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Sera from OspA-Vaccinated Dogs, but Not Those from Tick-Infected Dogs, Inhibit In Vitro Growth of *Borrelia burgdorferi*

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Dogs were challenged with *Borrelia burgdorferi* by exposure to ticks, with or without prior protection from infection by recombinant OspA (rOspA) vaccination. Sera from these dogs were tested for their capability to inhibit the growth of *B. burgdorferi* in vitro. Bacterial growth was detected by a color change in the culture medium, and the optical density was measured with a spectrophotometer in microtiter plates. By growth inhibition, which was complement dependent, the color change was lacking after 5 days of incubation. Over a 1-year study, nonvaccinated dogs infected by exposure to ticks showed high antibody titers to *B. burgdorferi* by kinetic enzyme-linked immunosorbent assay (KELA). The same sera did not inhibit spirochetal growth or did so only at a low dilution. These results corresponded to the lack of OspA and OspB antibodies seen in Western blots (immunoblots), and these dogs were not protected from infection or disease. In contrast, dogs immunized with rOspA prior to challenge with infected ticks produced high antibody titers, as determined by KELA, but their sera also had high growth-inhibiting antibody titers. Western blot analysis showed a strong band in the 32-kDa region when the sera of these dogs were tested. When adjuvant was administered with rOspA, antibody titers by both KELA and growth inhibition were higher and persisted longer in the immunized dogs. All dogs immunized with rOspA were protected from infection and disease.

Lyme disease is caused by *Borrelia burgdorferi* and is transmitted by *Ixodid* ticks. It is a common disease in humans and animals and has been reported in dogs (21, 22), horses (9), cattle (30), and cats (27). There are similarities between the disease in humans and that in dogs (1).

The common serological tests for Lyme disease are immunofluorescence assays, enzyme-linked immunosorbent assay (ELISA), and Western blotting (immunoblotting). Although these tests are valuable for the diagnosis of Lyme disease in humans and dogs, there is no correlation between elevated titers and resistance to infection and disease. In contrast, tests for serum antibodies which can inhibit the growth of *B. burgdorferi* are good indicators of protection from infection (18, 40). In earlier studies various counting techniques were used to determine the numbers of live and killed spirochetes (5, 19, 20, 24, 32, 38). The color change in the culture medium induced by live spirochetes was found to be a reliable and less labor intensive method of measuring growth-inhibiting antibodies (26, 36, 37). Recently, it was shown that flow cytometry is a useful tool for identifying infected patients apparently with small quantities of borreliacidal antibodies (6).

Both complement-dependent and -independent growth inhibition of *B. burgdorferi* has been found in studies with sera from humans (5, 38) and animals experimentally infected with *B. burgdorferi* (24, 26, 32) and sera with polyclonal and monoclonal antibodies to OspA and OspB (36–38). Sera from patients with chronic Lyme disease, which contain antibodies to OspA and OspB, protected hamsters (5) and mice (13) from infection with *B. burgdorferi*. Several investigators described a lack of an early antibody response to OspA and OspB after natural infection by exposure to ticks in humans (3, 11, 14, 47), dogs (1, 17), hamsters (35), mice (16), and rhesus monkeys (2). However, recent studies by more sensitive techniques suggest that there is a specific antibody response to OspA during the early stage of infection (41). The roles of antibodies to other outer surface proteins are unclear. Gerbils immunized with pC (20-kDa protein) were protected from infection by a strain expressing abundant quantities of pC (32). Additionally, the vaccination of hamsters with a mutant *B. burgdorferi* strain lacking OspA and OspB was protective against infection with the nonmutant, virulent parent strain (29). These data provide evidence that other antigens may stimulate the immune system to produce protective antibodies, but further studies with wild-type borreliae are needed.

Since a vaccine should provide protection against a variety of different strains, not only against the parent strain, we used recombinant OspA (rOspA), a promising candidate for vaccines, in our studies. Vaccination of dogs with rOspA protects them against infection and joint disease caused by wild-type *B. burgdorferi* inoculated into the dogs by ticks (7a). We report here a correlation between serum-induced growth inhibition of *B. burgdorferi* in vitro and protection against infection in dogs.

MATERIALS AND METHODS

Dogs. Specific-pathogen-free beagles from the James A Baker Institute for Animal Health colony were used. All dogs were kept in P2 isolation units and were fed a commercial ration and water ad libitum. All dogs were observed daily for clinical signs, and body temperature was recorded daily. Body weights were measured weekly.

