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Ability of Canine Lyme Disease Vaccine To Protect Hamsters against Infection with Several Isolates of *Borrelia burgdorferi*

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We used flow cytometry to determine levels of borreliacidal antibodies in hamsters after vaccination with a commercially available canine Lyme disease vaccine. In addition, we evaluated the ability of vaccinated hamsters to resist infection with several isolates of *Borrelia burgdorferi*. Borreliacidal antibodies could be detected 1 week after a primary vaccination, peaked at weeks 3 to 5, and then rapidly declined. One week after a booster vaccination, borreliacidal activity was detected at a dilution of 1:10,240, and it decreased fourfold by week 10 after the booster vaccination. Vaccinated hamsters were protected against infection with $\leq 10^6$ *B. burgdorferi* 297 organisms during the peak borreliacidal response (5 weeks after primary vaccination or 2 weeks after booster vaccination). However, hamsters were not fully protected from development of Lyme arthritis when the titer of borreliacidal antibodies was <1:5,120. In addition, no significant borreliacidal activity was induced against *B. burgdorferi* C-1-11, LV4, or BV1, which belong to three other seroprotective groups. These studies demonstrate that vaccination with the canine Lyme disease vaccine induces protective antibodies against *B. burgdorferi* 297. However, significant levels of borreliacidal antibodies are not produced until 5 weeks after vaccination, and protection is short-lived. In addition, no borreliacidal activity was induced against other isolates of *B. burgdorferi*. Because of this, the incorporation of multiple isolates or protein subunits may be necessary to increase the effectiveness of future vaccines.

Lyme disease is a multisystem disorder caused by infection with the spirochete *Borrelia burgdorferi* (27), which is most commonly transmitted to humans and animals by the bite of *B. burgdorferi*-infected ticks of the *Ixodes ricinus* complex (2). Lyme disease in humans is often characterized by an expanding skin lesion, erythema migrans, and constitutional symptoms such as headache, fatigue, and malaise (26). If the disease is untreated or treated inappropriately, cardiac, neurologic, or joint abnormalities may also occur (13, 20, 28). Since its discovery, Lyme disease has become the most common human tick-associated illness in the United States.

Canine Lyme disease is also common in many areas of the country (17). While a classical Lyme disease scenario in dogs has been more difficult to document, it is clear that infection of these animals with *B. burgdorferi* can cause significant morbidity manifested by many of the same symptoms associated with human disease. These have included fever, anorexia, fatigue, and, most commonly, limb and joint disorders (18).

We have previously demonstrated that inoculation of *B. burgdorferi* into hamsters induced borreliacidal antibodies which provided protection against subsequent challenge with *B. burgdorferi* (16, 23). Other investigators have also shown that vaccination of animals with *B. burgdorferi* or its components induced a protective humoral immune response (7, 8, 10, 24). Collectively, these investigations have begun to clarify the role of antibody-mediated resistance to infection and have assisted in efforts to develop effective animal or human Lyme disease vaccines. Currently, several companies have conducted or are preparing to conduct human trials of Lyme disease vaccines made from *B. burgdorferi* proteins which have been shown to induce protective antibodies in animals. A vaccine preparation for prevention of Lyme disease in dogs, composed of chemically inactivated *B. burgdorferi* organisms, has been commercially available for the past several years (6).

The irradiated-hamster model of Lyme arthritis is a rapid assay system to determine the efficacy of immune serum obtained from immunocompetent hamsters infected with the Lyme disease spirochete (22, 23). Using this animal model, we demonstrated that passive immunization with serum from immunocompetent hamsters infected with live *B. burgdorferi* organisms can prevent infection and induction of Lyme arthritis in irradiated recipient hamsters challenged with the same isolate (22). The recipient hamsters failed to develop hind paw swelling or histologic evidence of arthritis, and spirochetes were not recovered from their tissues. Additionally, we developed an in vitro assay for borreliacidal activity and showed that the presence of borreliacidal antibodies correlated with the ability of immune serum to protect against challenge (23). Borreliacidal antibodies were present 7 days after infection, peaked at 3 to 5 weeks after infection, and declined thereafter. Borreliacidal antibodies can also be isolate specific. Serum containing borreliacidal antibodies induced against a *B. burgdorferi* isolate could not protect hamsters from infection with other isolates (15).

