INFECTION AND IMMUNITY, Jan. 1999, p. 36–42 Vol. 67, No. 1
0019-9567/99/$04.00+0

Specificity of Infection-Induced Immunity among *Borrelia burgdorferi* Sensu Lato Species

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Received 27 July 1998/Returned for modification 16 September 1998/Accepted 15 October 1998

The specificity of infection-induced immunity in mice infected with cultured or host-adapted *Borrelia burgdorferi* sensu lato, the agent of Lyme disease, was examined. Sera obtained from mice following infection with high and low doses of cultured *B. burgdorferi* sensu stricto, transplantation of infected tissue (host-adapted spirochetes), or tick-borne inoculation all showed protective activity in passive immunization assays. Infection and disease were similar in mice infected with cultured spirochetes or by transplantation. Thus, the adaptive form of inoculated spirochetes did not influence the immune response during active infection. Mice infected with *B. burgdorferi* sensu stricto and then cured of infection with an antibiotic during early or late stages of infection were resistant to challenge with high doses of homologous cultured spirochetes for up to 1 year. In contrast, actively immune mice infected with different *Borrelia* species (*B. burgdorferi* sensu lato, *B. burgdorferi* sensu stricto cN40, *Borrelia afzelii* PKo, and *Borrelia garinii* PBi) and then treated with an antibiotic were resistant to challenge with cultured homologous but not heterologous spirochetes. Similar results were achieved for actively immune mice challenged by transplantation and by passive immunization with sera from mice infected with each of the *Borrelia* species and then challenged with cultured spirochetes. Arthritis and carditis in mice that had immunizing infections with *B. afzelii* and *B. garinii* and then challenged by transplantation with *B. burgdorferi* sensu stricto were equivalent in prevalence and severity to those in nonimmune recipient mice. These results indicate that protective immunity and disease-modulating immunity that develop during active infection are universal among species related to *B. burgdorferi* sensu lato but are species specific.

The laboratory mouse is a useful model for the study of host immune responses during active infection with *Borrelia burgdorferi* sensu lato, the agent of Lyme disease. In studies with a cloned strain (cN40) of *B. burgdorferi* sensu stricto, active infection, although persistent (7, 10), elicits strong protective immunity and disease (arthritis and carditis)-modulating immunity. Protective immunity can be measured by challenge of mice that were passively immunized with small amounts of serum from actively infected donor mice (immune serum) (4, 5, 8) or by challenge of actively immune mice that were previously actively infected and then cured of infection with an antibiotic (4). Although a strong protective immune response is elicited by active infection, it is unable to clear infection once infection is established (5, 7). Furthermore, persistently infected mice develop immune system-mediated resolution of arthritis and carditis (2, 6–8, 10, 11).

The infecting dose and adaptive state (culture, tick, or host) of the immunizing infection are important considerations. Inoculation with high doses of in vitro-cultured spirochetes elicits an immune response to the input inoculum that includes antigens, such as outer surface protein A (OspA), that are expressed principally in vitro (under culture conditions) or in the midgut of flat (unfed) ticks but are not expressed to a significant degree in the mammalian host or after ticks begin to feed (9, 15, 17). When low doses ($\leq 10^4$) of cultured spirochetes are used to infect mice or when mice are infected by tick-borne inoculation, the input antigenic load is sufficiently small that the immune response that ensues from the active infection reflects the host response to antigens expressed in vivo by spirochetes that have replicated and disseminated in the host (9, 43). As determined with immunoblots against cultured *B. burgdorferi* lysate antigen, the antibody response in mice infected following inoculation with low doses of spirochetes is similar to that in mice infected with tick-borne spirochetes as well as that in mice infected with host-adapted spirochetes introduced by transplantation of infected tissue (9, 15, 27, 38, 43). Since it is well known that protective immunity can be induced by OspA immunization and immunization with high doses of killed spirochetes containing abundant OspA (21, 22, 25, 44), high-dose inoculation may elicit protective OspA responses that mask host responses to antigens expressed in vivo. On the other hand, immunization with OspA or killed spirochetes does not elicit disease-modulating immune effects, which can be evoked only by active infection (6, 8, 9).

The purpose of this study was to carefully investigate the species specificity of low-dose infection-induced protective and disease-modulating immune responses among selected species related to *B. burgdorferi* sensu lato and with different adaptive states of spirochetes. The mouse model lends itself to such an investigation in that mice can be experimentally infected with a variety of species related to *B. burgdorferi* sensu lato and isolates (14).