Ticks. Adult ticks (*Ixodes scapularis*) were collected by flagging in North Salem, Westchester County, N.Y., in May and November. They were kept at 10°C in 94% relative humidity until they were used. The infectivity rate of the ticks was determined by grinding and culturing 20 male ticks individually in BSK II medium containing 8 μg of kanamycin per ml and 50 μg of rifampin per ml (BSK II + KR) as reported previously (1). A minimum of 60% of the ticks were infected with *B. burgdorferi.*

Vaccination of dogs. Beginning at 6 weeks of age, dogs were immunized twice at a 3-week interval. Six dogs received intramuscularly 100 μg of rOspA, prepared as described previously (8), in combination with QuilA (100 μg), two dogs

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received rOspA (100 μg) and ISA 25 (50 μl), and two dogs received rOspA (100 μg) with QuilA (100 μg) and ISA 25 (50 μl) as adjuvants. Eight dogs were immunized intramuscularly with 100 μg of rOspA without adjuvant. rOspA (10 μg) in alum (10 μg) as the adjuvant (supplied by Connaught Laboratories) was administered to four dogs subcutaneously. At 3 to 5 weeks after the last vaccination, all except one group of six dogs were challenged with infected ticks; however, one group of six dogs was challenged 5 months after immunization (two dogs were immunized with 100 μg of rOspA and 100 μg of QuilA, two dogs were immunized with 100 μg of rOspA and 50 μl of ISA 25, and two dogs were immunized with 100 μg of rOspA and 100 μg of QuilA with 50 μl of ISA 25). Our rOspA preparation and those supplied by Connaught Laboratories were produced by the same protocol by using the genetic information for strain B31. All rOspA preparations were lipidated.

Exposure of dogs to ticks. Fifteen female ticks and seven male ticks were placed onto the clipped chest of each dog as reported previously (1). In addition to the vaccinated dogs, 28 6-week-old, 14 12-week-old, and 4 19-week-old dogs were exposed to infected ticks.

Isolation of *B. burgdorferi*. To establish whether vaccinated and nonvaccinated animals were infected following exposure to ticks, isolation of *B. burgdorferi* from dog tissues was attempted. During the experiments, with the dogs under local anesthesia, 4-mm-diameter skin punch biopsy specimens were taken monthly near the site of the tick bite. When the dogs were euthanized 3 to 12 months after challenge, samples of different tissues (skin, proximal limb muscles, joint capsules of shoulders, elbows, stifles, fascias of the fore and hind legs, pericardium, peritoneum, meninges, and brain) were collected and used for borrelia isolation.

Skin biopsy specimens and postmortem tissues were homogenized in BSK II medium with a tissue homogenizer (Stomacher; Teckmar, Cincinnati, Ohio) and 3 ml of the suspension was placed into 27 ml of BSK II + KR. During 6 weeks of incubation at 34°C the medium was examined weekly for the presence of *B. burgdorferi* by dark-field microscopy. Spirochetes were identified as *B. burgdorferi* by indirect immunofluorescence assay with rabbit anti-*B. burgdorferi* antibodies.

Serology. Blood samples were taken before the vaccination or challenge and then at 2-week intervals thereafter. Serological tests were performed for 3 to 6 months and, for a few animals, for up to 1 year. Sera were stored at −20°C until they were tested. Sera were tested for *B. burgdorferi* antibodies by ELISA, Western blots, and growth inhibition assays.

ELISA and Western blots. Computerized kinetic ELISA (KELA) and Western blot analyses were performed as described previously (1). French-pressed whole *B. burgdorferi* B31 lysate was used as the antigen in both tests. Dog sera were used in the first reaction, and goat anti-dog immunoglobulin G conjugated to horseradish peroxidase (Cappel Research Products, Durham, N.C.) was used in the second reaction.

Growth inhibition test. High-passage-number (passages 42 to 49) and low-passage-number (passage 3) *B. burgdorferi* isolates (isolates were from infected dogs) were grown in BSK II + KR at 34°C at the logarithmic growth phase for 2 and 3 days, respectively. Serum samples were tested against high-passage-number borreliae. High- and low-passage-number borreliae showed comparable results in the growth inhibition test (Fig. 1). We chose to test serum samples against high-passage-number borreliae because they replicated more quickly, allowing the test to be conducted in a shorter time period, and because the color contrast was more pronounced. Serum samples were heat inactivated at 56°C for 45 min, centrifuged at 13,000 × *g* for 10 min, and filtered through a 0.45-μm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.). The test was performed in flat-bottom 96-well microtiter plates (Corning, Corning, N.Y.). All samples were tested in duplicate and were serially diluted in 100 μl of BSK II + KR containing an additional 40 μg of phenol red (Sigma, St. Louis, Mo.) per ml. The coefficient of variation within a duplicate set of samples was always less than 10%. The serum dilution range was from 1:20 to 1:2,560. Bacteria were counted with a Petroff-Hausser counting chamber (Baxter Scientific Products, Edison, N.Y.), and the concentration was adjusted to 10^6 spirochetes per ml with BSK II + KR, resulting in a total number of 10^5 spirochetes in 100 μl per well. After 30 min of incubation at room temperature, 10 μl of guinea pig complement with a hemolytic titer of 200 50% hemolytic complement U/ml (Sigma) was added to each well, and the contents were mixed. The plates were then covered with adhesive plate sealers (Marsch Biomedical Products, Rochester, N.Y.), the lids were then placed onto the plates, and the plates were sealed with Scotch Brand tape. The plates were wrapped in Saran Wrap, placed into an airtight box, and incubated at 37°C for 5 days. After 3, 4, and 5 days of incubation, the absorbance of each well was measured with a microplate reader (Bio-Tek Instruments, Winooski, Vt.) at 570/630 nm. Bacterial growth was measured as a function of the pH, indicated by a color change in the medium from red to yellow. Data from the 3-, 4-, and 5-day readings were compared. The reading with the largest difference between the optical density (OD) of the red medium and that of the yellow medium was used for the determination of the growth inhibition antibody titer.

