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Homologous and Heterologous *Borrelia burgdorferi* Challenge of Infection-Derived Immune Rabbits Using Host-Adapted Organisms

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We have recently found that strain B31 infection-immune rabbits are completely protected against homologous challenge with large numbers ($>10^6$) of host-adapted *Borrelia burgdorferi* (HAB) (E. S. Shang, C. I. Champion, X. Wu, J. T. Skare, D. B. Blanco, J. N. Miller, and M. A. Lovett, Infect. Immun. 68:4189–4199, 2000). In this study, we have extended these findings to determine whether B31 strain infection-immune rabbits are also protected against heterologous HAB challenge. Infection-immune rabbits challenged with large numbers ($>10^6$) of homologous HAB strain B31 were completely protected from erythema migrans (EM) and skin and disseminated infection. In contrast, infection-immune rabbits challenged with heterologous HAB strains N40 and Sh-2-82 were completely susceptible to EM and skin and disseminated infection; challenge with strain 297 also resulted in EM and infection of the skin and viscera, but clearance of infection occurred 3 weeks postchallenge. These findings confirm that immunity elicited in rabbits by B31 strain infection confers complete protection against large-dose homologous HAB challenge but not against a heterologous strain.

Lyme disease in humans, caused by tick transmission of the spirochete *Borrelia burgdorferi*, is known to result from different strains of *B. burgdorferi* sensu stricto and sensu lato found in different geographic regions of the world. While outer surface protein A (OspA) of *B. burgdorferi*, a surface protein expressed during tick adaptation, is currently being used for human vaccination (39, 43), it has been shown to possess antigenic heterogeneity among many strains (26, 29, 35, 48). Moreover, it is now recognized that OspA is downregulated during mammalian infection and that its mechanism of protective immunity works by inhibiting *B. burgdorferi* growth in the tick following a blood meal from a vaccinated individual (15, 37). Furthermore, organisms that have become host adapted and no longer express OspA are not susceptible to killing by OspA-specific antibodies (7). Thus, the search for additional cross-protective immunogens has attracted considerable interest for future adjuncts to the OspA vaccine.

Several experimental animal models for the study of Lyme disease pathogenesis and immunity have been utilized, including the rat (8), hamster (25), mouse (5), rabbit (18, 47), guinea pig (41), dog (10, 21), and monkey (33). With the exception of rabbits, mammalian infection with *B. burgdorferi* results in chronic infection in these animals (3, 6, 16, 31, 34). In the mouse model, Barthold has demonstrated that chronic infection elicits complete protection against homologous challenge and partial protection against heterologous challenge using cultivated organisms (4). However, protection studies in mice challenged with organisms acquired from infected tissues which no longer express OspA, called host adapted by Barthold and which we now refer to as host-adapted *Borrelia* (HAB), show only partial protection against homologous challenge and little to no protection against heterologous challenge (4). Recently, Hansen and coworkers have demonstrated that immunization of mice with decorin-binding protein A (DbpA) from strain 297, a surface protein which has been implicated in spirochetal adhesion and which is upregulated during mammalian infection, protected against homologous challenge with in vitro-cultivated *B. burgdorferi* but not against heterologous challenge (23). Further, mice immunized with DbpA were only partially protected against homologous HAB challenge (11). Thus, in experimental mouse protection studies, only limited partial protection has been achievable when using host-adapted organisms for homologous and heterologous challenge.

We have previously demonstrated that the rabbit model of Lyme disease has unique features relevant to the immunobiology of *B. burgdorferi*, including the highly reproducible development of erythema migrans (EM) (18). Of particular significance, infected rabbits clear the infection after several months, resulting in complete protection against challenge reinfection using large numbers ($>10^7$) of cultivated organisms (18). Passive immunization of rabbits with infection-immune rabbit serum results in complete protection against homologous challenge with cultivated organisms (13), suggesting that antibody is the major factor in this protection. We have also previously reported that infection-immune rabbits were resistent to challenge using numbers of organisms that were manyfold greater than that which could be demonstrated following immunization with either OspA (19, 38) or outer membrane vesicles derived from cultivated, virulent *B. burgdorferi* (38). We have also recently found that B31 infection-immune rab-

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bits are completely protected against homologous HAB challenge using greater than 10^6 organisms (38).