MATERIALS AND METHODS

Mice. Randomly chosen (with regard to sex) 3- to 4-week-old C3H/HeN mice were purchased from the NCI Animal Production Program of Frederick Cancer Research Center, Frederick, Md. Outbred Crl:CD-1(ICR) (CD-1) mice were purchased from Charles River Breeding Laboratories, Wilmington, Mass. Mice were killed with carbon dioxide gas, followed by exsanguination by cardiocentesis.

***Borrelia* species.** Selected members of various *Borrelia* species were used in this study. The index *B. burgdorferi* sensu stricto strain was *B. burgdorferi* cN40, a cloned, low-passage, pathogenic strain (7). *Borrelia afzelii* PKo, *Borrelia garinii* PBi, and *B. burgdorferi* sensu lato 25015 were cloned by 3-fold limiting dilution and mouse passage as described for *B. burgdorferi* cN40 (7). *B. burgdorferi* sensu

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lato 25015 is a North American variant that is not related to *B. burgdorferi* sensu stricto, *B. afzelii*, or *B. garinii* (36). Species identification of the cloned strains was confirmed by David H. Persing (Mayo Clinic, Rochester, Minn.) by using genomic macrorestriction analysis as described previously (36). Spirochetes were grown in modified Barbour-Stoenner-Kelly (BSKII) medium (3) and enumerated with a Petroff-Hauser bacterial counting chamber. Samples of blood, spleen, urinary bladder, ear, and inoculation sites (syringe injection site or transplant site) were cultured in BSKII medium as described previously (7).

Each of these strains, particularly *B. afzelii* PKo, was less infectious for laboratory mice than *B. burgdorferi* cN40. When 3-week-old C3H mice were initially inoculated with 10^6 *B. afzelii* PKo spirochetes, none of the mice became infected. One-week-old mice were therefore inoculated with 10^6 *B. afzelii* PKo spirochetes, and spirochetes were isolated from the urinary bladder and then serially passaged three times in 3-week-old mice. *B. garinii* PBi and *B. burgdorferi* sensu lato 25015 were capable of infecting 3-week-old mice at the original passage but were each passaged so that a majority of mice became infected following inoculation with 10^4 spirochetes. However, the rate of infection at this dose was not absolute with these strains.

Unless otherwise noted, a standardized dose of 10^4 spirochetes was used in all experiments to induce immunizing infections for the generation of immune sera, to establish infection of donor mice for ear tissue transplantation, as a means of primary active immunization for challenge studies, and for challenge inoculation. Although the infectious dose of *B. burgdorferi* cN40 is considerably lower than 10^4 (ca. 10) (9), the infectivities of *B. afzelii* PKo, *B. garinii* PBi, and *B. burgdorferi* sensu lato 25015 are even lower (see above). Rather than equilibrating the dose to the median infectious dose, I selected the maximal dose (10^4 spirochetes) that was below the OspA antibody-inducing threshold (9) yet would optimize the chance of infection with all *Borrelia* species and would equilibrate the input antigenic load for each *Borrelia* species. Although inoculation with 10^4 spirochetes did not result in infection of all mice with all strains, it was the highest dose chosen for these experiments because this dose of input inoculum has been shown not to elicit an OspA response (9). Thus, under these circumstances, the infectivities of *B. afzelii* PKo and *B. garinii* PBi were less than optimal at the dose of 10^4 spirochetes (see Table 2).

Passive immunization. *B. burgdorferi* sensu lato-specific immune sera were generated by intradermal inoculation of C3H mice with *B. burgdorferi* cN40, *B. afzelii* PKo, *B. garinii* PBi, or *B. burgdorferi* sensu lato 25015. At 30 days after inoculation, infection of mice was verified by culturing, and immune sera from culture-positive mice were pooled by species. Passive immunity was assessed with 1-week-old CD-1 mice by subcutaneous injection of 100 μl of 1:10-diluted immune serum (10 μl, neat) or normal mouse serum into the dorsolateral thorax. Mice were challenged intradermally 18 h later on the contralateral dorsal thorax with 10^4 spirochetes. These doses of immune serum and spirochetes were used because infant mice can be protected against challenge inoculation of up to 10^7 homologous spirochetes with 50 μl of immune serum and against challenge inoculation of 10^4 homologous spirochetes with less than 1 μl of immune serum (8). Infection status was assessed at 2 weeks after challenge by culturing of tissues.

