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OspA Vaccination of Mice with Established *Borrelia burgdorferi* Infection Alters Disease but Not Infection

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C3H mice were actively immunized with outer surface protein A (OspA) at different intervals after infection with *Borrelia burgdorferi* to determine the effect of postexposure vaccination on the course of murine Lyme borreliosis. Mice were vaccinated with an OspA-glutathione transferase fusion protein or glutathione transferase (control) in complete Freund's adjuvant; vaccination was followed by two weekly booster injections in incomplete adjuvant. Two weeks after the final booster injection, organs were cultured for *B. burgdorferi* (blood, spleen, skin, and bladder) and examined for histopathology (joints and hearts). When vaccination was commenced in the early stages (5 to 14 days) of infection, active immunization with OspA partially cleared spirochetes from the bloodstream but did not eliminate them from other tissues or alter the course of joint or heart disease. Commencement of vaccination at 60 days after infection (at which time joint or heart disease is resolving), however, reduced both the number of mice and individual joints with arthritis, a result suggesting an acceleration of the resolution phase of the disease. Postexposure immunization with OspA may partially alter the course of murine Lyme arthritis but does not eliminate infection.

The spirochete *Borrelia burgdorferi* causes Lyme borreliosis, which can result in acute and chronic diseases (23). In the early stages of Lyme borreliosis, erythema migrans and flu-like symptoms predominate (23). Persistent *B. burgdorferi* infection, however, sometimes causes chronic disease, mostly involving the joints and nervous system (23). Treatment of the later stages of Lyme disease with antibiotics is sometimes unsuccessful, a fact that makes necessary the development of additional methods of prevention of and therapy for *B. burgdorferi* infection.

The chronic nature of Lyme borreliosis and the difficulty in recognizing the initial tick bite have led to research directed towards the development of a Lyme disease vaccine by use of hamster and mouse models of *B. burgdorferi* infection (1, 3, 4, 6, 14, 15, 18). Research to determine the *B. burgdorferi* antigens important in eliciting protective immunity has used models of Lyme disease in C.B.17 mice with severe combined immunodeficiency (scid) and immunocompetent C3H mice. These models are useful, for both types of mice develop arthritis and carditis; however, immunocompetent C3H mice can be used for active immunization studies (8). Indeed, the course of *B. burgdorferi* infection in C3H mice partially mimics human disease (1, 5). Time points of 5, 14, and 60 days after infection represent specific stages in the evolution of Lyme borreliosis in C3H mice. Five days after an intradermal inoculation, mice are spirochetemic. Fourteen days after an intradermal inoculation, spirochetes can be cultured from the blood, spleen, bladder, and other sites, and polyarthritis and carditis are evident. Sixty days after an intradermal inoculation, joint or heart disease is resolving, but mice remain infected. Joint or heart lesions at this stage have subsided, with lymphoplasmacytic infiltrates rather than the acute inflammation seen at 14 days. Disease resolution is apparently immune system mediated, as scid mice develop progressive disease (6). Furthermore, disease resolution is not permanent, as a fraction of mice develop recurrent bouts of acute joint or heart disease at intervals of up to 1 year after inoculation, while virtually all remain persistently infected (4).

Using the C3H mouse model of Lyme borreliosis, we showed that outer surface proteins A and B (OspA and OspB, respectively) are vaccine candidates for Lyme disease (8, 10). Our studies showed that vaccination of C3H mice with recombinant OspA from *B. burgdorferi* N40 protected animals against infection. Further time course experiments indicated that the protection was long lasting (9). Studies then showed that N40 OspA-mediated protection extended to selected *B. burgdorferi* strains from the northeastern and midwestern United States and to *B. burgdorferi* strains isolated from both ticks and patients (8, 11). Protection did not extend, however, to *B. burgdorferi* 25015, a variant strain from New York that differs from N40, mostly in the amino acids in the carboxyl-terminal regions of OspA (11). We also showed that vaccination of mice with OspB protected against challenge with *B. burgdorferi* (10).