In this study, we have tested whether the high degree of homologous protective immunity that occurs following infection in rabbits also extends to heterologous challenge using HAB. HAB organisms were also used for challenge in order to assess protective immunity against *Borrelia* antigens other than OspA, which is present in both tick-infecting and cultivated organisms. The results show that B31 infection-immune rabbits are completely protected against homologous challenge with HAB but not heterologous challenge with HAB strains N40 and Sh-2-82. While B31 infection-immune rabbits challenged with HAB strain 297 also developed EM and skin infection, these animals completely cleared the infection 3 weeks postchallenge, as assessed by the absence of organisms in skin and internal tissues cultured in BSKII medium. These findings indicate that the high degree of complete protective immunity against high-dose homologous HAB challenge does not extend to heterologous HAB strain challenge.

Homologous and heterologous challenge of *B. burgdorferi* B31 infection-immune rabbits with HAB. To generate *B. burgdorferi* B31 infection-immune rabbits, 20 New Zealand White rabbits were inoculated intradermally at eight sites with 10^7 in vitro-cultivated *B. burgdorferi* strain B31 passage 1 for a total of 8×10^7 organisms per rabbit, as previously described (38). All rabbits developed typical EM lesions and were shown to have culture-positive skin infection in BSKII medium at 7 days postinoculation and clearance of skin infection 4 months later, as previously reported (18). Infection-immune rabbits were challenged 5 months after the initial inoculation as described below.

In order to generate HAB for homologous and heterologous implant challenge, a single donor rabbit was inoculated with 10^7 in vitro-cultivated *B. burgdorferi* of either strain B31, N40, 297, or SH2-82 at eight sites, yielding a total of 8×10^7 organisms/rabbit as described above. Rabbits inoculated with each strain developed typical EM lesions at each site 7 days postinoculation. At this time point, skin punch biopsies of EM were obtained for implantation into recipient rabbits or for culture in BSKII medium and quick frozen in dry ice-ethanol for quantitative PCR analysis. For challenge, the 20 *B. burgdorferi* B31 infection-immune rabbits and 20 naive control rabbits were bled and divided into four groups of five rabbits so that each group of five naive and five infection-immune rabbits were implant challenged with one of the four HAB strains of *B. burgdorferi*. For skin implantation, donor and recipient rabbits were first anesthetized with ketamine and xylazine as previously described (19). Each 5-mm skin punch from a donor rabbit (subsequently shown to be culture positive) was dissected into five 1-mm^2 pieces. Each of four sites per rabbit was implanted subcutaneously with five of these 1-mm^2 pieces (38). At 7 and 21 days following implant challenge, the rabbits were bled and punch biopsies were taken near the site of challenge for culture in BSKII medium as previously described (18). All rabbits were sacrificed 3 weeks following challenge with the exception of two naive rabbits from each group. The skin, right and left popliteal lymph nodes, right and left stifle joint tissues, and spinal cord were cultured in BSKII medium with antibiotics as previously described (18). All cultures were examined once a week for a total of 5 weeks for growth of *B. burgdorferi*.

TABLE 1. Homologous and heterologous HAB challenge of B31 infection-immune rabbits[a]

Rabbit group	Challenge strain	No. with EM lesions/total no.	No. with positive cultures/no. tested		
			Skin		Tissue, 3 wk
			8 days	3 wk	
B31 immune	B31	0/5	0/5	0/5	0/5
	N40	5/5	5/5	5/5	5/5
	297	5/5	5/5	0/5	0/5
	Sh2-82	5/5	5/5	5/5	3/5
Naive	B31	5/5	5/5	5/5	3/3
	N40	5/5	5/5	5/5	3/3
	297	5/5	5/5	5/5	3/3
	Sh-282	5/5	5/5	5/5	3/3

[a] Rabbits were challenged with four EM skin biopsies containing HAB. Rabbits were scored positive if any one of spinal cord, joint, or node tissues was culture positive.