Antibiotic treatment. Mice were cured of infection by subcutaneous injection of 16 mg of ceftriaxone (Rocephin; Hoffmann-La Roche Inc., Nutley, N.J.) per kg twice daily for 5 days as described previously (37). Each experiment contained controls to assess the efficacy of antibiotic treatment.

Transplant challenge. For transplant challenge inoculation, donor C3H mice were inoculated intradermally with spirochetes of the *B. burgdorferi* sensu lato-related species of interest, and then ears were collected at 30 days of infection. Infection of ears from donor mice was confirmed by culturing, in addition to culturing of other tissues. Ear tissues were cut into 1.5-mm^2 squares in saline and then immediately transplanted into the subcutis of recipient mice through a small stab incision in the skin of the back as described previously (9). Each experiment contained controls to assess the infectivity of transplants.

Histology. Rear limbs and hearts were fixed in neutral buffered formalin (pH 7.2) and processed by routine methods for histology. Arthritis (herein defined as inflammation of synovium of joints, bursae, and/or ligament sheaths) was evaluated with sagittal sections of both knees and tibiotarsi from each mouse. Prevalence of arthritis was recorded for the four joints (two knees and two tibiotarsi) examined for each mouse, and arthritis severity was assessed by scoring the degree of inflammation in the tibiotarsal joints of each mouse. The highest tibiotarsal arthritis score for each mouse was used for analysis. Tibiotarsal arthritis was scored on a scale of 0 (negative) to 3 (severe) as described previously (6, 11). Carditis (positive or negative) was evaluated with sagittal sections through the heart, including the aortic valve (2). All slides were blinded as to experiment and treatment group.

RESULTS

Protective antibody induced by different adaptive states of spirochetes. In order to verify that protective antibody is elicited by infection not only with cultured spirochetes but also with host-adapted and tick-borne spirochetes, I infected C3H mice by subcutaneous transplantation of ear tissue from infected donor mice or by allowing five infected *Ixodes scapularis* nymphs to feed upon mice. Ticks were infected by allowing uninfected larvae to feed upon mice experimentally infected with *B. burgdorferi* cN40 and then to molt and harden into nymphs. Ticks were derived from a *B. burgdorferi*-free colony in its second generation from field-collected adults as described previously (15). In addition, normal mouse serum and immune sera obtained from mice infected following inoculation with 10^2 or 10^4 cultured *B. burgdorferi* cN40 spirochetes were tested. All sera were collected at 2 weeks after infection, and only sera from culture-positive mice were tested. Pools of each type of serum (transplant, tick, 10^2, 10^4, or normal mouse) were diluted 1:10, 1:20, and 1:40, and then 100 μl of diluted serum (10, 5, and 2.5 μl, neat) was used to passively immunize groups of two infant CD-1 mice. All of the serum pools from infected mice, regardless of the mode of infection, had protective activity at a dilution of $\geq$1:20. Normal mouse serum was not protective ($<$1:10). Thus, active infection of mice, regardless of the infecting form of the spirochetes, elicited similar protective immune responses; inoculation with 10^4 cultured spirochetes (the standardized dose) was therefore a suitable means of eliciting protective immunity analogous to that elicited by tick, transplant, or low-dose infection.

Arthritis resolution and adaptive states of spirochetes. Previous studies determined that mice infected with cultured spirochetes via a syringe develop arthritis and carditis, which peak at 2 to 4 weeks and then undergo resolution over the course of persistent infection (2, 7, 10). Although it is likely that a similar course of events transpires regardless of the adaptive state of the inoculum, I inoculated groups of eight C3H mice with cultured *B. burgdorferi* cN40 or with host-adapted spirochetes by transplantation of ear tissue from *B. burgdorferi* cN40-infected donor mice. At 30 days, all four mice in each of the two groups were culture positive and had arthritis of equivalent severity (mean $\pm$ standard deviation tibiotarsal arthritis severities were 2.0 ± 0 for culture-inoculated mice and 1.8 ± 0.5 for transplant-inoculated mice), and all mice had active carditis of equivalent severity. At 90 days, all mice in both groups were culture positive but showed resolution of their arthritis and carditis, as previously described (2, 7, 10). In unrelated studies, similar results were obtained for mice infected by tick-borne inoculation (data not shown). Thus, the adaptive form of the spirochete inoculum did not influence arthritis and carditis evolution or resolution in actively infected mice.