All tests included sera from healthy dogs as negative controls and dog sera with known growth inhibition titers (determined in previous experiments) as positive controls and as controls for reproducibility. Serial dilutions of spirochetes in medium with or without guinea pig complement served as controls for bacterial growth.

Wells were considered negative for growth inhibition when the color of the medium turned to yellow. To distinguish between positive and negative wells in a standardized manner, the ratio of a well's OD (OD_n) and the optical density of the previous well in a serum dilution series (OD_{n-1}) was calculated. If the ratio exceeded 0.95 and the medium in the well was red, the well was considered positive for growth inhibition. The smallest ratio in a dilution series was indicated by a color change of the medium to yellow. When the following equation was true, the serum dilution lost its capability to inhibit the growth of *B. burgdorferi*: $OD_n/OD_{n-1} < 0.95$, with n equal to 2, 3, . . ., 8 (well number).

When the test was conducted, bacterial counts were performed in selected tests to determine the number of borreliae in wells with and without growth inhibition. Counts were performed with a Petroff-Hausser counting chamber.

High-passage-number borreliae replicated faster than low-passage-number borreliae, and so a higher spirochetal cell count per volume was achieved. However, the color change was not dependent on the total number of spirochetes. The medium's color change was detectable when the bacterial counts of the wells with growth inhibition was at least 10-fold lower than those of wells with growth. This observation is consistent with the results of Sadziene et al. (37), except that the addition of extra phenol red increased the sensitivity of the test.

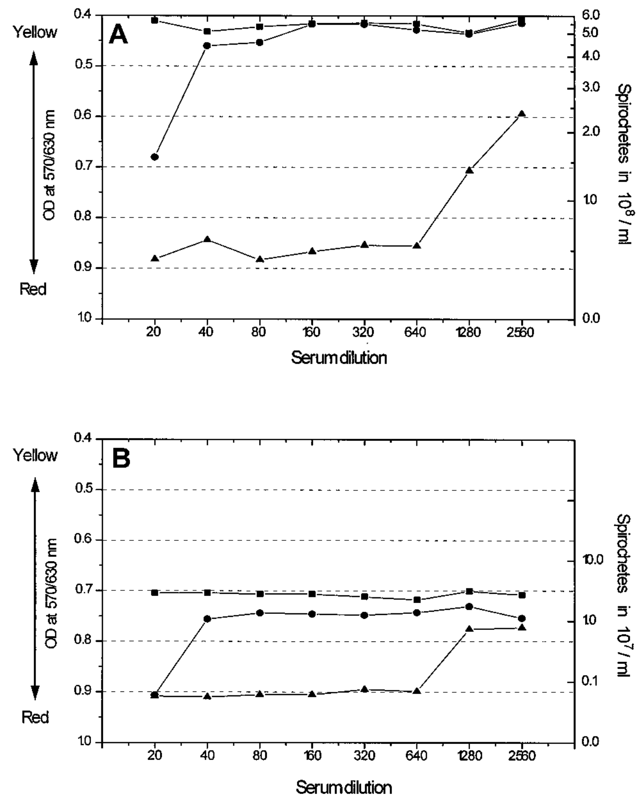

FIG. 1. Growth inhibition of high- and low-passage-number *B. burgdorferi* by canine serum. A constant number of spirochetes was cultured in BSK II medium containing different concentrations of dog serum (see Material and Methods). After 4 days (high passage number) or 5 days (low passage number) the OD of the medium was measured, and the growth inhibition titer, indicated by a significant decrease in the OD, was determined. In addition, the number of spirochetes was determined by dark-field microscopy. (A) Growth of high-passage-number *B. burgdorferi*; the organisms were cultured with serum from an OspA-immunized dog (▲), serum from a naturally infected dog (●), and serum from a healthy dog (■). (B) The same serum samples described for panel A were tested against low-passage-number *B. burgdorferi*. Identical results were achieved in both systems. Serum from the vaccinated dog had a titer of 1:640, serum from the naturally infected dog had a titer of 1:20, and no growth inhibition was obtained with serum from the healthy dog.