In this investigation, the kinetics of the borreliacidal antibody response in hamsters after vaccination with a canine Lyme disease vaccine were determined. In addition, the ability of these antibodies to protect hamsters against infection and subsequent Lyme arthritis was determined by using isolates of *B. burgdorferi* from different seroprotective groups. We also showed that the assay for borreliacidal activity can determine the immune status of vaccinated animals.

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MATERIALS AND METHODS

Animals. Five- to 10-week-old LVG or LSH hamsters were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) and from our breeding colony located at the Wisconsin State Laboratory of Hygiene. Hamsters were housed three or four per cage at an ambient temperature of 21°C.

Organisms. *B. burgdorferi* (we will refer to all genospecies as *B. burgdorferi*) 297 (genospecies I; from human cerebrospinal fluid; United States), C-1-11 (genospecies I; from *Microtus pennsylvanicus*; Illinois), LV4 (genospecies II; from human cerebrospinal fluid; Europe), and BV1 (genospecies III; from human blood, Europe) were grown in Barbour-Stoenner-Kelly (BSK) medium (1, 4) at 32°C to a concentration of approximately 10^7 organisms per ml. Following incubation, 500-μl aliquots of each *B. burgdorferi* isolate were dispensed into sterile 1.5-ml microcentrifuge tubes (Sarstedt, Inc., Princeton, N.J.) and frozen at −70°C until used. The isolates were obtained from S. M. Callister, R. C. Johnson, and G. Stanek and represent three genospecies and four distinct seroprotective groups (15; unpublished data).

Vaccination of hamsters and collection of serum. Hamsters were vaccinated intramuscularly in the left hind thigh with 0.5 ml or in both hind thighs with 0.2 ml of a canine Lyme disease vaccine (Fort Dodge Laboratories, Inc., Ft. Dodge, Iowa; serial no. 140111B) (6). In some experiments, hamsters were given a booster three weeks after the primary vaccination. At 1, 2, 3, 4, 5, 6, 7, and 52 weeks after the primary vaccination or at 1, 2, 3, 4, 5, 7, and 10 weeks after the booster, groups of three hamsters each were mildly anesthetized by inhalation of ether contained in a nose-and-mouth cup and bled by intracardiac puncture. The blood was allowed to clot, and the serum was separated by centrifugation at 500 × *g*. Serum was pooled, divided into 250-μl aliquots, and stored at −70°C until used. In addition, hamster sera from three nonvaccinated hamsters were pooled and used as a normal serum control.

Borreliacidal antibody. In vitro borreliacidal antibodies were detected by use of a modification of a previously described procedure (16). Briefly, hamster sera were serially diluted (1:20 to 1:20,480) in fresh BSK medium. One-hundred-microliter aliquots of each dilution were transferred to 1.5-ml microcentrifuge tubes (Sarstedt) and heat inactivated at 56°C for 10 min. Individual cultures of each *B. burgdorferi* isolate were diluted to approximately 10^6 organisms per ml, and 100-μl amounts of these suspensions were added to microcentrifuge tubes containing diluted sera. Subsequently, 10 μl of sterile guinea pig serum (>210 50% hemolytic complement units per ml) (Sigma Chemical Co., St. Louis, Mo.) was added. Assay suspensions were mixed by gentle vortexing and incubated for 18 to 24 h at 32°C.

After incubation, a 100-μl amount was removed from each microcentrifuge tube and diluted 1:5 in sterile phosphate-buffered saline (0.01 M, pH 7.2), and 50 μl of acridine orange (5.4×10^{-9} M) was added (12). Borreliacidal activity was detected with a FACScan single-laser flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Events were acquired from each sample for 90 to 120 s in the list mode and were analyzed with LYSYS II research software (Becton Dickinson). Forward scatter, side scatter, and acridine orange fluorescence were logarithmically amplified and converted to a linear scale for comparison after analysis.

Challenge with *B. burgdorferi*. Three or five weeks after the primary vaccination and two weeks after the booster with the canine vaccine, vaccinated hamsters and nonvaccinated controls were exposed to 600 rads of gamma irradiation with a ^{60}Co irradiator (Picker Corp., Cleveland, Ohio). Hamsters survived this level of radiation without reconstitution with normal bone marrow cells. A suspension of *B. burgdorferi* 297 containing approximately 5×10^7 organisms per ml was diluted with fresh BSK medium to 5×10^6, 5×10^5, 5×10^4, or 5×10^3 organisms per ml, and groups of three hamsters were challenged subcutaneously in each hind paw with 0.2-ml portions of these suspensions. In other experiments using different isolates of *B. burgdorferi*, hamsters were challenged subcutaneously with 5×10^6 spirochetes.