Duration and strength of protective immunity in mice immunized by active infection and then challenged with the homologous *B. burgdorferi* sensu stricto strain (cN40). In this experiment, I sought to determine the duration of challenge immunity to cultured spirochetes induced by active infection, as well as the ability of mice to resist various challenge doses of cultured spirochetes at various intervals after antibiotic treatment. I examined challenge immunity over the course of 1 year for mice treated with antibiotics early (day 30) or late (day 165 or 345) in the course of infection. This approach was used because levels of passive immunizing antibodies have been shown to peak during early infection (30 days) and then to progressively wane at later intervals of persistent infection up to 1 year (5). Based upon previous studies with *B. burgdorferi* N40 in C3H mice, virtually all non-antibiotic-treated mice remain persistently infected for at least 1 year (7). Ninety-six C3H mice were divided into three groups (A, B, and C) of 36, 24, and 36 mice, respectively (Table 1).

Group A was used to examine the waning of challenge immunity following treatment with an antibiotic during early infection (day 30). Mice were inoculated with cultured *B. burgdorferi* cN40 and then treated with ceftriaxone on day 30.

TABLE 1. Duration of challenge immunity in adult C3H mice immunized by active infection, treated with antibiotic, and then challenged with different doses of homologous *B. burgdorferi* sensu stricto cN40

Group and challenge dose	No. of culture-positive mice[a]/no. tested 2 weeks after challenge on day:		
	45	180	360
A[b]			
10^2	0/3	0/4	0/4
10^4	0/4	1/4	0/4
10^6	0/4	0/4	0/4
B[c]			
10^2	ND	0/4	0/4
10^4	ND	0/4	0/4
10^6	ND	0/4	1/3
C[d]			
10^2	4/4	3/4	1/3
10^4	4/4	3/3	4/4
10^6	4/4	3/3	3/3

[a] Culture results for blood, urinary bladder, spleen, ear, and inoculation site. A mouse was scored positive if any single site was positive. ND, not determined.
[b] Infected at time zero with cultured *B. burgdorferi* cN40, treated with ceftriaxone on day 30, and then challenged on day 45, 180, or 360 with 10^2, 10^4, or 10^6 cultured *B. burgdorferi* cN40 spirochetes.
[c] Infected at time zero with cultured *B. burgdorferi* cN40, treated with ceftriaxone on day 165 or 345, and then challenged on day 180 or 360 (respectively) with 10^2, 10^4, or 10^6 cultured *B. burgdorferi* cN40 spirochetes.
[d] Sham inoculated at time zero, treated with ceftriaxone on day 30, 165, or 345, and then challenged on day 45, 180, or 360 (respectively) with 10^2, 10^4, or 10^6 cultured *B. burgdorferi* cN40 spirochetes.

Following antibiotic treatment, subgroups of 12 mice each were challenged with cultured *B. burgdorferi* cN40 on day 45, 180, or 360 and then necropsied 15 days later. In each subgroup of 12 mice, 4 mice each were challenged with 10^2, 10^4, or 10^6 cultured *B. burgdorferi* cN40 spirochetes. None of the infected and day 30-treated mice challenged with 10^2, 10^4, or 10^6 *B. burgdorferi* cN40 spirochetes at 45 days and then necropsied at 60 days was infected. One of the infected and day 30-treated mice challenged with 10^4 spirochetes (but none of the others) at 180 days and then necropsied at 195 days was infected. None of the infected and day 30-treated mice challenged with 10^2, 10^4, or 10^6 *B. burgdorferi* cN40 spirochetes at 360 days and then necropsied at 375 days was infected. Thus, nearly all mice remained strongly resistant against reinfection with high challenge doses of the homologous *B. burgdorferi* strain for 1 year (Table 1).

Group B was used to examine the waning of challenge immunity following recent antibiotic treatment at various intervals of persistent infection. Group B mice were infected and then subgroups of 12 mice were treated with an antibiotic on day 165 or 345 relative to inoculation. In each subgroups of 12 mice, 4 mice each were challenged with 10^2, 10^4, or 10^6 cultured *B. burgdorferi* cN40 spirochetes on day 180 or 360, respectively, and then necropsied 15 days later. None of the persistently infected and day 165-treated mice challenged with 10^2, 10^4, or 10^6 *B. burgdorferi* cN40 spirochetes at 180 days and then necropsied at 195 days became infected. One of the infected and day 345-treated mice challenged with 10^6 spirochetes (but none of the others) at 360 days and then necropsied at 375 days became infected. Thus, protective immunity did not significantly wane during the course of persistent infection (Table 1).