Passive immunization studies have indicated that humoral immunity is important in OspA-mediated protection. Passive immunization with either monoclonal antibodies or polyclonal antibodies to OspA, in addition to anti-*B. burgdorferi* sera, protected both immunocompetent C3H and C.B.17 scid mice from infection (10, 17, 20). Mapping studies have indicated that protective monoclonal antibodies bind to regions within the carboxyl terminus of OspA, a result suggesting that epitopes important in eliciting a protective immune response are within these regions (19). While passive immunization is protective prior to exposure to the spirochete, it has not been shown to be effective after infection. Studies with hamsters showed that passive immunization of animals with anti-*B. burgdorferi* sera immediately after *B. burgdorferi* infection was not capable of clearing spirochetes (15). Similarly, postinfection immunization of C.B.17 scid mice with antibody just following *B. burgdorferi* challenge did not eradicate the infection (20).

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Recent studies, however, have indicated that postexposure vaccination can alter the course of infection in other diseases. Redfield and colleagues suggested that postexposure vaccination of human immunodeficiency virus (HIV)-positive patients with recombinant gp160 can invoke new antibody responses and T-cell proliferative responses, as well as stabilize CD4 counts in infected individuals (16). Similarly, Gibbs et al., using postexposure vaccination of HIV-seropositive and -seronegative chimpanzees, showed that therapeutic vaccination decreases the ability to recover virus from infected animals (13). Redfield et al. (16) and Gibbs et al. (13) both suggested that postexposure vaccination can influence the course of HIV infection. In Lyme borreliosis, humans are initially seronegative for OspA and OspB (12). Therefore, postexposure vaccination with the Osp proteins and the production of Osp-specific antibodies could potentially alter the course of disease. We have now performed postexposure active immunization studies by using a murine model of bacterial arthritis. In this study, we actively immunized *B. burgdorferi*-infected C3H mice with OspA at intervals after infection to determine whether therapeutic vaccination influences the course of murine Lyme borreliosis.

MATERIALS AND METHODS

Mice. Three-week-old female virus antibody-free C3H/HeJ mice were obtained from Jackson Laboratory, Bar Harbor, Maine. They were shipped in filtered crates and housed in microisolator cages. Food and water were provided ad libitum. Mice were killed with carbon dioxide gas.

B. burgdorferi. *B. burgdorferi* N40 is a low-passage in vitro isolate with previously proven infectivity and pathogenicity in C3H mice (1, 3, 5, 6). The spirochetes were grown to the log phase in modified Barbour-Stoenner-Kelly (BSK II) medium and counted in a hemocytometer under dark-field microscopy.

Recombinant OspA fusion protein. Recombinant OspA was expressed and purified as a fusion protein with glutathione transferase (GT) as previously described (8). In brief, the OspA gene from *B. burgdorferi* N40 was cloned into plasmid PGEX-2T (Pharmacia) in frame with the gene for GT. The recombinant OspA fusion protein was expressed in *Escherichia coli* DH5α and purified from a bacterial lysate by use of a glutathione column (Pharmacia). Recombinant GT prepared in *E. coli* with plasmid PGEX-2T was used as a control antigen (8).

Histology. Hearts and rear legs were immersion fixed in neutral buffered formalin (pH 7.2). Bones were decalcified and tissues were paraffin embedded and processed as previously described (1, 5). Heart sections were oriented through the aortic valve, atria, and ventricles. Knees and tibiotarsal joints were sagitally sectioned. The incidence of active arthritis in both tibiotarsal joints of all mice was tabulated. All sections were examined blindly as to interval and treatment group.