All naive rabbits challenged with each of the four *B. burgdorferi* HAB strains (five rabbits/strain) developed typical EM lesions, had culture-positive skin biopsies at 7 days and 3 weeks postchallenge, and had disseminated infection based on positive BSKII cultures (Table 1). By comparison, all five B31 infection-immune rabbits challenged with HAB strain B31 were completely protected from EM and from skin and disseminated infection (Table 1), corroborating our recent findings (38). In contrast, B31 infection-immune rabbits challenged with HAB strains N40 and Sh-2-82 developed typical EM as well as skin and disseminated infection, similar to the naive controls. Interestingly, while B31 infection-immune rabbits challenged with HAB strain 297 also developed EM lesions and had skin infection 7 days postchallenge, none of the rabbits showed evidence of skin or disseminated infection 3 weeks postchallenge. Thus, while HAB of strain 297 infected the B31 strain-immune animals, the rapid resolution of this infection suggests that some cross-immunity may be present. This rapid resolution of infection has not been observed previously in other animal models following heterologous challenge.

QPCR of donor skin implants. In order to demonstrate that differences in protection between the strains were not due to variable numbers of HAB in the skin punches used for implant, quantitative PCR (QPCR) was performed as previously described on the donor skin punches using *B. burgdorferi* gyrase B (*gyrB*) as the target DNA and a nonhomologous internal standard (PCR MIMIC) as the competitor (38). To first determine if the gene copy number of *gyrB* was the same in *B. burgdorferi* strains B31, N40, 297, and Sh-282, QPCR was performed on DNA extracted from 10^8 in vitro-cultivated *B. burgdorferi* of each strain enumerated by dark-field microscopy. As shown in Table 2, each strain of 10^8 organisms was found to have 1.25×10^9 to 2.5×10^9 copies of *gyrB*. The discrepancy between the number of organisms and the *gyrB* copy number determined by QPCR is likely due to multiple copies of the chromosome (38). Although a recent study using PCR analysis of cultured *B. burgdorferi* showed that the chromosomal gene *recA* was consistent with a single *recA* per bacterium (32), our findings are more consistent with those for *Borrelia hermsii*, in that organ-

TABLE 2. Quantitation of HAB challenge inocula by PCR[a]

Inoculum	Strain	No. of *gyrB* copies
In vitro cultivated *Borrelia*	B31	2.5×10^9
	N40	1.25×10^9
	297	2.5×10^9
	Sh-2-82	2.5×10^9
HAB	B31	7.3×10^6
	N40	1.71×10^7
	297	9.96×10^6
	Sh-2-82	3.6×10^6

[a] In vitro-cultivated *Borrelia* (10^8) were enumerated by dark field; HAB were quantified using a 5-mm skin punch biopsy from a 7-day-infected rabbit used for HAB challenge. QPCR was performed as previously described (38) using *B. burgdorferi gyrB* as the target DNA and a nonhomologous internal standard as the competitor. Average number of *gyrB* gene copies per sample is based on duplicate QPCR.

isms isolated from mice have 13 to 18 chromosome copies per cell (27). Thus, the comparison of the number of organisms determined by QPCR to that enumerated by dark-field microscopy (Table 2) indicates that in vitro-cultivated *B. burgdorferi* has 12 to 25 chromosome copies per cell. Our findings also demonstrate that the four *B. burgdorferi* strains have relatively comparable copy numbers of *gyrB*, validating the use of this gene to determine relative copy numbers of organisms in each strain present in infected tissue.

QPCR using *gyrB* was then performed on DNA extracted from each of two donor skin punches taken from different EM sites. All donor skin implants had relatively comparable copy numbers of *gyrB*, ranging from 3.6×10^6 to 1.71×10^7 copies per 5-mm skin biopsy (Table 2). The exact number of copies of the chromosome in host-adapted organisms is unknown at this time but is not likely to be greater than 25 copies/cell. These results demonstrate that any differences in protection against the different *B. burgdorferi* strains were not due to variable numbers of HAB used for implant challenge.

