Vaccination with the ospA- and ospB-Negative *Borrelia burgdorferi* Strain 50772 Provides Significant Protection against Canine Lyme Disease

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Beagles received placebo or ospA- and ospB-negative *Borrelia burgdorferi* before a tick challenge. A total of 28 (41%) ticks and skin biopsy specimens from each control dog (n = 10) contained *B. burgdorferi*. In contrast, 12 (19%) ticks recovered from the vaccine recipients (n = 10) were infected (P = 0.0077), and 5 dogs yielded spirochetes from the skin biopsy specimens (P = 0.0325). In addition, 9 (90%) placebo recipients and 4 (40%) vaccine recipients developed joint abnormalities (P = 0.0573). Therefore, vaccination with the ospA- and ospB-negative spirochete provided significant protection against Lyme disease.

Lyme disease, due primarily in the United States to the transmission of *Borrelia burgdorferi* from infected *Ixodes* ticks, causes significant morbidity in canines. However, infected dogs rarely develop acute illness (1); instead, the illness manifests as chronic subclinical polyarthritis and/or periarteritis (2, 3) that may progress to frank arthritis (4, 5). In addition, Labrador retrievers, golden retrievers, and Shetland sheepdogs appear more susceptible to kidney nephropathy (6).

Canine vaccines that provide protection by inducing anti-Ospa borreliacidal antibodies to kill *B. burgdorferi* in the midgut as the infected tick ingests blood (7, 8) have been commercially available for several decades, and the approach has been partially effective (9, 10). However, vaccinated dogs may still become infected, because the expression of OspA is downregulated immediately after the infected tick begins taking a blood meal (11), and the ticks may also transmit ospA-negative Lyme spirochetes (12).

In addition, borreliacidal antibodies specific for Ospa are genus-specific (13, 14), which is less problematic in the United States, where *B. burgdorferi* predominates, but significantly impacts efficacy in Europe and Asia, where other genospecies, such as *Borrelia garinii*, may infect canines (15, 16).

Researchers therefore sought to overcome the shortcomings of the traditional vaccines by developing a bivalent bacterin comprising a traditional OspA-expressing *B. burgdorferi* strain and a unique ospA- and ospB-negative *B. burgdorferi* strain that expressed high levels of OspC. Subsequent studies (17, 18) confirmed that the approach provided a high level of protection against canine Lyme disease for at least 1 year. In addition, the investigators demonstrated that the bacterin induced significant amounts of anti-OspC borreliacidal antibodies (17) and postulated that the high level of protection was likely due at least in part to the inclusion of the OspC-producing spirochete. We therefore examined this possibility more critically by evaluating the protection afforded by vaccination with only the ospA- and ospB-negative *B. burgdorferi* strain.

Vaccination and tick challenge. Two groups (n = 10 in each group) of 8-week-old laboratory-reared beagle puppies were randomized without regard to sex, vaccinated by subcutaneous injection with a 1-ml dose of bacterin or placebo, and Boostered after 21 days by subcutaneous injection with an additional 1-ml dose. The bacterin was prepared by growing the ospA- and ospB-nega-

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dogs were housed in individual cages during the tick challenge but housed communally in groups of 4 to 6 thereafter.

**Borreliacidal antibody responses after vaccination.** Blood samples were collected immediately prior to the tick challenge (7 days after booster) and tested for anti-OspA or anti-OspC borreliacidal antibodies, as described previously (17, 21). Briefly, 5 × 10^5 low-passage-number *B. burgdorferi* strains S-1-10 (OspA) or 50772 (OspC) were combined with serum and guinea pig complement, and the suspension was incubated at 35°C for 16 to 24 h. Following incubation, 100 μl of each assay suspension was combined with phosphate-buffered saline (PBS) and acridine orange, and the spirochetes were monitored for killing by using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). The borreliacidal antibodies were detected indirectly by monitoring the increased fluorescence intensity that occurs when the acridine orange intercalates into blebbled, nonmotile spirochetes. A ≥13% shift in the mean fluorescence intensity compared to a normal serum control was considered positive (21). The presence of blebbled, nonmotile *B. burgdorferi* was confirmed by dark-field microscopy. A positive control was also included, and serum samples from individual animals were assayed concurrently.

