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Long-Term Protection of Mice from Lyme Disease by Vaccination with OspA

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Mice vaccinated with recombinant outer surface protein A (OspA) have been shown to be protected from infection with *Borrelia burgdorferi*, the agent of Lyme disease, when sacrificed 14 days after challenge with an intradermal inoculum of the spirochete. To determine whether infection was not merely delayed and that protection was long-lasting, we sacrificed vaccinated mice 60, 120, and 180 days after challenge; and to determine whether vaccinated mice retained their immune state over long periods, we challenged mice with *B. burgdorferi* 60, 90, 120, and 150 days after vaccination. The results of both groups of experiments show that the mice remained free from infection and disease and extend the usefulness of OspA as a vaccine candidate for Lyme borreliosis.

Lyme borreliosis is a multisystem infection caused by the spirochete *Borrelia burgdorferi*, which is transmitted to humans and animals mostly by *Ixodes dammini* ticks and can cause disease involving the joints, heart, and nervous system (22). An immunocompetent model of Lyme borreliosis has been developed in the C3H/He mouse which partly resembles human infection (6). Using this model, mice intradermally inoculated with 10^4 *B. burgdorferi* N40 spirochetes develop arthritis and carditis which is evident within 14 days after infection, at which time *Borrelia* spirochetes can be readily cultured from the blood and spleen (8).

Outer surface protein A (OspA), the major outer membrane protein of *B. burgdorferi* (4), plays a role in protective immunity to spirochete infection. We have shown that C3H/He mice actively immunized with a preparation of recombinant OspA from *B. burgdorferi* N40 are protected from infection and disease when the animals are sacrificed 14 days after challenge inoculation with *B. burgdorferi* N40 (12). Protection also extended to *B. burgdorferi* B31 and CD 16, suggesting that there is some degree of cross protection (12). Antibody to OspA is important in protective immunity, as serum from mice actively immunized with recombinant OspA-N40 prevents infection when transferred to naive mice; and in both the C3H/He and CB.17 severe combined immunodeficient (*scid*) mouse, passive immunization with monoclonal antibodies to OspA is protective (12, 16, 20).

While the initial vaccination experiments are encouraging, it is unclear whether immunization with OspA induces long-lasting protective immunity, because it has been noticed that *B. burgdorferi* can persist in its hosts for long periods. In human disease, spirochetes have been visualized or cultured from blood, synovial fluid, cardiac tissue, cerebrospinal fluid, and dermatologic lesions at various time points after infection (9–11, 15, 21). In one case report, organisms were isolated from a patient with acrodermatitis chronica atrophicans 10 years after the initial infection (1). Similarly, in experimental hamster and mouse models of Lyme borreliosis, animals may remain chronically infected (6, 7, 17, 18). In the C3H/He mouse, spirochetes may be isolated from cultures of the blood, spleen, and bladder up to 1 year after infection (5).

It has also been reported that a closely related spirochete, *Borrelia hermsii*, the agent of relapsing fever, has the capacity to alter the antigens present on its outer membrane by using mechanisms similar to those of the trypanosomes (3, 14). Antigenic variation enables *B. hermsii* to evade the host immune response and survive, accounting in part for the relapsing bouts of fever that occur during infection. While it has not been shown that *B. burgdorferi* has the ability to alter its surface membrane in a similar manner, the chronic nature of borreliosis in both animals and humans suggests that the protection afforded mice vaccinated with OspA when sacrificed 14 days after challenge might not necessarily extend to later time points. We therefore conducted vaccination studies in which mice were sacrificed at later time points to determine whether immunization with OspA induces long-lasting protective immunity in the C3H/He mouse. Furthermore, mice were challenged with *Borrelia* spirochetes up to 150 days after vaccination to determine whether vaccinated animals retain their immune status over time.

MATERIALS AND METHODS

Mice. Three-week-old C3H/HeJ mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. They were shipped in filter crates and housed in microisolator cages. Three to five mice were placed in individual cages. Food and water were provided ad libitum. Mice were killed with carbon dioxide gas.