Antigenic comparison of cultivated *B. burgdorferi* strains by two-dimensional Western blot analysis of Triton X-100-solubilized outer membrane proteins. In order to determine if antigenic differences in outer membrane proteins between the different strains could account for the lack of HAB heterologous cross-protection, two-dimensional Western blot analysis using B31 strain-immune rabbit serum was performed. In vitro-cultivated *B. burgdorferi* strains B31, N40, 297, and SH-2-82 (4×10^8 organisms) were incubated in 2% Triton X-100 for 2 h at 4°C, which has been shown previously to solubilize the spirochetal outer membrane and enrich for outer membrane-associated proteins (14). Following detergent treatment, samples were centrifuged at 20,000 $\times$ *g* for 15 min to remove protoplasmic cylinders, and the outer membrane-enriched soluble supernatants were precipitated with 10% trichloroacetic acid, washed with acetone, and suspended in isoelectric focusing sample buffer as previously described (40). The solubilized proteins were separated by two-dimensional nonequilibrium pH gel electrophoresis and transferred to a polyvinylidene difluoride (Immobilon P) membrane as previously described (40). The blots from each strain were probed with pooled prechallenge immune rabbit serum from the 20 B31 infection-immune rabbits at a dilution of 1:1,000, and antibody-antigen interactions were identified with the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, Ill.) as previously described (40).

Analysis of the amido black-stained protein profile of each blot showed that all four strains were almost identical with the exception of strain N40, which did not possess the major 32-kDa OspB protein migrating just above OspA, and strain 297, which possessed a prominent 67-kDa protein which is likely albumin contamination (Fig. 1A). Western blot analysis demonstrated that B31 strain-immune rabbit serum reacted strongly with many similar migrating proteins among the different strains, although some differences were apparent which may be relevant to the lack of heterologous cross-protection (Fig. 1B). In addition, a strong anti-OspA reaction was present in Western blots of all four strains (Fig. 1B). Taken together, these findings suggest that many of the highly cross-reactive outer membrane antigens between these different strains cultivated in vitro, including OspA, do not provide cross-immunity when challenged with HAB. This finding for OspA is not surprising given the downregulation of this protein following mammalian host adaptation of *B. burgdorferi*.

We have recently found that B31 infection-immune rabbits are completely protected against high-dose homologous challenge with HAB, an observation that has not been reported previously in other animal models of Lyme disease (38). Our findings in the rabbit model differ from the results of two recent studies of homologous HAB challenge in the mouse model (4, 11). Actively infected mice which were cured with antibiotics and challenged with $\leq$1,280 homologous HAB organisms resulted in protection against disseminated infection but only partial protection against skin infection (4). In studies by Cassatt et al., mice immunized with DbpA and challenged with $\leq$10 homologous B31 and N40 HAB resulted in infection of 3 of 10 and 1 of 10 mice, respectively, indicating only partial protection in both cases (11). Of particular consideration is the small numbers of HAB organisms used for challenge in these studies. The dose of $\leq$1,280 HAB organisms used for challenge was determined by limiting-dilution QPCR and did not consider the potential for multiple chromosome copy numbers (7). Due to the inability to isolate sufficient numbers of host-adapted organisms, the HAB chromosome copy number is not known at this time. However, given a potential chromosome copy number of 25 per cell, based on the findings in this study, it is conceivable that considerably fewer HAB organisms were used for challenge of these mice. Thus, the small numbers of HAB used for challenge in these mouse studies (7, 11) were 100- to 1,000-fold less than in vitro-cultivated organisms used for standard challenge inoculation (10^3 to 10^4 organisms), yet mice were only partially protected against homologous HAB challenge. By comparison, homologous HAB challenge using infected rabbit skin allows for markedly greater numbers of organisms to be used for challenge (5.8×10^5 [Sh-2-82] to 2.7×10^6 [NP40] organisms per rabbit; 1.5×10^5 to 6.8×10^5 organisms at each of four sites). Moreover, infection-immune rabbits challenged with high-dose homologous HAB challenge are completely protected. The number of HAB organisms used for this challenge was at least 10 times greater than the standard challenge of rabbits using 6×10^4 in vitro-cultivated organisms (38). This was also at least 400 times more HAB than used for the challenge of mice. Thus, the relative greater