Evaluation of arthritis. The volume of each hind paw was measured prior to challenge and every other day thereafter for 2 or 3 weeks with a plethysmograph (Buxco Electronics, Sharon, Conn.). Measurements were obtained by lightly anesthetizing hamsters, immersing each hind paw in a column of mercury, and recording the amount (in milliliters) of mercury displaced. Mercury displacement was standardized with a volume calibrator.

Recovery of *B. burgdorferi* from tissues. Fourteen or 21 days after challenge, hamsters were sacrificed by inhalation of carbon dioxide. The left kidney, spleen, bladder, and heart were aseptically removed and forced through 3- or 5-ml syringes (without a needle) into tubes containing 6 ml of fresh BSK medium. Cultures were vortexed, and 600 μl of supernatant was transferred into an additional 5.4 ml (10%, vol/vol) of BSK medium. Cultures were incubated at 32°C for 3 weeks and examined weekly for spirochetes by dark-field microscopy. Samples which remained negative after 3 weeks were concentrated by centrifugation at 5,000 × *g* for 5 min, and pellets were examined for spirochetes. In other experiments 0.5-ml portions of cultures were transferred to fresh BSK medium (5 ml) and examined weekly for 3 weeks for spirochetes.

Removal of IgG antibodies. Removal of immunoglobulin G (IgG) antibodies from sera of normal and vaccinated hamsters was performed by combining 0.5 ml of serum with 0.5 ml of swelled protein A agarose beads (Sigma) and incubating, with mixing, for 2 h at 25°C. To confirm removal of IgG antibodies, dot blots were performed by use of a modification of a previously described procedure (21). Briefly, protein A-treated normal or vaccinated hamster serum was serially diluted (1:150 to 1:9,600), and 5-μl amounts were spotted onto nitrocellulose strips and allowed to air dry. The nitrocellulose strips were then blocked for 1 h at 25°C with 3% nonfat dry milk. The strips were rinsed and incubated at 25°C for 2 h with rabbit anti-hamster IgG antibodies contained in 5 ml of 15 mM Tris–0.5 M NaCl–0.05% Tween 20 buffer, and 2 ml of peroxidase-labeled goat anti-rabbit IgG antibodies was added. Strips were incubated at 25°C for an additional 2 h and rinsed with distilled water, and 2 ml of 4-chloro-1-naphthol was added. Reactions were considered complete when color development was similar to that observed with normal hamster serum.

Statistical analyses. The *t* test (25) was used to examine pairs of means. The alpha level was set at 0.05 before the experiments were started.

RESULTS

Borreliacidal antibody response after vaccination. Sera from normal and vaccinated hamsters were tested for borreliacidal activity against *B. burgdorferi* isolates from four seroprotective groups (14, 15). After 1 week, vaccination induced borreliacidal antibodies against *B. burgdorferi* 297 which were detectable to a serum dilution of 1:640, peaked at week 5 (1:5,120), and declined by week 6 (1:640) (Fig. 1). Hamsters receiving primary and secondary vaccinations developed higher levels of borreliacidal antibody. One week after the

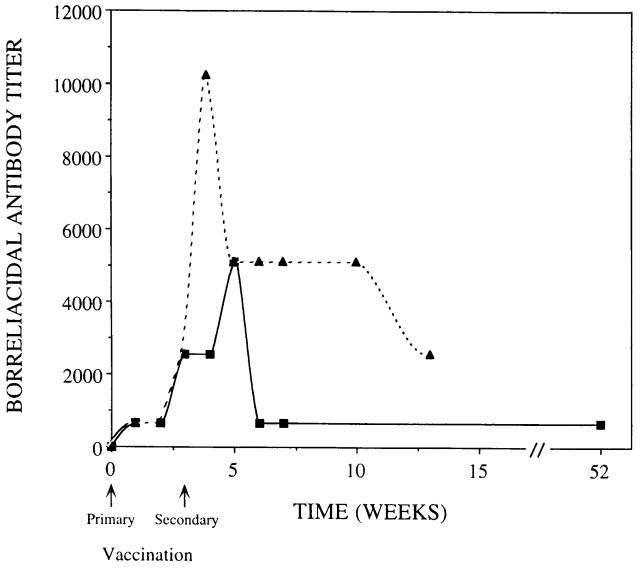

FIG. 1. Borreliacidal antibody titers against *B. burgdorferi* 297 after primary (■) and secondary (▲) vaccinations.

booster (4 weeks after primary vaccination), borreliacidal antibodies were detectable at a serum dilution of 1:10,240, remained elevated (1:5,120) from 2 to 7 weeks after secondary vaccination, and declined (1:2,560) by 10 weeks (13 weeks after primary vaccination). In contrast, no significant borreliacidal activity was detected when *B. burgdorferi* C-1-11, LV4, or BV1 was tested with these sera.