B. burgdorferi. Isolates of *B. burgdorferi* N40 and B31 with proven infectivity and pathogenicity in C3H/He mice were used. These *B. burgdorferi* isolates had been passaged in vitro three times. The spirochetes were grown to the log phase in modified Barbour-Stoenner-Kelly (BSK) medium (2) at 32°C and diluted to the appropriate concentration (10^5 spirochetes per ml) in BSK medium.

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Recombinant OspA. The recombinant OspA-N40 glutathione transferase fusion protein was expressed and purified from transformed *Escherichia coli* as previously described (12).

Vaccination of C3H/He mice. In the first series of experiments, immunized mice, challenged with spirochetes, were sacrificed at different time points to determine whether protection was long-lasting. Four-week-old mice were immunized intradermally with (0.1 ml) 20 μg of purified recombinant OspA glutathione transferase fusion protein in complete Freund's adjuvant. Mice were boosted with a similar preparation of OspA in incomplete Freund's adjuvant on days 14 and 28. Control mice were immunized with purified recombinant glutathione transferase in an identical fashion. Mice were challenged with an intradermal syringe inoculation (0.1 ml) of 10^4 *B. burgdorferi* N40 or B31 spirochetes in BSK medium 14 days after the final boost and sacrificed 60, 120, or 180 days later.

In the second series of experiments, designed to determine whether vaccinated mice retain their immune status over time, OspA-immunized and control mice were challenged with an identical inoculum of N40 spirochetes 60, 90, 120, and 150 days after the final boost and sacrificed 14 days later.

In both series of experiments, cultures of the blood, spleen, and bladder were incubated in BSK medium at 32°C for 2 weeks. Random cultures examined at 2 weeks were then reexamined at 4 weeks to ensure that spirochetes were detected in all positive cultures. A 100-μl sample of blood, obtained by cardiac exsanguination, was placed in 7 ml of BSK medium. The spleen was homogenized in 3 ml of BSK medium in a ground glass tissue grinder, and one-half of the suspension was placed in 5.5 ml of BSK medium. Bladder cultures were prepared in an identical manner as the spleens. Cultures of skin were also obtained from mice sacrificed 180 days after challenge with spirochetes. An ear punch from each mouse was placed in 70% ethanol for 1 s and then washed in BSK medium for 5 s. Individual ear punches were then placed in 7 ml of BSK medium and cultured for 2 weeks. One or two cultures of each specimen (blood, spleen, bladder, and skin) were examined by dark-field microscopy. A 100-μl sample of the BSK culture medium was placed on a slide and covered with a coverslip, and 20 high-power fields were scanned per slide. Positive cultures had a total of 1 to 100 spirochetes in 20 high-power fields and typically contained 15 organisms, while negative cultures had no organisms.

The right and left knees, both tibiotarsal joints, and the heart from each mouse were collected, processed for histology, and blindly examined microscopically for evidence of inflammation. Inflammation in any of the joints or hearts examined was considered evidence of disease.

Serum collection and immunoblots. *B. burgdorferi* N40 extract was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, using a 12.5% acrylamide gel. Briefly, 30 μg of spirochetes boiled in SDS sample buffer was loaded into each of 10 wells in a gel which was 16 cm long and 1.5 mm thick, using a Hoefer apparatus. The protein was transferred to nitrocellulose (Millipore Corp., Bedford, Mass.) overnight at 4°C. The blots were cut into strips for probing, and adjacent strips were used for immunoblotting. Mouse serum obtained at various time points was diluted 1:100 to 1:500,000 and incubated with the nitrocellulose strips. The strips were washed, incubated with a 1:5,200 dilution of alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (Amersham), and developed with nitroblue tetrazolium and 5-bromo-4-chloro-indolyl phosphate.