Infection of C3H/HeJ mice with *B. burgdorferi* and postexposure immunizations. Groups of five mice were infected with an intradermal inoculation of 10^4 *B. burgdorferi*. At selected time points (representative of the stages of evolution of Lyme borreliosis in C3H mice) after infection (5, 14, and 60 days), the mice were actively immunized with 10 μg of recombinant OspA fusion protein in complete Freund's adjuvant and given booster injections with the same amount of protein in incomplete Freund's adjuvant twice at weekly intervals. Five days after *B. burgdorferi* inoculation represents initial infection, 14 days after infection is acute disseminated disease, and at 60 days after infection, arthritis and carditis are resolving. Control mice were actively immunized with GT in an identical manner. The OspA and GT groups were inoculated simultaneously with the same *B. burgdorferi* preparation within each experiment. Additional groups of mice were passively immunized with 0.1 ml of anti-*B. burgdorferi* hyperimmune sera, prepared in rabbits as previously described (8), at the same time points as the actively immunized groups to determine whether the transfer of antibody produced effects similar to those produced by vaccination.

Seven days after the last booster injection (26, 35, and 81 days after infection), the mice were killed. Sections of joints (both knees and tibiotarsal joints) and hearts were examined for inflammation. Arthritis was blindly graded on a scale from 0 to 3: grade 0 represented a lack of inflammation, grades 1 and 2 indicated mild inflammation and moderate inflammation, respectively, and grade 3 signified severe inflammation. Only active arthritis was scored and tabulated. Resolved lesions with focal lymphoplasmacytic infiltration and synovial scarring but without active exudation were not included. Carditis was tabulated as active when there was acute neutrophilic inflammation of the aortic root and chronic when there was lymphoplasmacytic infiltration of the aorta and connective tissue of the heart base as described previously (1). Blood, spleen, skin, and bladder were collected from the mice and cultured in BSK II medium as described previously (8). Cultures were incubated for 2 weeks and examined by dark-field microscopy. Twenty high-power fields were scanned per culture.

Immunoblots. *B. burgdorferi* extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 12.5% acrylamide gel as described previously (8). The protein was transferred to nitrocellulose and cut into strips for probing. Mouse serum obtained 7 days after the final immunization was diluted from 1:100 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, and developed with nitroblue tetrazolium and 5-bromo-4-chloro-indolyl phosphate.

RESULTS

Groups of 5 C3H mice were injected with an intradermal inoculation of 10^4 *B. burgdorferi*. At 5, 14, and 60 days after infection with *B. burgdorferi*, the mice were actively immunized with 10 μg of recombinant N40 OspA in complete Freund's adjuvant and given two boosters at weekly intervals. At 5 days after infection, the initial spirochetemia was apparent; at 14 days, disseminated acute disease was evident; and at 60 days, inflammation was resolving. These time points represent the evolution of disease in C3H mice. Seven days after the final booster, the OspA-immunized mice produced high titers of OspA antibody, detectable at a dilution of 1:10,000 on immunoblots (data not shown). Seven days after the final immunization, the mice were sacrificed, specimens were cultured for spirochetes, and the joints and hearts were examined microscopically for inflammation. The results represent a combination of data from research experiments, all of which yielded similar data.

Active immunization with OspA at 5, 14, or 60 days did not eliminate spirochetes in the infected mice (Table 1). Active immunization at 5 and 14 days after infection resulted in a partial clearance of spirochetes from the bloodstream and a decrease in the overall numbers of spirochetes cul-

TABLE 1. Spirochete positive C3H mice, immunized with OspA or anti-*B. burgdorferi* sera, after infection with *B. burgdorferi*[a]

Immunization	Days after *B. burgdorferi* infection	No. of positive cultures/no. of cultures examined for:					
		Blood	Spleen	Skin	Bladder	All four tissues	*B. burgdorferi*-positive mice[b]
Active							
OspA	5	2/13	4/13	5/9	5/8	16/43	9/13
	14	2/9	3/10	4/10	2/10	11/39	7/10
	60	0/17	0/15	2/8	5/17	7/57	7/17
GT (control)	5	7/13	6/13	7/8	6/8	26/34	12/13
	14	4/10	4/11	2/7	6/10	16/38	9/10
	60	1/17	1/16	5/9	7/16	14/58	11/17
Passive, anti-*B. burgdorferi* sera	5	1/8	5/7	5/6	6/7	17/28	9/9
	14	0/5	4/5	1/3	0/5	5/18	4/5