RESULTS

Clinical signs. Sporadic elevated body temperature for 1 or 2 days was found in most of the 46 nonvaccinated dogs after exposure to infected ticks. None of the vaccinated dogs showed elevated temperature. Weight loss was not seen in any of the dogs.

Of the 28 dogs that were exposed to infected ticks at 6 weeks

of age, 23 showed lameness of one limb for a 3- to 4-day period. The lameness was recurrent with up to three episodes at 2- to 4-week intervals. Only 2 of 14 dogs exposed at 12 weeks of age and 1 of 4 dogs exposed at 19 weeks of age showed lameness. Lameness was accompanied by joint swelling and local pain. None of the vaccinated dogs became lame.

Infection of dogs. Because the study used dogs from 6 to 19 weeks of age and the dogs were euthanized 3 to 12 months after challenge, the number of dogs in the study changed from month to month. Following exposure to infected ticks, *B. burgdorferi* was isolated from skin biopsy specimens. During the first month 81.5% of the skin biopsy specimens were positive, during the second month 88.2% were positive, during the third month 84.8% were positive, during the fourth month 71.0% were positive, during the fifth month 78.6% were positive, and during the sixth month 100% were positive. During the sixth month, the longest period after challenge that dogs were tested, only four dogs remained in the studies. In total, 35 of 37 nonvaccinated, tested dogs showed persistent infection, as demonstrated by isolation of *B. burgdorferi* from skin biopsy specimens.

B. burgdorferi was also isolated at necropsy from at least one tissue of 45 of 46 nonvaccinated dogs between 3 and 12 months after exposure. Isolation was most frequently from muscle or joint tissues or from connective tissues such as the pericardium or peritoneum. *B. burgdorferi* was not isolated from skin biopsy specimens or tissues obtained postmortem from any of the vaccinated dogs.

Serology. (i) KELA. Antibody titers greater than 100 KELA units are indicative of infection with *B. burgdorferi* or of immunization with borrelia antigen (44). By KELA a consistent antibody response was seen among nonvaccinated dogs, regardless of age, after exposure to infected ticks. Within 30 to 60 days after infection a continuous rise in antibody titers was seen, reaching maximal levels at about 3 to 5 months (Fig. 2A). Antibody levels remained constant thereafter for up to 360 days postinfection, the longest time period tested. Persistent high antibody levels determined by KELA were always accompanied by persistent borrelia infection and were also present in dogs with clinical disease (lameness and joint swelling). In contrast, antibodies in vaccinated dogs determined by KELA were always present by 3 weeks after the first dose of OspA vaccination, regardless of whether adjuvant was administered or whether an intramuscular or subcutaneous route was used. Titers reached maximal levels by 3 to 4 weeks after the second vaccination. Titers declined gradually thereafter, regardless of whether the dogs were challenged at that time (Fig. 3A). Neither the type of adjuvant used (alum, ISA 25, QuilA) nor the concentration of rOspA (10 or 100 μg) caused significant differences in the production of antibodies determined by KELA. However, titers were lower and declined more rapidly in dogs vaccinated with OspA alone without adjuvant (Fig. 4A).

(ii) Western blots. Bands specific for *B. burgdorferi* were not seen in nonvaccinated, tick-exposed dogs until at least 4 weeks after infection. The densities of specific bands increased until approximately 8 weeks after infection and remained constant thereafter. Consistent with previous experiments, there was no antibody response or there was a minimal detectable antibody response to OspA and OspB in the 32- and 34-kDa regions throughout the 1-year observation period (Fig. 5). In contrast, dogs vaccinated with OspA developed antibodies by 3 weeks after the first vaccination, and the antibodies were expressed in a wide band over the 32- to 33-kDa region. By 3 weeks after the second vaccination, the bands had higher densities. Challenge by tick exposure at that time did not alter the pattern. The densities of the 32-kDa bands gradually decreased thereafter,