TABLE 1. Protection of mice vaccinated with OspA, sacrificed 60 to 180 days after challenge, from infection with *B. burgdorferi*

Challenge	Sacrifice (days)	No. positive/no. examined (controls)		
		Culture	Arthritis	Carditis
N40	60	1/24 (9/21)	1/19 (15/18)	1/19 (18/18)
N40	120	0/10 (4/9)	1/10 (6/10)	2/10 (9/10)
B31	120	0/9 (3/9)	0/9 (6/10)	2/9 (10/10)
N40	180	0/10 (4/9)	0/10 (6/9)	0/10 (9/9)
B31	180	0/5 (4/6)	0/5 (2/6)	0/5 (6/6)

RESULTS

We first determined whether mice vaccinated with OspA were protected from infection with *B. burgdorferi* N40 or B31 when sacrificed 60, 120, or 180 days after challenge with the spirochetes. The results in Table 1 indicate that at these time points, the vaccinated animals were protected from both infection and disease. At 60 days, only 1 of 24 vaccinated mice was culture positive, while spirochetes were cultured from 9 of 21 control animals ($P < 0.001$). At this time point, 1 of 19 vaccinated animals had arthritis and carditis, while disease manifestations were evident in all control animals ($P < 0.001$). At 60 days, the inflammatory response consisted mostly of a lymphoplasmacytic infiltrate indicative of a resolving process. In the experiments, random samples of the cultures examined at 2 weeks were reexamined at 4 weeks to determine whether additional positive cultures could be identified. In all cases, the results of cultures examined at 2 and 4 weeks were identical.

At 120 days, the vaccinated mice were also protected from challenge with spirochetes, as none of the vaccinated animals were culture positive, whereas 7 of 18 control animals were infected ($P < 0.05$). Similarly, the OspA-immunized mice were protected from disease, as 4 of 19 vaccinated animals had evidence of arthritis or carditis, whereas 19 of 20 control animals had disease ($P < 0.001$). More specifically, two vaccinated mice challenged with B31 and two mice challenged with N40 spirochetes showed evidence of carditis, and one of the mice challenged with N40 also had arthritis.

At 180 days, the vaccinated animals were fully protected from both infection ($P < 0.05$) and disease ($P < 0.001$), compared with 8 of 15 control mice that were culture positive, 8 of 15 with arthritis, and 15 of 15 with carditis. At this time point, the disease manifestations were consistent with a resolving process, and in some cases chronic scarring without active inflammation was the only evidence of disease.

Serum samples from six different vaccinated mice drawn 60 and 180 days after challenge with *B. burgdorferi* showed antibodies to OspA (31 kDa) but not to other spirochetal antigens, including flagellin (41 kDa) and OspB (34 kDa). In contrast, serum samples from six different control mice drawn at the same time had antibodies to numerous spirochetal proteins. Interestingly, serum samples from *B. burgdorferi*-infected control mice did not contain antibodies to OspB and only had a weak response to OspA. A representative immunoblot with sera obtained from an OspA-vaccinated mouse and a control mouse 180 days after challenge is shown in Fig. 1. Sera from normal uninfected mice had no reactivity with *B. burgdorferi* on immunoblots and is therefore not shown.

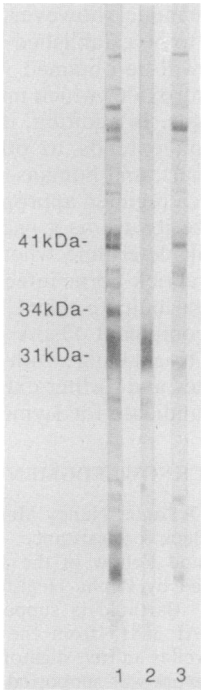

FIG. 1. Immunoblot of a protein extract of *B. burgdorferi* N40 probed with serum from OspA-vaccinated or control mice, drawn 6 months after challenge with *B. burgdorferi* N40. Lane 1, probed with rabbit anti-*B. burgdorferi* N40 serum (diluted 1:200). Lane 2, probed with serum from an OspA-vaccinated mouse (diluted 1:200). Lane 3, probed with serum from a control mouse (diluted 1:200). The band at 31 kDa is OspA, the band at 34 kDa is OspB, and the band at 41 kDa is flagellin.