[a] At various time points (days after *B. burgdorferi* infection), the animals were actively immunized with OspA or passively immunized with anti-*B. burgdorferi* and given two boosters at weekly intervals. Control mice were actively immunized with GT in an identical fashion. One week after the final booster, the mice were sacrificed and various tissues and fluids were cultured for *B. burgdorferi*. Cultures contaminated with common bacterial growth were excluded from the analysis.

[b] Number of mice for which *B. burgdorferi* could be cultured from at least one site/number of mice examined.

tured from various sites. Significantly fewer mice vaccinated with OspA at 5 days were spirochetemic, in comparison with GT-vaccinated controls (chi-square test; $P < 0.05$). For these mice, 16 of 43 cultures for all sites (blood, spleen, skin, and bladder) were positive for the experimental group and 26 of 42 cultures were positive for the control group (chi-square test; $P < 0.05$). Similarly, for mice vaccinated at 14 days after infection, 11 of 39 total cultures were positive for the experimental group and 16 of 33 cultures were positive for the control group, but this difference was not statistically significant (chi-square test). For mice vaccinated at 60 days after infection, spirochetes were most readily isolated from the skin and bladder. For mice vaccinated with OspA at all intervals, the number of positive cultures for one or more sites was lower than that for GT-vaccinated controls ($P < 0.05$), and the combined blood culture data for all intervals indicated fewer spirochetemic mice in the OspA-vaccinated group than in the control group ($P < 0.05$).

Additional mice were passively immunized with hyperimmune anti-*B. burgdorferi* sera 5 or 14 days after challenge with *B. burgdorferi* and sacrificed 7 days after the final immunization to determine the effect of antibody transfer. Passive immunization resulted in a partial clearance of spirochetes from the blood (Table 1). The persistence of *B. burgdorferi* in the other organ sites, however, was not affected.

Arthritis and carditis were present in most mice, regardless of interval or vaccine treatment (Table 2). In mice immunized at 5 and 14 days, disease of tibiotarsal joints and tendon or ligament sheaths (herein collectively termed arthritis) involved exudation of fibrin and neutrophils into their lumina, and the synovium showed hypertrophy and was hyperplastic (Fig. 1). All mice and nearly all of their tibiotarsal joints showed active disease, with moderate inflammation (grade 2), in both vaccine groups at these time points. In contrast, fewer mice vaccinated at 60 days had arthritis, positive mice had fewer affected joints, and disease was mild (grade 1 inflammation) (Fig. 2), indicative of resolution. However, both the number of mice with arthritis and the number of affected joints were smaller in OspA-vaccinated mice than GT-vaccinated mice (chi-square test; $P < 0.05$). The histopathology for GT-vaccinated mice at all time points was identical to that seen for nonvaccinated infected mice that we previously examined (3, 4, 5). Vaccinated noninfected mice did not develop disease. These data suggest that OspA vaccination accelerated arthritis resolution. There were no differences in the rate or nature of heart lesions among treatment groups.

DISCUSSION

Our data show that postexposure active vaccination with OspA or passive immunization with *B. burgdorferi* with antibodies is unable to eliminate spirochete infection in C3H mice. As expected, mice vaccinated with OspA prior to *B. burgdorferi* infection were protected from infection (data not shown). This result is consistent with those of postinfection passive immunization studies performed with anti-*B. burgdorferi* sera for hamsters and C.B.17 scid mice (15, 17). Therapeutic vaccination with OspA is, however, capable of

TABLE 2. Disease manifestations in C3H mice, immunized with OspA or anti-*B. burgdorferi* sera, after infection with *B. burgdorferi*[a]