Ability of borreliacidal antibodies to protect hamsters from infection with *B. burgdorferi* and from arthritis. To determine whether borreliacidal antibodies could protect hamsters from *B. burgdorferi* infection and from arthritis, vaccinated and nonvaccinated hamsters were irradiated and challenged with 10^6, 10^5, 10^4, or 10^3 *B. burgdorferi* 297 organisms 3 and 5 weeks after primary vaccination or 2 weeks after secondary vaccination. The onsets and degrees of swelling of hind paws of 3-week-vaccinated and nonvaccinated hamsters challenged with *B. burgdorferi* were similar. Arthritis peaked in severity 10 or 12 days after challenge with 10^6 or 10^5 *B. burgdorferi* organisms (Fig. 2). When 3-week-vaccinated hamsters and

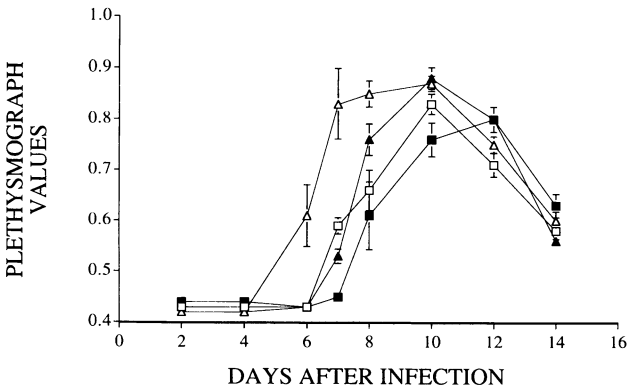

FIG. 2. Hind paw swelling of vaccinated (closed symbols) or nonvaccinated (open symbols) hamsters challenged with 10^6 (▲) or 10^5 (■) *B. burgdorferi* organisms 3 weeks after primary vaccination.

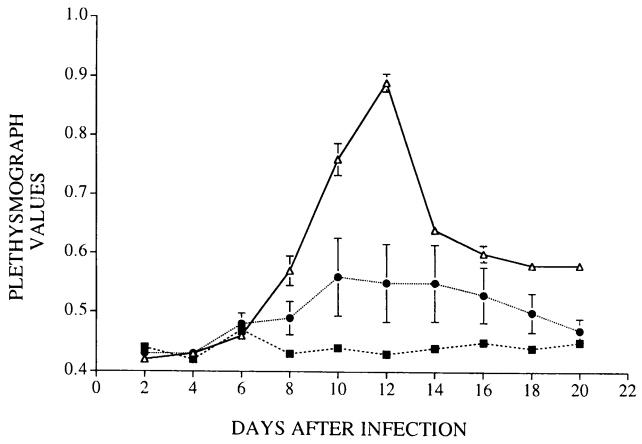

FIG. 3. Hind paw swelling of nonvaccinated (△), vaccinated (5 weeks) (●), or boosted (secondary vaccination) (■) hamsters following challenge with 10^6 *B. burgdorferi* organisms.

nonvaccinated hamsters were challenged with 10^4 or 10^3 spirochetes, hind paw swelling peaked at 14 days. In contrast, hamsters challenged 5 weeks previously or given a booster vaccination failed to develop hind paw swelling after irradiation and challenge with 10^6 or fewer *B. burgdorferi* 297 organisms (Fig. 3).

Similar results were obtained when recovery of *B. burgdorferi* from hamster tissues (left kidney, spleen, bladder, and heart) was used to assay the effectiveness of vaccination. *B. burgdorferi* organisms were recovered from three (100%) normal hamsters challenged with 10^6, 10^5, 10^4, or 10^3 spirochetes and from three (100%) hamsters vaccinated 3 weeks prior and challenged with 10^6 or 10^5 spirochetes. *B. burgdorferi* was also recovered from two (67%) hamsters after challenge with 10^4 or 10^3 spirochetes. In contrast, spirochetes were not recovered from any hamsters challenged 5 weeks after primary vaccination or 2 weeks after secondary vaccination.

