received encapsulated, oral (PO) vaccine (SRB51 with alginate or SRB51 with VpB) at the same dose. Control group received empty capsules. Results are expressed as mean counts per minute (cpm)±standard deviation (SD). * $P<0.005$ are statistically different from the control by analysis of variance (ANOVA).

serves to modify the uptake and processing of antigen (Eyles et al., 2001; Sun et al., 2003). Also, it has been suggested that prolonged persistence of the vaccine strain in the host is needed for the development of a suitable anti-*Brucella* immunity (Kahl-McDonagh and Ficht, 2006). In an effort to develop a more efficient way to present the current vaccine SRB51 to the lymphoid tissue and to increase the exposure time of the organism to the host cells, we developed a controlled-release strategy in which SRB51 was encapsulated into alginate-VpB composite microspheres. We were successfully able to entrap SRB51 and develop uniform spherical batches of capsules, even when VpB was added to the formulation. By adding this component to the capsules, we modified the efficiency of the capsule as demonstrated by the difference in cellular and humoral responses observed in animals that received this formulation.

Live vaccines are more efficacious than vaccination with heat-killed organisms or cellular extracts and provide a significant level of immunity for protection against brucellosis (Zhan et al., 1995; Schurig et al., 2002). During microsphere formulation, SRB51 was exposed to relatively mild conditions that preserved bacterial viability (95%). This is in contrast to many standard encapsulation procedures, which employ harsh conditions to affect polymerization, including direct exposure of the bacteria to organic solvents, shear stress, and ultrasound homogenization (Lima and Rodrigues, 1999).

Oral bait administration of vaccines is the most practical and cost-effective method to vaccinate wildlife, and successful techniques and strategies for PO immunization of foxes (*Vulpes vulpes*) against rabies have been extensively demonstrated (Schneider, 1995; Pastoret and Brochier, 1996). Many pathogens, includ-

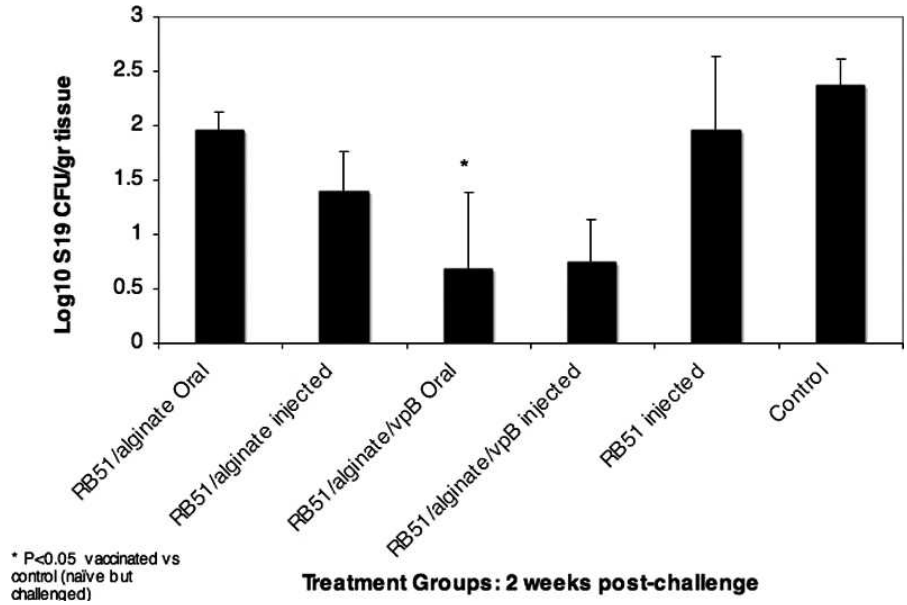

FIGURE 4. Host response against S19 challenge. Red deer received either oral (PO) or subcutaneous (SC) vaccine, and 7 mo postvaccination animals were conjunctivally infected with 1×10^9 colony-forming units (cfu) of S19. At 2 wk postinfection, animals were euthanized, and spleens were harvested. Values are reported as the mean $\log_{10}$ recovery of S19 from spleens. Difference in colonization between the vaccinated and control animals was determined by analysis of variance (ANOVA). * $P<0.05$.