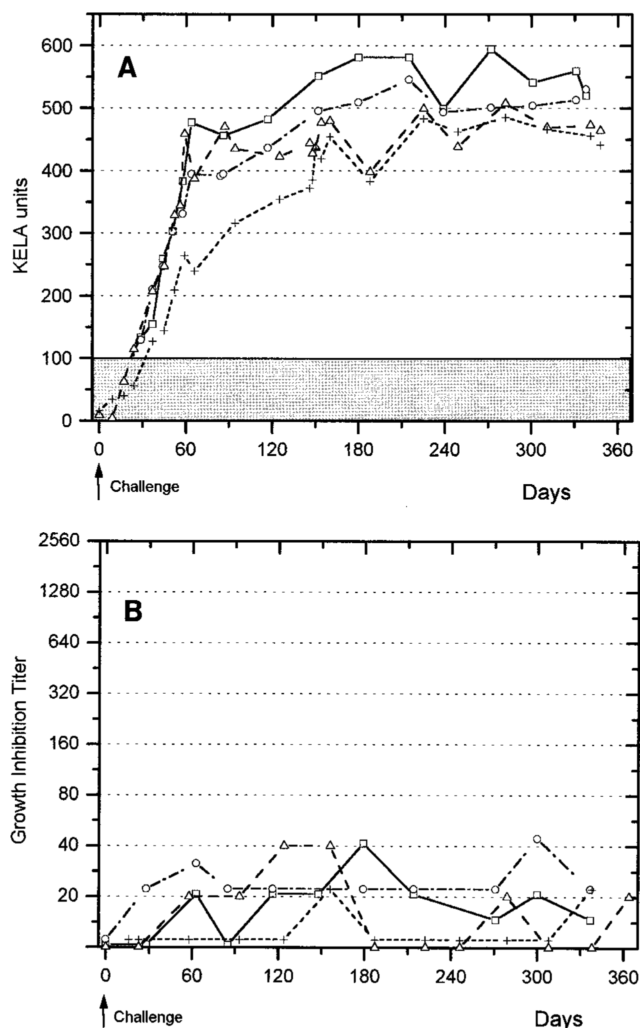

FIG. 2. (A) Development of antibody titers against *B. burgdorferi* after challenge with infected adult ticks (*I. scapularis*) during a time period of 360 days determined by ELISA. The antibody responses of four individual dogs are shown. An antibody titer of 100 KELA units indicates the cutoff point for a positive antibody response to *B. burgdorferi* (shaded area). (B) Growth-inhibiting antibody titers against *B. burgdorferi* from the same four dogs (see panel A). The dogs were challenged on day 0 with infected adult ticks (*I. scapularis*) and were monitored for 1 year.

regardless of whether adjuvant was administered (Fig. 5), and correlated with a drop in KELA values. In addition to the 32-kDa band, bands appeared in the 20- and 62-kDa regions by 3 weeks after the second vaccination. The use of monoclonal antibodies against OspA showed that these bands may represent degradation products and dimers of OspA (Fig. 5). Similar responses were seen in dogs vaccinated with 100 or 10 μg of OspA and were not altered by adjuvants or the route of inoculation.

(iii) Growth inhibition test. All 46 nonvaccinated dogs were challenged with infected ticks, and 45 of 46 dogs became infected. Sera from these 46 dogs were tested for the presence of growth-inhibiting antibodies. All nonvaccinated dogs developed minimal or no growth-inhibiting antibodies during an observation period of up to 360 days after tick exposure. If antibodies were present, they were detectable 30 to 60 days after challenge, but their titers always remained lower than 1:80 (Fig. 2B). In addition, 22 dogs that were immunized with