We next confirmed that borreliacidal activity was restricted to one seroprotective group, primarily isolate 297. When hamsters vaccinated 5 weeks prior or boosted were challenged with 10^6 *B. burgdorferi* organisms of isolate C-1-11, LV4, or BV1, significant hind paw swelling occurred (Fig. 4). In

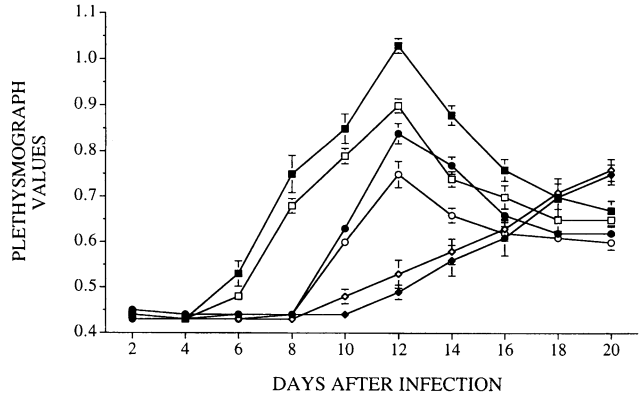

FIG. 4. Hind paw swelling of nonvaccinated (open symbols) and secondary-vaccinated (closed symbols) hamsters after challenge with 10^6 *B. burgdorferi* organisms of isolate C-1-11 (■), BV1 (●), or LV4 (◆).

addition, *B. burgdorferi* organisms were recovered from tissues of all vaccinated hamsters challenged. These results correlated with our in vitro findings. No significant killing of isolate C-1-11, LV4, or BV1 was detected with serum from hamsters vaccinated 5 weeks earlier compared with serum from nonvaccinated hamsters.

Effect of IgG removal on borreliacidal activity. Hamsters vaccinated 3 weeks previously were not protected against challenge with *B. burgdorferi*. In contrast, hamsters were fully protected 5 weeks after primary vaccination. IgM borreliacidal antibodies present in serum 3 weeks after vaccination may not be as protective (21). Subsequently, IgG antibodies were removed from sera of hamsters vaccinated 3 and 5 weeks previously and were assayed for borreliacidal activity with *B. burgdorferi* 297. The removal of IgG antibodies by treatment with protein A caused 97 and 99% decreases in borreliacidal activities of 3- and 5-week vaccination sera, respectively.

DISCUSSION

We recently developed an in vitro assay for detecting borreliacidal antibody which has facilitated investigations characterizing the protective humoral immune response (5, 16, 23). In the current study, this method was used to evaluate the ability of a whole-cell canine Lyme disease vaccine to induce a protective humoral immune response against *B. burgdorferi* isolates from 4 seroprotective groups (14, 15). In addition, the kinetics of the borreliacidal antibody response and ability to protect hamsters from infection with *B. burgdorferi* and subsequent Lyme arthritis were determined.

A humoral immune response against *B. burgdorferi* 297 developed rapidly in vaccinated hamsters, and the kinetics of the response were similar to those obtained in live challenge experiments (16, 23). Borreliacidal antibodies could be detected 1 week after primary vaccination, peaked at weeks 3 to 5, and then rapidly declined, although borreliacidal activity was still detectable 52 weeks after vaccination. The borreliacidal antibody titers also reached higher levels after primary vaccination than those observed during infection with live *B. burgdorferi* organisms (16, 23).

Surprisingly, vaccinated hamsters were not completely protected from challenge with a small inoculum (10^3) of *B. burgdorferi* 297 3 weeks after primary vaccination, even though borreliacidal activity could be detected when serum was diluted 1:2,560. It is unknown whether this titer would be protective against lower numbers of spirochetes obtained, for example, during tick challenge. In contrast, hamsters were protected against a challenge of 10^6 organisms 5 weeks after vaccination, when borreliacidal activity was detectable at a dilution of 1:5,120. We hypothesized that this may be due to an incomplete shift from IgM to IgG antibodies, specifically IgG2, which more effectively confers protection in hamsters (21). However, removal of IgG from 3- and 5-week vaccination sera caused significant (>97%) decreases in killing activity, indicating that borreliacidal antibodies were primarily IgG. These results suggest that IgG borreliacidal antibodies induced by using killed *B. burgdorferi* organisms may be of different or lesser quality than antibodies produced against live spirochetes. This is not unexpected, since important epitopes on *B. burgdorferi*, which could have been structurally altered by the chemical inactivation process (6), may have prevented a typical immunologic response from occurring. If this is the case, induction of higher titers of borreliacidal antibodies with vaccination would appear to be necessary to ensure complete protection.

