

## Vaccination against Lyme Disease Caused by Diverse *Borrelia burgdorferi*

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### Summary

Diversity and mutations in the genes for outer surface proteins (Osps) A and B of *Borrelia burgdorferi sensu lato* (*B. burgdorferi*), the spirochetal agent of Lyme disease, suggests that a monovalent OspA or OspB vaccine may not provide protection against antigenically variable naturally occurring *B. burgdorferi*. We now show that OspA or OspB immunizations protect mice from tick-borne infection with heterogeneous *B. burgdorferi* from different geographic regions. This result is in distinct contrast to in vitro killing analyses and in vivo protection studies using syringe injections of *B. burgdorferi* as the challenge inoculum. Evaluations of vaccine efficacy against Lyme disease and other vector-borne infections should use the natural mode of transmission and not be predicated on classification systems or assays that do not rely upon the vector to transmit infection.

Vaccination with outer surface proteins (Osps)<sup>1</sup>A or B from a *Borrelia burgdorferi sensu lato* (*B. burgdorferi*) isolate (designated N40) protected C3H/HeJ mice from infection when challenged with the same strain (1, 2). Humoral immunity was sufficient for protection as the passive transfer of OspA or OspB antibodies protected C.B.-17 *scid* or C3H mice from *B. burgdorferi* infection (1–4). OspA and OspB variability, including amino acid differences or truncations of the COOH terminus of the Osps, however, allowed *B. burgdorferi* to survive in the presence of protective antibody in vitro and in vivo, predicting a lack of vaccine efficacy (2, 5–11). In addition, spirochetes have been identified that lack the 49-kb plasmid encoding *ospA* and *ospB* or have chimeric Osps due to homologous recombination between *ospA* and *ospB*, suggesting that OspA or OspB mediated immunity would not be protective in the event of infection by these mutants (7, 12). The infectivity of these mutant spirochetes in selected hosts has not yet been delineated. RFLP, multilocus enzyme electrophoresis, or nucleic acid hybridization studies distinguished as many as four groups of *B. burgdorferi*, with the

description of three new proposed species—*B. burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* (13–17). Similarly, reactivity with OspA mAbs divided *B. burgdorferi* into seven serogroups which upon detailed analysis (several of the serotypes were variants of the three species that arose from *osp* recombination) corresponded to the three new species (13). *B. burgdorferi sensu stricto* is prevalent in North America whereas European sites may contain all three species. In vivo passive immunization studies demonstrated that antiserum against spirochetes from individual groups protected *scid* mice or hamsters against challenge with homologous, but not heterologous *B. burgdorferi* (8, 18).

The in vivo protection studies used single isolates of *B. burgdorferi*, and syringe inoculations as the method of spirochete challenge, and therefore do not mimic natural transmission of the agent. Accordingly, we have shown that vaccination with OspA (from *B. burgdorferi* N40) protected mice when fed upon by *Ixodes dammini* that were experimentally infected with *B. burgdorferi* N40 or by naturally infected ticks collected on Nantucket, MA (19, 20). We observed a dual mode of vaccine action—killing of the spirochetes within the host and direct destruction of spirochetes within the vector. The prevalence of *B. burgdorferi* with variable Osps, the rate of *osp* mu-

<sup>1</sup> Abbreviation used in this paper: Osp, outer surface protein.

tations within ticks, the number of different *B. burgdorferi* isolates and their relative concentrations in individual ticks, and the infectivity and pathogenicity of different isolates, represent variables that may influence monovalent vaccine efficacy. We now determine whether OspA or OspB immunization protects mice from *B. burgdorferi* infection transmitted by ticks infected with spirochetes isolated from diverse geographic locations and within different taxonomic groups.

## Materials and Methods

**Mice.** 4-wk-old female C3H/HeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The animals were shipped and housed in micro-isolator cages. The water was provided daily, and the animals were euthanized with carbon dioxide.

*B. burgdorferi* isolates were grown to log phase in modified Barbour-Stoenner-Kelly II medium, and counted using a hemocytometer under darkfield microscopy.

**Immunoblot Analysis.** 3  $\mu$ g of boiled *B. burgdorferi* were separated by SDS-PAGE and transferred to nitrocellulose. *B. burgdorferi*, OspA, and OspB antiserum or mAbs to OspA or OspB were diluted (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 (or rabbit) IgG, and devel-

oped with nitroblue tetrazoleum and 5-bromo-4-chloro-3-indolyl-phosphate, as described (5).