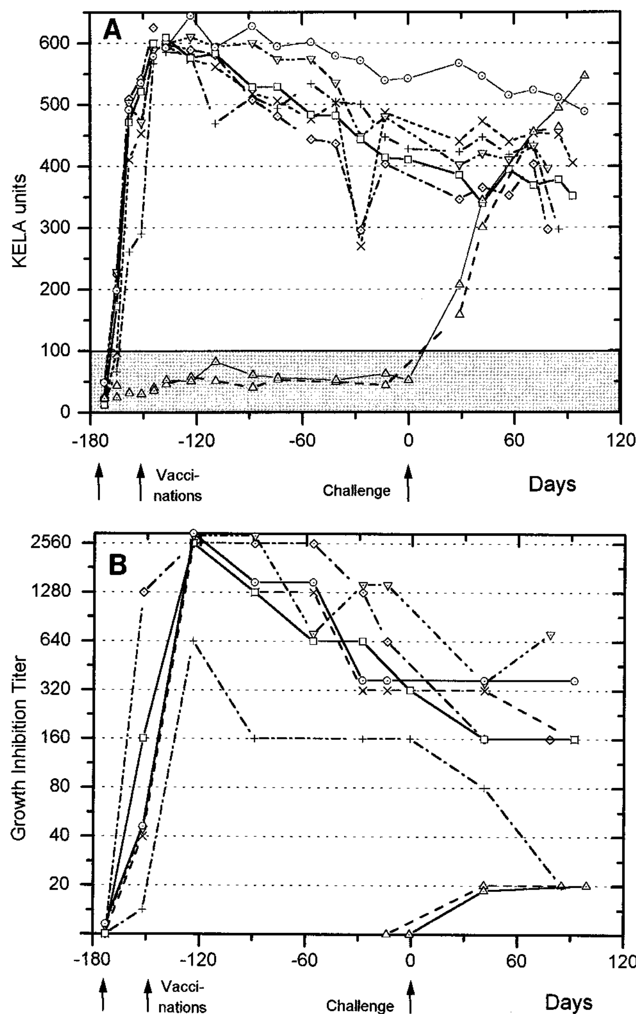

FIG. 3. (A) Antibody titers against *B. burgdorferi* in sera from six vaccinated and two unvaccinated control dogs determined by ELISA. Dogs were vaccinated twice with 100 μg of rOspA in combination with adjuvants (100 μg of QuilA or 50 μl of ISA 25) intramuscularly at a 3-week interval and were challenged with infected adult ticks (*I. scapularis*) 6 months later. The unvaccinated control dogs (◬, △) were kept together with the vaccinated dogs and were challenged at day 0. Arrows indicate the time of vaccination and the time of challenge. An antibody titer of 100 KELA units indicates the cutoff point for a positive antibody response to *B. burgdorferi* (shaded area). (B) Growth-inhibiting antibody titers of sera from the vaccinated and unvaccinated control dogs (see panel A). Arrows indicate the time of vaccination and the time of challenge.

rOspA and challenged with infected ticks were tested. All dogs immunized with rOspA showed maximum growth-inhibiting antibody titers at the time of challenge (≥1:2,560). These titers stayed high for 3 months but gradually declined to values of between 1:640 and 1:20. The growth-inhibiting antibody responses in dogs challenged 6 months after vaccination with OspA and adjuvant were similar to the responses in dogs challenged 3 to 4 weeks after vaccination (Fig. 3B). The antibody response was independent of the dose of rOspA or of the type of adjuvant. In contrast, there was a significant difference between dogs which did or did not receive adjuvants. If OspA was administered without adjuvant, maximum titers peaked only at titers of 1:1,280 and declined rapidly afterward. Three weeks after the second vaccination at the time of challenge, a titer of 1:160 was present in eight of eight dogs tested. Three months after challenge, minimal growth-inhibiting antibody activity was detectable (Fig. 4B).

DISCUSSION

The in vitro growth inhibition or borreliacidal test appears to be an indicator of the protection of animals against experimental *B. burgdorferi* infection. Borreliacidal activity has been reported to be present in sera containing anti-OspA and anti-OspB antibodies (7, 13, 36–39, 46). However, gerbils immunized with OspC appeared to be protected from challenge infection (32), and monoclonal antibodies to a 39-kDa protein of *B. burgdorferi* (42) were borreliacidal in vitro. In addition, Aydintug et al. (2) found borreliacidal activity in sera from rhesus monkeys which were absorbed with nonlipidated OspA and OspB.

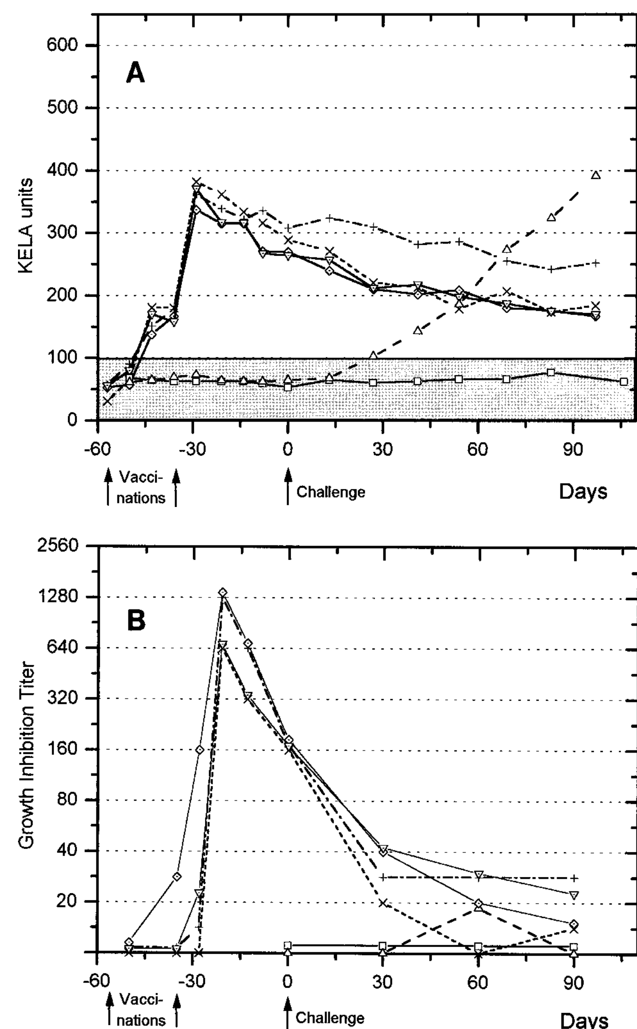

FIG. 4. (A) Antibody titers against *B. burgdorferi* in sera from dogs vaccinated with rOspA without adjuvant and control dogs determined by ELISA. Four dogs of this group received 100 μg of rOspA intramuscularly. Dogs were vaccinated twice at a 3-week interval and were challenged 5 weeks after the second vaccination. Unvaccinated control dogs were kept in the same isolation units. One of these dogs was infected (△) and one dog was not infected with *B. burgdorferi* (□). An antibody titer of 100 KELA units indicates the cutoff point for a positive antibody response to *B. burgdorferi* (shaded area). (B) Growth-inhibiting antibody titers in sera from dogs vaccinated with rOspA without adjuvant and control dogs (see panel A). Arrows indicate the time of vaccination and the time of challenge.