Group C mice were control mice for the above two groups. They were sham inoculated (BSKII medium) and treated with an antibiotic on day 30, 165, or 345; subgroups of 12 mice were challenged with 10^2, 10^4, or 10^6 cultured *B. burgdorferi* cN40 spirochetes on day 45, 180, or 360 and then necropsied 15 days later. With the exception of a few mice challenged with 10^2 spirochetes, all of the group C mice were infected, verifying that antibiotic treatment had no residual effect upon the effectiveness of challenge and verifying the infectivity of the challenge inoculum (Table 1).

TABLE 2. Challenge immunity in adult C3H mice immunized by active infection with *B. burgdorferi* sensu stricto cN40, *B. afzelii* PKo, or *B. garinii* PBi, treated with antibiotic, and then challenged with homologous or heterologous *Borrelia* species

Immunizing inoculum[c]	No. of culture-positive mice[a]/no. tested with the following challenge inoculum[b]:			
	cN40	PKo	PBi	Sham
cN40	0/5	4/5	4/5	0/5
PKo	5/5	0/5	3/3	0/5
PBi	4/5	4/5	0/5	0/5
Sham	5/5	3/5	3/5	0/5

[a] Culture results for blood, urinary bladder, spleen, ear, and inoculation site. A mouse was scored positive if any single site was positive.
[b] Mice were challenged on day 45 with cultured *B. burgdorferi* cN40, *B. afzelii* PKo, or *B. garinii* PBi or BSKII medium (sham) after immunizing infection and antibiotic treatment.
[c] Mice were immunized by active infection following inoculation with cultured *B. burgdorferi* cN40, *B. afzelii* PKo, or *B. garinii* PBi or BSKII medium (sham) and treatment with ceftriaxone on day 30.

Cross-protective immunity among actively immunized mice infected with different *Borrelia* species. Groups of five C3H mice were inoculated with cultured *B. burgdorferi* cN40, *B. afzelii* PKo, or *B. garinii* PBi or sham inoculated with an equivalent volume (100 μl) of BSKII medium. On day 30, all of the mice were treated with ceftriaxone. On day 45 (10 days after completion of antibiotic treatment), mice were challenged with cultured *B. burgdorferi* cN40, *B. afzelii* PKo, or *B. garinii* PBi or with BSKII medium (controls). On day 60 (15 days after challenge), mice were necropsied, and cultured tissues were examined for carditis or arthritis. None of the mice originally inoculated with *B. burgdorferi* cN40, *B. afzelii* PKo, *B. garinii* PBi, or BSKII medium, treated with antibiotic, and then sham challenged remained infected, as determined by culturing. None of the mice originally infected with any of the *Borrelia* species became reinfected upon challenge with the homologous species, but most became infected when challenged with heterologous species. The infectivity of *B. afzelii* PKo and *B. garinii* PBi appeared to be less than complete, but data still demonstrated significant homologous protection and lack of cross-protection (Fisher's exact test: $P < 0.05$ for all comparisons between homologous and heterologous treatment groups) (Table 2).

Cross-protective immunity among mice passively immunized with immune sera from mice actively infected with different *Borrelia* species. Pools of immune sera from C3H mice with culture-verified infection with *B. burgdorferi* cN40, *B. afzelii* PKo, or *B. garinii* PBi were generated. For this particular experiment, treatment groups which included (i) mice treated with immune serum obtained from mice infected with *B. burgdorferi* sensu lato 25015 and (ii) mice challenged with this strain were added. *B. burgdorferi* sensu lato 25015 was added because it is a North American *B. burgdorferi* sensu lato strain from the same geographic region as *B. burgdorferi* cN40 (36). Groups of three 1-week-old CD-1 mice were administered 10 μl of immune serum or normal mouse serum and then challenged with

TABLE 3. Challenge immunity in infant CD-1 mice passively immunized with serum from mice actively infected with *B. burgdorferi* sensu stricto cN40, *B. afzelii* PKo, *B. garinii* PBi, or *B. burgdorferi* sensu lato 25015 and then challenged with homologous or heterologous *Borrelia* species

Serum treatment[c]	No. of culture-positive mice[a]/no. tested with the following challenge inoculum[b]:			
	cN40	PKo	PBi	25015
cN40	0/3	2/3	2/2	2/2
PKo	3/3	0/3	3/3	3/3
PBi	3/3	1/3	0/3	3/3
25015	2/3	2/3	2/3	0/3
Sham	1/1	3/3	3/3	3/3

[a] Culture results for blood, urinary bladder, and spleen. A mouse was scored positive if any single site was positive.
[b] Mice were challenged with cultured *B. burgdorferi* cN40, *B. afzelii* PKo, *B. garinii* PBi, or *B. burgdorferi* sensu lato 25015 18 h after passive immunization.
[c] Mice were passively immunized 18 h prior to challenge inoculation with 10 μl of immune serum from mice infected with *B. burgdorferi* cN40, *B. afzelii* PKo, *B. garinii* PBi, or *B. burgdorferi* sensu lato 25015 or with normal mouse serum (sham).