Immunization	Days after *B. burgdorferi* challenge	No. of mice with the following active disease/no. examined:	
		Carditis	Arthritis (no. of individual tibiotarsal joints with active arthritis/no. examined)
Active			
OspA	5	13/13	13/13 (24/26)
	14	10/10	10/10 (19/20)
	60	14/15	8/16 (10/32)
GT (control)	5	13/13	13/13 (23/26)
	14	10/10	10/10 (18/20)
	60	16/18	16/18 (22/36)
Passive, anti-*B. burgdorferi* sera	5	9/9	9/9 (18/18)
	14	5/5	5/5 (9/10)

[a] Mice were immunized and sacrificed as described in Table 1, footnote *a*.

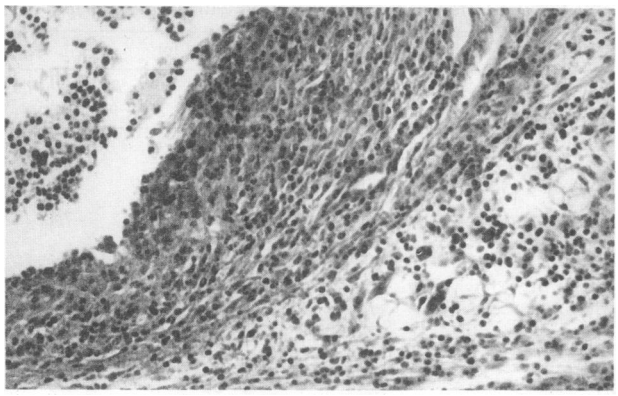

FIG. 1. Tibiotarsal synovium of a GT-vaccinated control mouse, typical of mice subjected to either OspA or GT vaccination commencing on day 5 or 14 after inoculation. The synovium is thickened, with a fibrinopurulent exudate in the lumen and intense inflammation of the surrounding connective tissue.

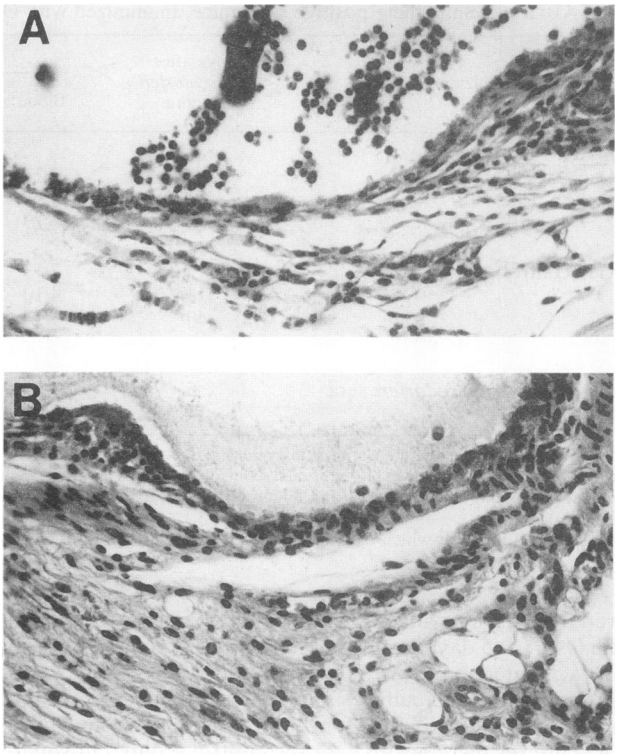

FIG. 2. (A) Tibiotarsal synovium of a GT-vaccinated control mouse, typical of mice subjected to the vaccination protocol commencing on day 60 after inoculation. Inflammation and synovial thickening are regressing. (B) Tibiotarsal synovium of an OspA-vaccinated mouse, typical of mice subjected to the vaccination protocol commencing on day 60 after inoculation. Synovial thickening and exudation are no longer present, but the periarticular connective tissue is sparsely infiltrated with mononuclear leukocytes.