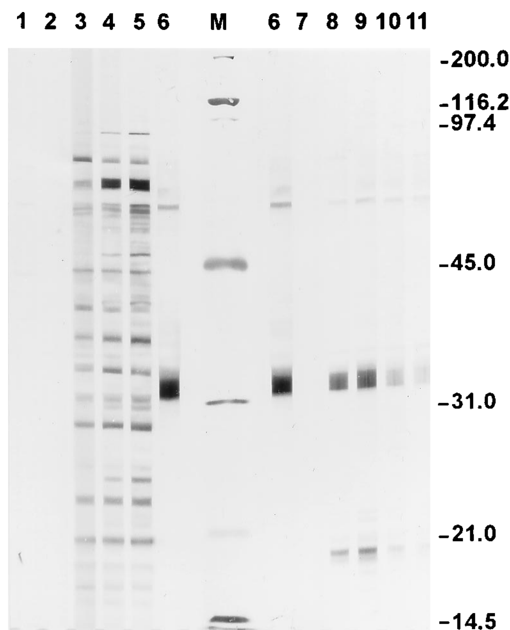

FIG. 5. Western blots of sera from a tick-infected dog and an rOspA-immunized dog. Lanes 1 to 5, immune response against *B. burgdorferi* at the time of challenge (lane 1) and 1, 3, 6, and 12 months after infection (lanes 2 to 5, respectively). Specific bands for OspA and OspB are missing from the 32- and 34-kDa regions. In contrast, lanes 7 to 11 show the immune response of an immunized dog at the time of the first and second vaccinations (lanes 7 and 8, respectively), time of challenge (lane 9), 6 and 12 weeks after challenge (lanes 10 and 11, respectively). A monoclonal antibody against OspA which specifically recognizes OspA in the 32-kDa region and dimers of OspA in the 64-kDa region was used in lane 6. A molecular mass marker is included in lane M. Numbers on the right are in kilodaltons.

In our experiments, low levels of growth-inhibiting antibody activity (<1:80) were found in all 46 nonvaccinated dogs tested for a period of up to 1 year after tick exposure (Fig. 2B). That finding corresponded well with the absence of OspA and OspB antibodies, as seen in Western blots (Fig. 5). Antibodies to borrelia antigens in the 19-, 23-, 26-, and 29-kDa regions were present in the sera, which may correspond to antibodies to OspE, OspC, OspF, and OspD, respectively. These antibodies could be responsible for low levels of growth inhibition, since antibodies against OspE and OspF can reduce the number of borreliae in ticks (28). However, high levels of antibodies, as determined by KELA, were present in the sera of nonvaccinated dogs up to 1 year after tick exposure (Fig. 2A) and were always accompanied by persistent infection of the animal.

In contrast, all dogs vaccinated with rOspA developed high levels of antibody titers, as determined by growth inhibition (Fig. 3B and 4B). The administration of adjuvants enhanced the immune response to rOspA such that dogs receiving a preparation of rOspA and adjuvant developed higher growth-inhibiting antibody titers than dogs receiving OspA only. Similar observations were made when saponin QS-21 was used as an adjuvant (25). All immunized dogs were protected from infection and disease when they were challenged with infected ticks. It is not known whether the inactivation of the spirochetes occurs in the host or in the tick, but there is evidence that the organism is destroyed within the tick (15, 28). In this way migration of borreliae from the midgut to the salivary gland may be inhibited when ticks are feeding on hosts with borreliacidal antibodies.

Borreliacidal activity seems to be complement dependent in sera from humans and hamsters (19, 20, 24), while rat sera and some monoclonal antibodies kill without complement (10, 31). However, Sadziene et al. (37) also found complement-independent growth inhibition with human and mouse sera. Canine antibodies required complement to elicit growth inhibition. Activated complement alone did not have any effect on the survival of *B. burgdorferi*, in accordance with other reports (7). As reported earlier for human and rodent sera (7, 24, 26, 32), growth inhibition by dog sera was not completely borreliacidal. When the plates were kept for extended time periods (more than 7 days), bacterial growth eventually occurred, as indicated by a change in the color of the medium.