TABLE 4. Challenge immunity in adult C3H mice immunized by active infection with *B. burgdorferi* sensu stricto cN40, *B. afzelii* PKo, or *B. garinii* PBi, treated with an antibiotic, and then challenged by transplantation of ear tissue from mice infected with homologous or heterologous *Borrelia* species

Immunizing inoculum[c]	No. of culture-positive mice[a]/no. tested with the following transplant challenge inoculum[b]:			
	cN40	PKo	PBi	Sham
cN40	1/5[d]	4/4	1/5	0/5
PKo	5/5	2/5[d]	3/5	0/5
PBi	4/5	4/4	1/5	0/5
Sham	4/5	5/5	3/5	0/5

[a] Culture results for blood, urinary bladder, spleen, ear, and inoculation site. A mouse was scored positive if any single site was positive.
[b] Mice were challenged on day 45 by transplantation of ear tissue from donor mice that were actively infected with *B. burgdorferi* cN40, *B. afzelii* PKo, or *B. garinii* PBi or from uninfected mice (sham).
[c] Mice were immunized by active infection following inoculation with cultured *B. burgdorferi* cN40, *B. afzelii* PKo, or *B. garinii* PBi or BSKII medium (sham) and treatment with ceftriaxone on day 30.
[d] Culture positive at site of inoculation (transplant) only.

cultured *B. burgdorferi* cN40, *B. afzelii* PKo, *B. garinii* PBi, or *B. burgdorferi* sensu lato 25015. At 15 days after challenge, infection status was determined by culturing. Mice resisted challenge with the homologous species but were susceptible to challenge with heterologous species (Table 3). There appeared to be some protection of mice treated with *B. garinii* PBi-generated immune serum and challenged with *B. afzelii* PKo, but differences were not statistically significant due to small group sizes. Despite the small treatment groups, the overall trend of homologous protection was clear.

Cross-protective immunity to transplant challenge with infected tissue among actively immunized mice infected with different *Borrelia* species. Passive immunization with immune serum was shown to be protective against homologous syringe challenge with cultured spirochetes but not against homologous challenge by transplantation of ear tissue from infected donor mice (15). In contrast, actively immune mice (infection induced) resisted homologous challenge by transplantation (4). I therefore examined cross-protective immunity among actively immune mice following homologous and heterologous transplant challenges. Groups of five C3H mice were inoculated with cultured *B. burgdorferi* cN40, *B. afzelii* PKo, or *B. garinii* PBi or BSKII medium (controls). Mice were treated with ceftriaxone on day 30 and then challenged by subcutaneous transplantation of ear pieces from donor mice infected with *B. burgdorferi* cN40, *B. afzelii* PKo, or *B. garinii* PBi or from uninfected donor mice. With the exception of a few mice in which the transplants at the inoculation site remained culture positive, mice resisted transplant challenge with the homologous species but were susceptible to transplant challenge with heterologous species (Table 4).

Assessment of arthritis and carditis in transplant-challenged mice. Based upon previous studies, active immunity should prevent or ameliorate the development of arthritis and carditis (6, 8, 11). Because mice in the above experiment became infected by transplantation of tissue from donor mice infected with heterologous species, I examined the joints and hearts of the infected mice to assess the prevalence and severity of arthritis and carditis. Even though mice could be readily infected with *B. afzelii* PKo and *B. garinii* PBi by transplantation, they developed inconsistent and mild arthritis and carditis because of the low pathogenicity of these strains in mice (data not shown). Therefore, I could only assess arthritis in mice that were immunized with *B. afzelii* PKo and *B. garinii* PBi but challenged with *B. burgdorferi* cN40, which has sufficient pathogenicity to assess arthritis and carditis (Table 5). Among mice initially infected with *B. afzelii* PKo or *B. garinii* PBi and then transplant infected with *B. burgdorferi* cN40, all had arthritis with prevalence and severity similar to those in sham-immunized mice transplant infected with *B. burgdorferi* cN40. These results suggested that immunizing infection with *B. afzelii* PKo or *B. garinii* PBi, which prevented syringe or transplant infection with the homologous species, did not confer resistance to the development of arthritis or carditis induced by challenge infection with a heterologous, pathogenic species, *B. burgdorferi* cN40.