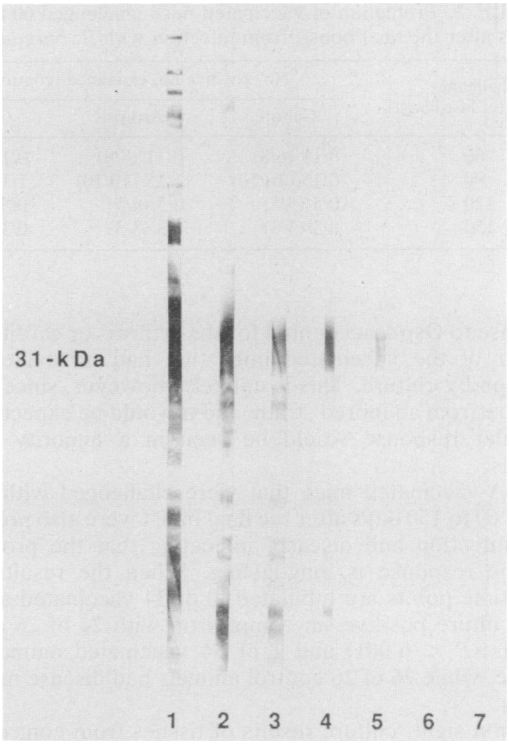

FIG. 2. Immunoblot of a protein extract of *B. burgdorferi* N40 probed with various dilutions of serum from a mouse immunized with OspA. The serum was drawn 2 months after the final booster immunization with OspA. Lane 1, probed with rabbit anti-*B. burgdorferi* N40 serum (diluted 1:100). Lanes 2 to 6, probed with serum from a mouse immunized with OspA at various dilutions: lane 2, 1:200; lane 3, 1:1,000; lane 4, 1:10,000; lane 5, 1:50,000; lane 6, 1:500,000. Lane 7, normal mouse serum (diluted 1:100). The band at 31 kDa is OspA.

To determine whether the immune response is long-lived and sufficient for protection, we challenged mice with *B. burgdorferi* 60, 90, 120, and 150 days after immunization. Sixty days after the second boost with recombinant OspA-N40, mice were bled and antibodies to OspA could be detected at a dilution of 1:50,000 when used to probe *B. burgdorferi* N40 on immunoblot (Fig. 2). At high titers, the OspA antibodies bind to lower-molecular-weight proteins as well as OspA in *B. burgdorferi*. At low titers (1:50,000 dilution), the serum only binds OspA. The titer decreased to 1:20,000 at 90 days and 1:10,000 at 120 and 150 days, indicating that a high antibody titer persisted. The results in Table 2 indicate that the vaccinated mice were protected from infection ($P < 0.001$) and disease ($P < 0.001$) compared with controls when sacrificed 14 days after challenge. The carditis and arthritis in these older control animals were identical to but less severe than those in younger mice as previously reported (12).

DISCUSSION

The results indicate that the protective effect of recombinant OspA administered in Freund's adjuvant to C3H/He mice is long-lasting, suggesting that the vaccinated animals were completely protected against challenge infection. Vaccinated mice which were sacrificed 60, 120, or 180 days after challenge with the spirochetes were also protected from both infection and disease. When the data from all the time points are combined, only 1 of 58 vaccinated animals was culture positive, while 24 of 55 control animals were culture positive, and only 5 of 54 vaccinated animals had disease manifestations, whereas 53 of 54 control animals had arthritis and/or carditis. Furthermore, serum from the OspA-vaccinated mice had antibodies to OspA but not to other spirochetal antigens, suggesting that the *Borrelia* spirochetes were eliminated before a strong immune response was elicited.