**Vaccination Studies.** Groups of female C3H/HeJ mice were immunized subcutaneously with 10  $\mu$ g of recombinant OspA or OspB (both expressed as glutathione transferase [GT] fusion proteins) in CFA and boosted at 14 and 28 d with the same amount in IFA (1). Control mice were immunized with 10  $\mu$ g of recombinant GT with the same regimen. For studies of *B. burgdorferi* infection of mice transmitted by ticks, animals were challenged with *B. burgdorferi*-infected ticks. 2 wk after the last booster immunization, five nymphal ticks were placed on each mouse. Ticks were allowed to feed to repletion (72–96 h), naturally detach, and were collected over a water bath as described (19). Mice were killed 2 wk after the retrieval of ticks from the water. For passive immunization studies, mice were immunized with 0.2 ml of selected OspA and OspB mAbs. Passively immunized mice were challenged intradermally with  $10^2$  *B. burgdorferi* 1 d after the immunization. Mice were killed 14 d after challenge inoculation.

**Immunofluorescence of Tick Lysates.** Ticks were examined for *B. burgdorferi* by direct immunofluorescence of tick lysates with rabbit anti-*B. burgdorferi* serum as described (20). Tick lysates (100  $\mu$ l) were placed on slides, immersed in acetone for 10 min, and then air dried for 10 min. FITC-conjugated rabbit anti-*B. burgdorferi* (10 mg/ml), used at a dilution of 1:100, was placed on the slides and allowed to incubate for 30 min at 37°C, and then washed

**Table 1.** Efficacy of OspA or OspB Vaccine Mediated Protection against Tick-transmitted Infection with *B. burgdorferi* from Different Geographic Regions

| <i>B. burgdorferi</i> origin | Immunogen | Culture | Disease | Infection | Chi square     |
|------------------------------|-----------|---------|---------|-----------|----------------|
| Northeast US                 | OspA      | 0/4     | 0/4     | 0/4       | $p \leq 0.001$ |
|                              | OspB      | 0/4     | 0/4     | 0/4       |                |
|                              | Control   | 4/5     | 5/5     | 5/5       |                |
| California                   | OspA      | 0/12    | 0/12    | 0/12      | $p \leq 0.01$  |
|                              | OspB      | 0/12    | 0/12    | 0/12      |                |
|                              | Control   | 4/10    | 5/10    | 5/10      |                |
| Sweden                       | OspA      | 0/12    | 0/12    | 0/12      | NS             |
|                              | OspB      | 0/11    | 0/11    | 0/11      |                |
|                              | Control   | 0/12    | 1/12    | 1/12      |                |
| Germany                      | OspA      | 0/10    | 0/10    | 0/10      | $p \leq 0.001$ |
|                              | OspB      | 4/10    | 4/10    | 4/10      |                |
|                              | Control   | 8/10    | 10/10   | 10/10     |                |
| JD-1                         | OspA      | 0/5     | 0/4     | 0/5       | $p \leq 0.001$ |
|                              | Control   | 4/5     | 5/5     | 5/5       |                |
| 20001                        | OspA      | 0/5     | 0/5     | 0/5       | $p \leq 0.05$  |
|                              | Control   | 3/5     | 3/5     | 3/5       |                |
| pGau                         | OspA      | 0/10    | 0/10    | 0/10      | NS             |
|                              | Control   | 0/10    | 0/10    | 0/10      |                |

We immunized mice with OspA or OspB GT fusion proteins or GT (controls). Mice were evaluated for infection by culture of the blood, spleen, bladder, and skin, and histopathology (arthritis and carditis). We tabulated the number of infected mice per number of mice examined, based on culture and disease.

**Table 2.** Destruction of *B. burgdorferi* within Ticks that Fed on Immunized Mice

| <i>B. burgdorferi</i> origin | Immunogen | No. infected ticks/No. ticks examined | Chi square     |
|------------------------------|-----------|---------------------------------------|----------------|
| Northeast US                 | OspA      | 0/6                                   | $p \leq 0.05$  |
|                              | OspB      | 1/9                                   |                |
|                              | Control   | 5/12                                  |                |
| California                   | OspA      | 0/14                                  | $p \leq 0.05$  |
|                              | OspB      | 0/14                                  |                |
|                              | Control   | 4/14                                  |                |
| Sweden                       | OspA      | 0/5                                   | $p \leq 0.05$  |
|                              | OspB      | 0/7                                   |                |
|                              | Control   | 4/9                                   |                |
| Germany                      | OspA      | 6/29                                  | $p \leq 0.001$ |
|                              | OspB      | 6/21                                  |                |
|                              | Control   | 22/30                                 |                |
| JD-1                         | OspA      | 1/12                                  | $p \leq 0.001$ |
|                              | Control   | 9/11                                  |                |
| 20001                        | OspA      | 0/7                                   | $p \leq 0.05$  |
|                              | Control   | 4/8                                   |                |
| pGau                         | OspA      | 1/8                                   | NS             |
|                              | Control   | 5/14                                  |                |

The ticks were evaluated for *B. burgdorferi* 10 d after engorgement to repletion. Ticks lysates were examined for *B. burgdorferi* by direct and indirect immunofluorescence, using FITC-labeled rabbit anti-*B. burgdorferi* sera or flagellin mAb H9724 as described (19, 20).

three times in water, after which the stained spirochetes were visualized by fluorescence microscopy.