DISCUSSION

It is known that hyperimmunization of hamsters with killed *B. burgdorferi* spirochetes or passive immunization with antiserum to killed spirochetes affords protective immunity against challenge inoculation (30–32). The protective immune response elicited against killed, cultured *B. burgdorferi* is likely to be generated largely against OspA, an immunogenic protein that is expressed abundantly by cultured spirochetes (18). It is

TABLE 5. Arthritis and carditis in adult C3H mice immunized by active infection with *B. afzelii* PKo or *B. garinii* (PBi), treated with an antibiotic, and then challenged by transplantation of ear tissue from mice infected with *B. burgdorferi* cN40

Immunizing inoculum[a]	Arthritis		Carditis[d]
	Prevalence[b]	Severity[c]	
PKo	3.6 ± 0.5	1.6 ± 0.5	5/5
PBi	2.8 ± 1.0	1.8 ± 1.0	4/4
Sham	3.5 ± 0.6	1.8 ± 0.5	4/4

[a] Mice were immunized by active infection following inoculation with cultured *B. afzelii* PKo or *B. garinii* PBi or BSKII medium (sham) and treatment with ceftriaxone on day 30. Mice were challenged on day 45 by transplantation of ear tissue from donor mice that were actively infected with *B. burgdorferi* cN40.
[b] Mean ± SD number of joints with arthritis among both knees and tibiotarsi (four joints) of each mouse examined.
[c] Mean ± SD severity score of tibiotarsal arthritis. The highest score, based upon a scale of 0 (negative) to 3 (severe) for both tibiotarsi of each mouse, was used to establish the overall score for each mouse.
[d] Number of hearts with inflammation/number of hearts examined.

now well established that passive or active immunity to OspA is protective (16, 20–22, 41, 42, 44, 46).

The passive immunization effects of serum from actively infected experimental animals was first demonstrated with hamsters, in which passive immunization with immune sera from actively infected donor hamsters conferred protection against challenge with cultured *B. burgdorferi* (47). Interpretation of that study, however, is complicated by the presence of OspA antibodies resulting from the high-dose inocula used to induce infection. It was previously shown that infection of mice following inoculation with $\geq 10^6$ spirochetes induces an OspA antibody response reflective of the input inoculum, whereas infection of mice following inoculation with $\leq 10^4$ spirochetes does not induce a significant OspA antibody response (5, 9). When mice are actively infected following inoculation with low doses of spirochetes, they develop a strong, OspA-negative, protective antibody response (5, 9). Notably, active infection induces a stronger protective humoral immune response than hyperimmunization against OspA or hyperimmunization against killed, cultured spirochetes (6).

Thus, inoculum dose is a critical element in the interpretation of protection assays when examining the biologic activity of *B. burgdorferi* antigens and immune responses to them during active infection. In addition, the adaptive state of the *B. burgdorferi* spirochetes is an important factor in susceptibility to immune responses. It was shown here that infection with cultured spirochetes, infection with host-adapted spirochetes by transplantation with infected tissue, or infection by tick-borne inoculation all induced protective antibodies in the serum of infected mice, as determined by challenge with cultured spirochetes. Thus, regardless of the means of infection, protective antibodies are induced during active infection. However, it was also shown that immune serum has protective effects against challenge with cultured spirochetes but not against host-adapted spirochete challenge (in the form of transplantation of infected tissue or injection of spirochetes grown within in vivo chambers) or against challenge by tick-borne inoculation (15). Thus, although active infection (regardless of the adaptive state of the inoculum) elicits protective immune responses, host-adapted and tick-borne spirochetes seem to be relatively invulnerable to the protective effects of immune serum.

Despite the relative resistance of host-adapted and tick-borne spirochetes to the effects of passively transferred immune serum, even in relatively large amounts (15), active infection induces a much more complete protective immunity that is effective against culture-derived, host-adapted, and tick-borne spirochetes. It was previously shown that active infection followed by antibiotic treatment and then challenge with infected-tissue transplants prevents homologous transplant-borne infection (4). Likewise, a recently published study demonstrated that active infection followed by antibiotic treatment protects mice against tick-borne challenge infection with the same *B. burgdorferi* strain for over 1 year (39). This result is in keeping with my findings of durable (1-year) protective immunity against homologous challenge with cultured spirochetes, regardless of challenge dose (up to 10^6 spirochetes), and protective immunity against homologous transplant-borne challenge. These results suggest that although serum antibodies are important in protective immunity, other elements of the acquired immune response are important for the induction of protective immunity against host-adapted or tick-borne spirochetes.