In all the experiments in which mice were sacrificed at time points later than 14 days, the disease manifestations are a more sensitive indicator of infection than culture, and we hypothesize that the five vaccinated animals that had disease manifestations were infected with *Borrelia* spirochetes. Since all the vaccinated animals sacrificed at the latest time point, 180 days, were fully protected from both infection and disease, we believe that the five vaccinated mice with evidence of disease represent animal-to-animal experimental fluctuation and did not mount a strong antibody response to OspA. We had previously shown that animals immunized with *E. coli* expressing OspA developed antibodies to OspA (detected at a titer of 1:10,000) and were partially protected from disease. In contrast, mice immunized with recombinant OspA in Freund's adjuvant developed antibodies to OspA (detected at a titer of 1:64,000) and were fully protected from both infection and disease (12). Since the antibody titers were not measured for each individual mouse, it is possible that a few mice did not mount titers sufficient for full protection. An alternative explanation is that an immune

TABLE 2. Protection of vaccinated mice challenged 60 to 150 days after the final boost, from infection with *B. burgdorferi*

Challenge (days after last boost)	No. positive/no. examined (controls)		
	Culture	Arthritis	Carditis
60	0/11 (6/8)	0/11 (8/8)	0/11 (7/8)
90	0/15 (10/10)	1/15 (10/10)	1/15 (9/10)
120	0/5 (5/5)	0/5 (4/5)	0/5 (5/5)
150	0/3 (3/3)	0/3 (3/3)	0/3 (3/3)

response to OspA accounted for the arthritis or carditis seen in four of the vaccinated mice that had no evidence of infection by culture. This is unlikely, however, since all the mice are from an inbred strain, and it would be expected that a similar response would be seen in a majority of the animals.

OspA-vaccinated mice that were challenged with spirochetes 60 to 150 days after the final boost were also protected from infection and disease, indicating that the protective immune response is long-lasting. When the results from these time points are tabulated, 0 of 34 vaccinated animals were culture positive, in comparison with 24 of 26 control animals ($P < 0.001$) and 1 of 34 vaccinated animals had disease, while 26 of 26 control animals had disease manifestations.

At first sight, culture results of tissues from control mice suggest a lower rate of infection compared with disease results, for spirochetes are more readily cultured from various organs of control mice 14 days after challenge than at 60 to 180 days after challenge (5). In the first series of experiments, we cultured tissues and examined joints and hearts microscopically at 60, 120, and 180 days after challenge infection. The relatively low yield of culture-positive control mice in these experiments reflects the fact that as the duration of infection increases, mice clear themselves of spirochetemia and the rate of isolation from various target organs declines (5). Furthermore, the arthritis and carditis were also less pronounced at 60 days compared with earlier intervals, since these lesions peak at 14 to 30 days and then regress, with only residual lymphoplasmacytic infiltrates in target tissues at later intervals. In the second series of experiments, we cultured tissues and examined joints and hearts microscopically at 14 days after challenge in mice that had been vaccinated for 60 to 150 days. Culture results were more consistently positive for the reasons stated above.

The present report extends previous studies on the longevity of protection performed by Johnson et al. (13) and Schmitz et al. (19). Johnson et al. (13) showed that hamsters actively immunized with a single dose of inactivated *B. burgdorferi* were protected from challenge 30 days after vaccination; however, resistance to infection decreased considerably 90 days after vaccination. Passive immunization experiments by Schmitz et al. (19) indicated that serum from hamsters infected with *B. burgdorferi* for up to 1 year could confer complete protection on irradiated recipients challenged with the spirochete. High levels of borreliacidal activity were apparent early in infection and decreased over time. Our study indicates that active immunization with OspA elicits long-lasting protection. These superior results are most likely due to the use of a single recombinant antigen (OspA) administered in three doses with adjuvant rather than a single vaccination with a whole-cell extract of the organism or passive immunization with sera from naturally infected animals.

In our experimental model, however, Freund's adjuvant is used, and it remains to be established that such a sustained protective response will be obtained with other adjuvants, such as aluminum hydroxide, which may be used in domestic animals and humans. In addition, it must be shown that long-lasting protection extends to other strains of mice, other species of animals, and humans. Further studies with recombinant OspA in a regimen appropriate for humans will be needed to optimize its use as a vaccine in humans, and additional studies will determine whether vaccination protects mice from natural tick-borne infection with *B. burgdorferi*. The present data indicate that C3H/He mice actively immunized with recombinant OspA develop long-lasting protective immunity to challenge with an intradermal inoculum of 10^4 spirochetes and further extend the usefulness of OspA as a vaccine candidate for Lyme borreliosis.

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