Serum samples from the placebo-vaccinated controls did not contain detectable levels of borreliacidal antibodies (titer <1:80). In addition, the immune serum samples from the vaccine recipients did not contain anti-OspA borreliacidal antibodies that could be detected by using *B. burgdorferi* S-1-10. However, each serum sample contained high levels of borreliacidal activity (titer range, 1:640 to 1:20,480) that could be detected by using *B. burgdorferi* 50772. Moreover, passing 1-ml volumes of immune serum samples from four vaccine recipients over a column that contained recombinant OspC bound to Tetralink tetrameric avidin resin (Promega, Madison, WI) reduced the borreliacidal activity in each serum to <1:80. Additionally, significant levels (titer range, 1:320 to 1:1,280) of borreliacidal activity also remained detectable even after repeated adsorption with OspC. Therefore, the findings corroborated previous reports (17, 18) that vaccination with the spirochetes reliably induced significant amounts of anti-OspC borreliacidal antibodies and also showed that the bacterin induced borreliacidal antibodies specific for other unknown antigens expressed by *B. burgdorferi* 50772.

**Ability of vaccination to eliminate spirochetes from feeding ticks.** After the tick challenge, the midguts of the ticks recovered from the dogs were examined for spirochetes. Each midgut was fixed on a glass slide, overlaid with *B. burgdorferi*-specific rabbit polyclonal antibodies diluted 1:500 in PBS (pH 7.2), and incubated for 30 min at 37°C. After the slide was washed, fluorescein isothiocyanate-labeled IgG antibodies (Sigma-Aldrich, St. Louis, MO) diluted 1:200 in PBS were added, and the slide was reincubated for 30 min at 37°C. The slide was then washed and masked and examined by dark-field microscopy. The midguts from 28 (41%) of 68 ticks from the bacterin recipients contained *B. burgdorferi*; and at least 1 positive tick was recovered from each (100%) dog (Table 1). In contrast, spirochetes were detected in only 12 (19%) of 63 engorged ticks from the placebo recipients contained *B. burgdorferi*; and at least 1 positive tick was recovered from each (100%) dog (Table 1). In contrast, spirochetes were detected in only 12 (19%) of 63 engorged ticks from the bacterin recipients (*P* = 0.0077, Fischer’s exact test), but positive ticks were still recovered from 6 (60%) vaccinated dogs (*P* = 0.0867).

**Ability of vaccination to prevent colonization of skin.** At approximately 1, 2, and 4 months postchallenge, skin biopsy specimens were collected from each dog from areas adjacent to the tick bite sites. The skin was anesthetized with 0.5 ml of lidocaine (2%), and a biopsy specimen was removed with a disposable 4-mm dermal punch (Miltex, Inc., York, PA). Each skin biopsy specimen was then removed from the punch with sterile forceps and placed in a tube that contained BSK medium supplemented with gelatin (20%), rifampin (40 μg/ml), and kanamycin (8 μg/ml). The cultures were then incubated at 35°C and examined weekly by dark-field microscopy for 4 weeks. The ability of the BSK medium to support growth from an inoculum of one organism (19) was confirmed prior to culture. *B. burgdorferi* was recovered from at least one skin biopsy specimen from each (100%) placebo recipient. In contrast, skin biopsy specimens from 5 (50%) bacterin recipients yielded spirochetes (*P* = 0.0325).

**Ability of vaccination to prevent joint abnormalities.** The dogs were also observed daily for 8 months after the tick challenge for Lyme disease-related joint stiffness or lameness (1). To exacerbate the progression of disease, dexamethasone (Amtech, St. Joseph, MO) was injected intramuscularly at a dosage of 0.4 mg/lb of body weight for 5 consecutive days beginning at week 19 after the challenge (17, 18). At necropsy, samples of joint tissues were cultured by removing approximately 1-cm³ sections of the capsules from the left stifle, tarsus, elbow, and carpus. Half of the tissue sample was combined with 9 ml of BSK medium in a sterile bag and homogenized by passage through a laboratory blender (Stomacher 80; Seward Medical, London, United Kingdom). One milliliter of the suspension was then transferred to 9 ml of fresh BSK, and the culture was incubated at 35°C and examined weekly for 4 weeks by dark-field microscopy. The remaining sample was placed into 10% formalin, processed using routine methods for histopathology studies, stained with hematoxylin-eosin, and examined for cellular infiltrates and tissue damage caused by infec-