The current study demonstrated strong, long-term resistance to challenge with the homologous strain of *B. burgdorferi*, which set the stage for the examination of immunity to challenge with selected heterologous *Borrelia* species. I used *B. burgdorferi* sensu stricto cN40, *B. afzelii* PKo, and *B. garinii* PBi for these experiments. For one experiment, I also included *B. burgdorferi* sensu lato 25015. The results underscored the universality of protective immunity induced by active infection among species related to *B. burgdorferi* sensu lato, which could be measured by both active and passive immunization assays with mice. They also demonstrated that the responsible antigenic targets, whatever they may be, are not widely conserved or cross-protective among *Borrelia* species. The results support the work of Lovrich et al. (34, 35), who showed, with much larger numbers of uncloned *B. burgdorferi* isolates, that species related to *B. burgdorferi* sensu lato can be divided into seroprotective groups, as determined by in vitro borreliacidal assays and passive immunization of hamsters. I did not attempt to evaluate such a large array of isolates; rather, my purpose was to demonstrate the principle of species specificity of infection-induced immunity to both cultured and host-adapted spirochetes. The studies of Lovrich et al. (34, 35) were performed with immune sera from hamsters inoculated with high doses of spirochetes ($>10^6$), which could complicate results. Nevertheless, passive and active immunization of hamsters or mice against OspA has been shown not to protect against heterologous *Borrelia* genotypes (23, 33, 45). Thus, immunity to *B. burgdorferi* induced by active infection appears to be *B. burgdorferi* specific and generated against as-yet-undefined antigens expressed in vivo during the course of infection.

In addition to protective immunity, active infection induces immune-mediated resolution of arthritis and carditis, which evolve early during the course of infection and then undergo resolution, despite persisting infection (2, 7, 10). The current study demonstrated that arthritis and carditis evolve and regress similarly, regardless of the adaptive form of the infecting spirochetes. Cross-immunization studies were not feasible for examining species-specific immune effects on arthritis and carditis because of the limited pathogenicity of *B. afzelii* PKo and *B. garinii* PBi. However, analyses of joints and hearts of mice immunized by prior infection with *B. afzelii* PKo and *B. garinii* PBi and then challenged by transplantation with *B. burgdorferi* cN40, which is pathogenic, revealed that immune modulation of arthritis and carditis appears to be *B. burgdorferi* specific as well.

The *B. burgdorferi* antigens that elicited these infection-induced protective and disease-modulating immune responses remain unknown. A number of proteins that are expressed to some degree under culture conditions appear to be upregulated during infection; these include OspC, P39 (BmpA), and decorin binding proteins A/B (DbpA/B). These proteins elicit early antibody responses during active infection and are effective serologic markers for infection (19, 29, 48). A number of proteins are expressed predominantly in vivo; these include OspE-related proteins (1, 13, 49, 50), P35 and P37 (24), and others (51).

OspC immunization can elicit protective immunity, but it is highly *B. burgdorferi* strain specific (8, 12, 26, 40, 41). Furthermore, OspC does not elicit a protective or disease-modulating immune response with *B. burgdorferi* sensu stricto cN40 (8, 12). A recent report demonstrated that passive immunization of SCID mice with homologous OspC antiserum was therapeutic, eliminating both spirochetes and disease in mice infected with *B. burgdorferi* sensu stricto ZS7 (52), but this effect has not been found with *B. burgdorferi* N40 (8, 12) or other isolates. Indeed, although some degree of protective (but not disease-modulating) activity has been found with P35 and P37 combined (24), none of the above-mentioned proteins has been incriminated as a target of the strong protective immune re-

sponse generated by active infection with *B. burgdorferi* N40. A possible exception is DbpA. Active and passive immunization against recombinant DbpA has been shown to protect mice against syringe challenge with *B. burgdorferi* strains, including strain N40 (19, 28, 29), but has no disease-modulating effects (19). Thus, a number of in vivo-expressed, immunogenic proteins have been identified, but the antigens responsible for eliciting strong protective and disease-modulating immune responses remain undefined. Based upon findings in the current study, once the target antigens are defined, they are likely to be heterogeneous among species related to *B. burgdorferi* sensu lato.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-26815 and AI-45253 from the National Institute of Allergy and Infectious Diseases.

The technical assistance of Deborah Beck, Dane Mathiesen, and Gordon Terwilliger is greatly appreciated.

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Editor: D. L. Burns