We have used the change in color of phenol red to yellow, as a result of pH changes in the medium, as an indicator for metabolically active spirochetes. At the time of reading, yellow medium contained 10-fold more borreliae than red medium with growth-inhibiting antibodies when the medium was inoculated with the same number of borreliae at the beginning of the test. We have not observed a difference in growth inhibition of high-passage-number (passages 42 to 49) or low-passage-number (passage 3) *B. burgdorferi*. Although low-passage-number spirochetes replicated more slowly than high-passage-number spirochetes, the end results were comparable (Fig. 1).

There appears to be a correlation between the absence of OspA and OspB antibodies, low levels of borreliacidal antibodies, and the persistence of borreliae. When dogs were needle inoculated with low-passage-number (passage 2) *B. burgdorferi* isolates, they developed high levels of antibodies, as detected by KELA, as well as antibodies detected by growth inhibition and antibodies to OspA and OspB within 1 or 2 weeks after inoculation (data not shown). However, persistent infection was not established, joint disease did not occur, and antibody levels gradually declined over a 6-month period (1). In contrast, dogs that were exposed to infected ticks had persistent infections for at least 1 year. Antibody levels determined by KELA remained high throughout the 1-year observation period, but OspA and OspB antibodies were not detectable and growth-inhibiting antibody levels remained less than 1:80. The discrepancy in host antibody responses between needle and tick inoculation has been observed in a variety of species. It was first reported in dogs by Greene et al. (17) in 1988 and was found in mice (16), hamsters (35), and rhesus monkeys (2). In humans delayed antibody responses to OspA and OspB after tick exposure are well known (3, 11, 14, 47). It seems that the naive immune system is not able to eliminate low numbers of tick-inoculated borreliae. Once the infection with *B. burgdorferi* is established, the immune system has no means of changing the status quo. Even a vaccination with OspA after an established *B. burgdorferi* infection does not clear the infection (12).

Several explanations have been offered to explain why borreliacidal anti-OspA and anti-OspB antibodies are produced only by needle inoculation of borreliae in high numbers from cultures. Gern et al. (16) expressed the opinion that the failure of OspA and OspB recognition is merely a matter of the number of spirochetes and that needle inoculation of low numbers of *B. burgdorferi* corresponded to the numbers inoculated by tick infection. However, there is still no explanation why persistent infection with replication of *B. burgdorferi* in humans and dogs after exposure to ticks does not result in an appropriate, protective production of OspA and OspB antibodies. The host immune response may be altered by tick saliva (33, 34, 48), but it would not explain the selective suppression of OspA and OspB over extended time periods. Spirochetes may be altered when they migrate from the midgut of the tick to the salivary gland. The masking of OspA and OspB by host proteins, as suggested by Simon et al. (45), host feedback mech-

anisms, or suppression of OspA and OspB upon infection might be other explanations. However, OspA antigens have been found in borreliae isolated from salivary glands (4). Additionally, small quantities of anti-OspA and OspB immunoglobulin M antibodies have been detected in immune complexes by biotin-avidin Western blots during the early stage of Lyme disease (41).

We have not tested any possible cross-reactions (or failures) with other borrelia strains. Strain differences appear to be more of a problem in Europe and Asia than in North America, where the B31-type *B. burgdorferi* sensu stricto appears to dominate (23).

Lack of sensitivity resulting from the high spirochetal inoculum renders the colorimetric growth inhibition test inefficient for diagnostic purposes. As seen in our experimental dogs, the levels of growth-inhibiting antibodies were low or the antibodies were missing after exposure to ticks. Eventually, a more sensitive test based on flow cytometry used by Callister et al. (6) may provide a helpful tool for the diagnosis of early Lyme disease. At this time it is not clear whether this method will be applicable during the early stage of the disease, since it is not known when borreliacidal antibodies appear in detectable quantities. On the other hand, the color-based growth inhibition test is very useful for monitoring the level of protective antibodies after vaccination. In contrast to laboratory conditions, one must assume that people and animals in the field may be confronted with multiple exposures months after the immunization. It seems possible that a decrease in borreliacidal antibody levels over time can render individuals susceptible to infection, even if antibody levels determined by ELISA stay high. To increase the reliability of a vaccine, it appears necessary to establish guidelines which help to define the levels of protective antibodies. The growth inhibition test can help to determine the efficacy of a vaccine and should be used in further studies.

ADDENDUM

Since the manuscript was completed, results on two OspA-immunized and subsequently twice-exposed dogs became available. Both dogs were immunized twice with 100 μg of rOspA without adjuvant intramuscularly at a 3-week interval. Five weeks after the last vaccination the dogs were challenged with 15 female ticks and 7 male ticks. Monthly skin biopsy specimens from these dogs were culture negative for *B. burgdorferi* during the following 6 months. A second challenge 184 days after the first challenge with 15 female ticks and 7 male ticks resulted in persistent infection of one dog. Skin biopsy and tissue specimens from this dog were culture positive for *B. burgdorferi* within 1 month after exposure.

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