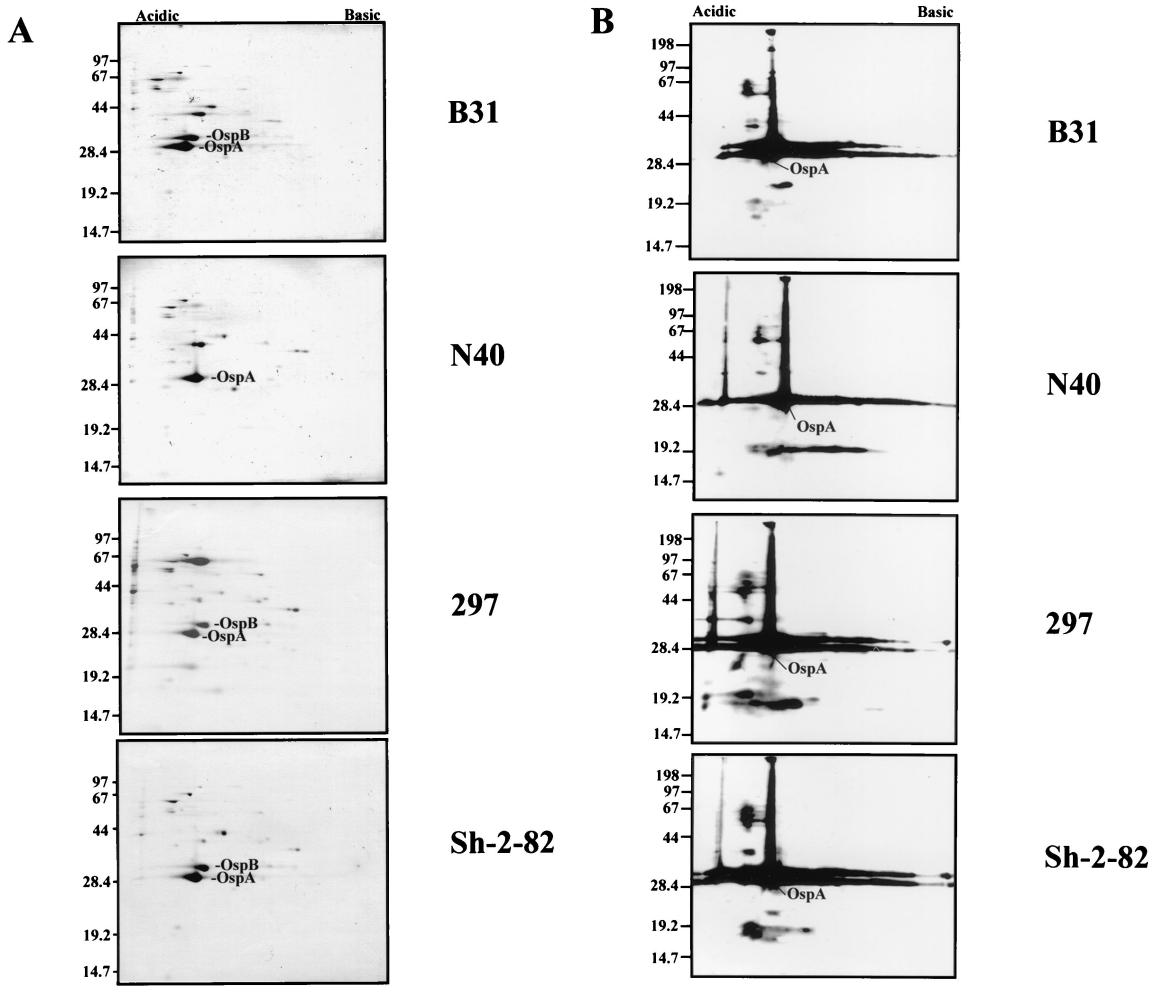

FIG. 1. Two-dimensional nonequilibrium gel electrophoresis of Triton X-100-solubilized proteins from 4×10^8 *B. burgdorferi* of strains B31, N40, 297, and Sh-2-82. (A) Amido black stain. (B) Immunoblot in panel A probed with pooled B31 infection-immune prechallenge sera. Molecular size markers are indicated to the left (in kilodaltons). Acidic and basic ends are indicated at the top.

degree of protection in immune rabbits against HAB compared to chronically infected and treated mice may reflect a greater quantitative and/or qualitative response to *Borrelia* antigens expressed during rabbit infection or, alternatively, the expression during rabbit infection of novel *Borrelia* antigens which are potent protective immunogens.