partially altering the course of murine Lyme borreliosis. Immunization with OspA reduced the overall recovery of spirochetes from the various organ sites that were evaluated. In addition, postexposure vaccination was able to partially accelerate the resolution stage of active arthritis in infected mice, thereby reducing the severity of disease in half of the OspA-immunized animals. In the early stages of murine disease, 5 and 14 days following spirochete infection, vaccination with OspA had no effect on the development of arthritis or carditis. In the later stages of murine disease, which may be somewhat analogous but not identical to the later stages of human Lyme disease, therapeutic vaccination with OspA was sufficient to diminish the degree of active arthritis but had no effect on heart disease.

These findings suggest that Lyme arthritis in C3H mice is driven by the presence of spirochetes and that a protective antibody that helps reduce the spirochete burden contributes to the resolution of arthritis. These hypotheses are supported by the evolution of Lyme borreliosis in C3H mice. Five days following infection, mice are spirochetemic, but arthritis is not yet present. Fourteen days following infection, spirochetes have fully disseminated and active arthritis is maximal. At this time, antibodies to OspA and OspB have not yet developed or are present in low titers. Indeed, the early antibody response to OspA is directed toward OspA epitopes that are not protective (19). Therefore, peak disease is present at times at which protective antibodies are not readily available. In the normal course of murine Lyme borreliosis, OspA antibodies that bind protective epitopes in the carboxyl terminus of OspA develop between 30 and 60 days after infection (19), times at which it is somewhat more difficult to culture *B. burgdorferi* from infected mice. At these times, arthritis begins to resolve, suggesting that the diminution of disease manifestations parallels the development of protective OspA immunity.

In the present study, the resolution of active arthritis was hastened by postexposure vaccination. Postexposure vaccination may accelerate or increase the production of protective antibodies, which seem to eliminate spirochetes from the synovium, thereby resulting in arthritis resolution. Our data suggest that the number of culture-positive sites, including blood, is significantly diminished by vaccination but is insufficient to sterilize infected mice. This suggestion is consistent with those of previous studies showing that postexposure passive immunization could not clear spirochetes and implies that the organism is able to sequester itself at sites within the host that antibodies cannot readily access or that it is able to evade immune responses by masking its outer surface, thereby becoming inaccessible to protective antibodies.

The murine model only partially mimics human infection. Nevertheless, this study suggests that therapeutic vaccination of infected humans with late-stage Lyme arthritis may help ameliorate the disease. This suggestion is based on the hypothesis that the chronic manifestations of Lyme arthritis are caused, at least in part, by persistent infection, caused by either a highly pathogenic organism or the inability of the host to clear the infection. This hypothesis is supported by the identification of *B. burgdorferi* in skin, joint, and cardiac tissue from patients with chronic disease (2, 7, 21, 22). Postexposure hyperimmunization, enhancing the protective immune response, would then help to reduce the spirochete burden and diminish the disease manifestations. Active immunization with OspA elicits protective antibodies, including antibodies that bind epitopes in the carboxyl terminus of OspA. In this study, OspA was administered with complete Freund's adjuvant, and it is not known whether a vaccination protocol with a different regimen would provide similar results. Indeed, the efficacy of OspA as a vaccine

candidate for Lyme disease will likely be related to the method of antigen administration.

Potential reasons for the chronic manifestations of Lyme borreliosis, other than *B. burgdorferi* persistence, must also be considered. A fraction of patients (those with DR2 and DR4 major histocompatibility haplotypes) are prone to chronic, unrelenting arthritis that may have an immunopathologic component (24). If chronic Lyme borreliosis is due in part to an autoimmune reaction between host tissues and the immune response to specific *B. burgdorferi* antigens, as has been suggested by several investigators (24, 25), then therapeutic vaccination would be unlikely to be effective. The murine model may be insufficient to resolve this issue. Further studies with other model systems and humans will help determine the utility of therapeutic vaccination with OspA as a mode of therapy for Lyme arthritis.

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