Significantly higher levels of borreliacidal antibodies occurred after a booster vaccination. One week after this secondary vaccination, borreliacidal antibodies could be detected when serum was diluted 1:10,240. However, borreliacidal activity had decreased fourfold (1:2,560) 10 weeks after secondary vaccination. In the current study, a borreliacidal antibody titer of 1:2,560 could not provide complete protection against challenge with $\geq 10^3$ *B. burgdorferi* organisms. We confirmed that this decrease in titer correlated with waning of borreliacidal antibodies by repeating the assay for borreliacidal activity with sera collected 2 or 10 weeks after secondary vaccination and evaluating killing activity against increasing amounts of *B. burgdorferi* organisms. Significant borreliacidal activity could be detected against 10^5 spirochetes with serum obtained 2 weeks after booster (1:5,120). However, borreliacidal activity could be detected only when 10^4 or fewer organisms were combined with serum collected 10 weeks after secondary vaccination (1:2,560). Additional studies are needed to clarify the duration of effective immunity provided by primary and secondary vaccinations. However, our results indicate that borreliacidal antibodies wane rapidly after booster vaccination and faster after primary vaccination. The inability of vaccination to induce long-term protection makes it necessary to closely monitor levels of protective antibodies in vaccinated animals, especially in areas where the disease is highly endemic. Vaccinated animals may also need close monitoring, since repeated vaccinations necessary to ensure high levels of borreliacidal antibodies may increase the potential for deleterious side effects, such as immune complex disease (11a).

Our results suggest that vaccination with the canine Lyme disease vaccine can provide protection against infection from some *B. burgdorferi* isolates, primarily those related to isolate 297. However, we also demonstrated that vaccine-induced borreliacidal antibodies do not provide protection against other isolates of *B. burgdorferi*. These findings were confirmed by challenging vaccinated hamsters, during peak anti-297 borreliacidal activity, with isolates from three other previously identified seroprotective groups (14, 15). The inability to protect against *B. burgdorferi* infection and Lyme arthritis correlated with results obtained by using the in vitro assay for borreliacidal activity. We (14, 15) and others (11) have previously confirmed the existence of distinct seroprotective groups by demonstrating that passive immunization of hamsters with sera from animals infected with *B. burgdorferi* does not provide recipient animals protection against all isolates. Our current results extend these findings by demonstrating that vaccination with a canine Lyme disease vaccine does not provide protection against all isolates of *B. burgdorferi*.

A number of investigators have shown that the majority of North American isolates belong to the single genospecies *B. burgdorferi* sensu stricto (3, 19). Since *B. burgdorferi* 297 is an isolate from this genospecies, it can be expected that vaccination with the canine vaccine will induce protection against many North American isolates. Isolates LV4 and BV1 are likely *Borrelia garinii* and *Borrelia afzelii*, respectively. These genospecies have not, to our knowledge, been recovered in the United States. In contrast, *B. burgdorferi* C-1-11 was recently identified as representative of a separate seroprotective group found in the United States (15). Recently, Fikrig et al. (9) identified a *B. burgdorferi* isolate which could not be protected against by an Osp A vaccine previously determined to be effective against isolate 297. The nucleotide sequence of *ospA* from this spirochete has strong homology with *B. burgdorferi* C-1-11 *ospA* (15). Additional studies are needed to determine the frequency distribution of this seroprotective group within the United States.

In conclusion, we have demonstrated that vaccination of hamsters with a canine Lyme disease vaccine induced protective borreliacidal antibodies. However, the duration of immunity appeared to be short, even after a booster vaccination. In addition, vaccination did not induce protection against the European genospecies *B. garinii* and *B. afzelii* or against an isolate representing another U.S. seroprotective group. To increase the effectiveness of future vaccines, it appears to be necessary to incorporate multiple *B. burgdorferi* isolates or their protein subunits. The assay for borreliacidal activity should be helpful for determining the effectiveness of these vaccines and monitoring the immune status of vaccinated animals or humans.

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