ing *Brucella* spp, access the body via the mucosal surfaces; neutralization of the microorganism at the mucosal site would be an ideal situation to prevent brucellae from infecting the host. Our data indicate that increased efficacy with current *Brucella* vaccine strains can be achieved if the antigens are presented PO in a controlled-release format. The composite microspheres may serve to enhance the viability of bacteria in the ruminant digestive tract while providing immunization in a controlled-release format. The capsule might also provide a vaccine package that could be combined with baits for easy delivery.

Humoral immunity was assessed within all the SRB51 vaccine formulations and routes of vaccination. Immunization with SRB51 induced elevated anti-*Brucella* IgG levels (total IgG); however, levels were lower in deer vaccinated with the nonencapsulated vaccine than those induced by the microcapsules. In PO vaccinates, increasing levels of IgG between 17 to 28 wk were observed, which may reflect the protective benefits of the capsule for the live vaccine during exposure to conditions in the digestive tract. It is important to mention that all groups except controls, had increased levels of anti-*Brucella* IgG. Only the PO RB51 vaccinated groups had statistically significant increase compared with deer vaccinated via SC ($P<0.05$). The overall increases observed in all groups may have been associated with the seasonal hormonal cycles in deer.

Induction of specific cell-mediated immune responses following immunization is a hallmark for the establishment of a protective immune response. In elk, both cellular and humoral responses might be needed to generate a strong immunity toward *Brucella* infections (Kreeger et al., 2002a). Our results with the encapsulated SRB51 indicated that by 12 wk alginate/VpB capsules administered SC or PO stimulated a statistically significant higher cellular response compared with nonencapsulated SRB51. These data suggest that by incorporating the SRB51 vaccine

into a delivery vehicle, the necessary conditions needed to trigger a protective cellular response are created. Moreover, a low cellular response elicited by nonencapsulated RB51 was observed, corroborating the results previously reported by other researchers (Cook et al., 2002; Kreeger et al., 2002a).

After the initial cellular and humoral responses were assessed, three or four animals from each group were challenged with S19 to determine the degree of protection conferred by the encapsulated vaccine to subsequent *Brucella* exposure. This strain was used because it has been previously shown that S19, by itself, is able to cause prolonged infection in deer and can be cultured from the spleen by 2 wk postinoculation. Using a challenge dose of 1×10^9 cfu, a significantly ($P<0.04$) lower infection rate was observed in animals that were immunized with the encapsulated vaccine. This was especially true for deer that received VpB in the formulations. In the case of PO vaccination, results not only corroborate the observed cellular and humoral responses but also support our idea that the capsule serves as a vehicle necessary for proper immunogenicity. The fact that reduction of infection was afforded to such a degree via PO delivery is highly promising and relevant to the current needs for a practical vaccination strategy. Further investigations with a higher number of animals, and actual challenge with wild-type organisms, are still needed.

In summary, our findings indicate that alginate-VpB encapsulation of live *Brucella* might be used not only to enhance vaccine efficacy in elk but also to provide a practical means of vaccination. Enhanced immune responses were observed in red deer that were vaccinated with encapsulated formulations, especially containing the VpB additive. Oral vaccination with VpB encapsulated formulations was able to invoke both humoral- and cell-mediated responses in red deer and to offer protection from a challenge of live S19, as evidenced by organism recovery from the spleen. These data support the hypothesis that an enhanced and prolonged host response because of a mucosal immune stimulation can be achieved via PO vaccination. Finally, the results observed using nonencapsulated RB51 were similar to those obtained in previous studies (Olsen et al., 2002) in which RB51, by itself, is not sufficient to induce a good cellular response or reduce infection in elk (Kreeger et al., 2002a) to a significant degree.

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