We have speculated that proteins specific to or upregulated in HAB are responsible for the high level of homologous protective immunity observed in infection-immune rabbits (38). Several proteins have been previously identified in the mouse model as being upregulated in vivo, including DbpA (11), OspC (37), and OspE and OspF (44). Proteins identified as uniquely expressed in vivo include EppA (12), p35 and p37 (17), the OspE-F homologue p21 (45), the OspF homologues bbk2.1 (2) and pG (46), and the proteins encoded on operon 2.9-71pB (1). While the concept that antigens unique to HAB are essential targets for protective immunity is appealing, many of the molecules currently known to be upregulated or uniquely expressed during mouse infection have not shown great potential as protective immunogens. OspC has been found to be highly variable among strains of *B. burgdorferi* and not protective against some homologous strains (9, 24). Recent findings also suggest that the protective OspC epitopes are conformation dependent (20). Active and passive immunizations with combined antisera to p35 and p37 were protective following challenge with 100 but not with 10,000 cultivated organisms (17). Recently, it has been reported that mice immunized with recombinant DbpA showed no protection following challenge with infected ticks (22). Thus, it is likely that the *B. burgdorferi* molecules that confer the high degree of complete protection observed following infection are HAB proteins that as yet have not been identified.

In this study we investigated whether this high degree of protection against large numbers of HAB also extends to heterologous challenge. Although B31 infection-immune rabbits were completely protected against homologous HAB challenge, they were not protected against HAB challenge using the heterologous strains N40, Sh-2-82, and 297. Interestingly, B31 strain-immune animals did show rapid clearance of infection following heterologous challenge with HAB strain 297. This difference following challenge between strain 297 and the other strains was not due to variations in the number of or-

ganisms used for challenge or to major differences in antibody recognition of in vitro-expressed membrane antigens. We have recently observed a similar rapid clearance of infection from skin in rabbits immunized with purified outer membrane derived from virulent B31 or B313 strains and challenged with homologous in vitro-cultivated organisms (38). This rapid clearance of infection may represent a degree of partial immunity and is impressive in view of the large numbers of HAB used for the heterologous challenge. However, this potential cross-protection against heterologous HAB challenge using strain 297 must be considered in the context of previous reports showing that cultivated organisms of these two strains are susceptible to killing by antibody raised against the other strain (29). Thus, whether the rapid clearance of infection observed in this study following heterologous HAB challenge is the result of common immunogens on host-adapted organisms, on both host-adapted and cultivated organisms, or on solely cultivated organisms is not known at this time.

Of further interest is the comparison of the strains used for this study. Strains B31, N40 (8), and Sh-2-82 (36) are all *Ixodes scapularis* isolates from New York State, while strain 297 is a human cerebrospinal fluid isolate from Connecticut (42). Despite isolation from the same species of tick and geographic location, no cross-protection was observed. Strains B31 and N40 have been shown by Mathiesen and coworkers, using genomic macrorestriction neighbor-joining analysis, to be closely related based on their OspA and 23S rDNA gene sequences (30). However, differences between strains B31 and N40 were noted by restriction fragment length polymorphism analysis (30), which in other studies have been associated with differences in human disease manifestations (49). It is therefore conceivable that the lack of protection observed among these strains translates to differences in upregulated HAB antigens that are strain specific.

In summary, we have corroborated our recent findings showing that infection-immune rabbits are completely protected against high-dose homologous challenge using HAB. However, this high degree of infection-derived protective immunity does not extend to other host-adapted strains used for heterologous challenge. This finding was somewhat surprising given the high degree of protection against high-dose homologous challenge and suggests that strain-specific molecules are the basis for infection-derived immunity. In this regard, we have recently isolated several novel HAB proteins from infected rabbit tissue that we have found to be upregulated relative to the amounts detected in cultivated organisms. We are currently investigating whether these proteins represent strain-specific or common antigens. It is hoped that determining the molecular basis of homologous infection-derived immunity will provide insights into the potential for heterologous cross-protection.

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