| Group and dog no. | Spirochetes in ticks | B. burgdorferi from skin* | Lameness | Synovitis | B. burgdorferi from joints |
|------------------|----------------------|--------------------------|----------|----------|--------------------------|
| Placebo          | 1                    | +                        | –        | +        | +                        |
|                  | 2                    | +                        | +        | +        | +                        |
|                  | 3                    | +                        | –        | +        | +                        |
|                  | 4                    | +                        | –        | –        | –                        |
|                  | 5                    | +                        | –        | +        | –                        |
|                  | 6                    | +                        | –        | –        | +                        |
|                  | 7                    | +                        | –        | –        | –                        |
|                  | 8                    | +                        | +        | +        | +                        |
|                  | 9                    | +                        | –        | +        | –                        |
|                  | 10                   | +                        | +        | +        | +                        |
| Vaccine          | 1                    | –                        | –        | –        | –                        |
|                  | 2                    | –                        | –        | –        | –                        |
|                  | 3                    | –                        | –        | –        | –                        |
|                  | 4                    | –                        | –        | –        | –                        |
|                  | 5                    | –                        | –        | –        | –                        |
|                  | 6                    | –                        | –        | –        | –                        |
|                  | 7                    | –                        | –        | –        | –                        |
|                  | 8                    | –                        | –        | –        | –                        |
|                  | 9                    | –                        | –        | –        | –                        |
|                  | 10                   | –                        | –        | –        | –                        |

* Spirochetes recovered at 1-, 2-, and/or 6-month biopsy time points.
tion with *B. burgdorferi* (2, 17, 18). After immunosuppression of the dogs, 9 (70%) placebo recipients developed one or more joint abnormalities, which included recovery of *B. burgdorferi* from the joints of 7 (70%) dogs, synovitis in the joint tissues from 8 (80%) dogs, and lameness in 3 (30%) dogs (Table 1). In contrast, joint abnormalities were detected in 4 vaccine recipients (*P* = 0.0573), which included spirochetes from the joints of 4 dogs (*P* = 0.3698), 2 joint-positive dogs with synovitis (*P* = 0.023), and one joint-positive dog that also developed lameness (*P* = 0.582).

**Conclusions.** Most canine Lyme disease vaccines provide less-than-ideal protection, because they induce only anti-OspA borreliacidal antibodies that kill *B. burgdorferi* in the midgut as the infected tick takes a blood meal (7, 8). However, we showed previously (17, 18) that a bivalent bacterin comprised of an OspA-expressing *B. burgdorferi* strain and the ospA- and ospB-negative strain 50772 used in this study provided complete protection from canine Lyme disease for up to 1 year. A shortcoming of the previous studies, however, was a lack of confirmation that the ospA- and ospB-negative isolate contributed significantly to the high level of protection. Therefore, we examined the ability of vaccination with only the ospA- and ospB-negative *B. burgdorferi* strain 50772 to provide protection from infection and clinical disease.

The results demonstrated that vaccination with only *B. burgdorferi* 50772 also provided protection against canine Lyme disease. Specifically, significantly (*P* = 0.0077) fewer numbers of ticks that fed on the vaccinees were infected with spirochetes, which then apparently resulted in significantly decreased transmission of spirochetes to the skin (*P* = 0.0325). In addition, Lyme disease-associated joint abnormalities were decreased to a level that was just short of significance (*P* = 0.0573). Therefore, vaccination with *B. burgdorferi* 50772 did not provide complete protection against infection, but the findings confirmed that the immune response contributed significantly to the complete protection observed in previous studies after vaccination with a combination of a traditional OspA-expressing *B. burgdorferi* and the 50772 strain (17, 18).

In addition, our findings support a previous report (17) that vaccination with *B. burgdorferi* 50772 reliably induces high levels of anti-OspC borreliacidal antibodies. Therefore, the anti-OspC borreliacidal antibody response was a likely contributor to the enhanced protection, especially since Lyme disease spirochetes express OspC in the tick midgut and salivary glands and during the early stages of infection in the mammalian host (22, 23). An important caveat, however, is that ospC is incredibly diverse, even among *B. burgdorferi* isolates from the same geographic region (24), so comprehensive protection would be dependent on inducing borreliacidal antibodies specific for a conserved region of OspC. While we did not evaluate this possibility in this study, a previous investigation (17) showed that vaccination with the *B. burgdorferi* 50772 isolate induced significant amounts of borreliacidal antibodies specific for a highly conserved region of OspC (25). In addition, our findings mimicked those of a previous report (17) that vaccination with *B. burgdorferi* 50772 also induced significant levels of borreliacidal antibodies specific for other unknown antigens. Therefore, additional studies to confirm the specific mechanism(s) of protection are ongoing.

In summary, canines can acquire Lyme disease despite vaccination with a bacterin comprising only OspA-expressing *B. burgdorferi* (9, 10). However, while additional studies to better characterize the specific mechanism(s) responsible for protection remain necessary, these findings confirm that vaccination with the ospA- and ospB-negative *B. burgdorferi* 50772 provides protection against Lyme disease. Therefore, a combination vaccine that contains a traditional OspA-expressing *B. burgdorferi* and *B. burgdorferi* 50772 provides more comprehensive protection.

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