**RFLP Analysis.** Total genomic DNA was extracted from *B. burgdorferi* strains as described (15) and 5  $\mu$ g of DNA was digested with 100 U of restriction endonuclease according to the manufacturer's recommendations (Boehringer Mannheim, Mannheim, Germany). Samples were subjected to electrophoresis on a 0.7% agarose gel and the DNA fragments were transferred to nitrocellulose. Hybridization with  $^{32}$ P-labeled probes was done overnight at 65°C in 0.5 M NaHPO<sub>4</sub>, 7% SDS, pH 7.2, at room temperature for 30 min, and dried. The membranes were developed by autoradiography. *OspA* and *flagellin* probes were prepared as previously described (15).

## Results and Discussion

We collected infected *Ixodes* ticks from selected distant geographic locations for use in vaccination studies. The *B. burgdorferi* infection rates in ticks from the Northeastern United States, California, Germany, or Sweden were 35, 5, 12, and 16% respectively. The low prevalence of spirochetal infection in host-seeking ticks from European and Californian sites precluded the possibility of using these field-collected ticks for the challenge experiments. Accordingly, we generated our challenge ticks by experimental infections that simulated the natural process of infection as closely as possible. Challenge ticks containing spirochetes from California, Sweden, or Ger-

many were derived by allowing nymphal *Ixodes pacificus* (from California) or *Ixodes ricinus* (Sweden or Germany) that had been swept from vegetation to feed on hamsters (California or Sweden) or gerbils (Germany), and these hosts then served as the source of infection for larval *I. dammini* (for California or Sweden) or *I. ricinus* (for Germany). In this manner, we were able to insure that spirochetes infected most of our challenge ticks; a median of 50% of all nymphs that were used in these experiments contained spirochetes. We also infected ticks with isolates representing *B. burgdorferi sensu stricto* (JD1 and 20001) or *B. afzelii* (pGau)-type spirochetes with different degrees of infectivity in the C3H mouse—JD1, 20001, and pGau are highly, moderately, and poorly infectious in this model system, respectively. *B. garinii* (i.e., isolate pBi) that we have tested are not infectious in C3H mice, therefore, we have not included these spirochetes in our protection studies. JD1 was maintained in continuous tick-mouse-tick passage since its derivation from a group of nymphal *I. dammini* collected from Crane Beach, MA (21), 20001 was isolated from a field swept *I. ricinus* in Brittany, France (22), and pGau was isolated from the skin of a patient with acrodermatitis chronica atrophicans (23).

We then determined whether vaccination with OspA or OspB (from *B. burgdorferi* N40) protected mice from infection with *B. burgdorferi* from different regions. Immunization with OspA fully protected mice from infection by ticks

containing *B. burgdorferi* from the Northeastern United States, California, or Germany (Table 1). The Northeastern United States and German isolates efficiently infected all control mice whereas the Californian isolates were recovered from 50% of the control mice. OspA-mediated protection extended to mice fed upon by ticks containing JD-1 or 20001 spirochetes, as well. OspB vaccination protected mice from infection by *B. burgdorferi* from the Northeastern United States or California, and provided partial protection against German *B. burgdorferi*. The Swedish or pGau spirochetes were not readily transmitted by ticks to C3H mice (only one control animal developed disease) so we could not directly evaluate protection.

Analysis of tick lysates by immunofluorescence showed that spirochetes were eliminated directly within ticks that fed on Osp-vaccinated mice, regardless of whether the *B. burgdorferi* originated from the Northeastern United States, California, or Sweden, or were JD-1 or 20001 spirochetes (Table 2). Significant destruction of German or pGau spirochetes occurred in ticks that fed on Osp-immunized mice. Thus the immune response to heterologous OspA or OspB is sufficient to kill *B. burgdorferi* within the vector regardless of their origin. Such destruction correlates strongly with protection and allows us to infer that protection with our monotypic antigens may be broadly protective.

We determined the extent of genetic heterogeneity within *B. burgdorferi* that had been cultured from ticks that had fed on the control mice by means of RFLP analysis (15) (Table 3). 21 United States tick isolates were tested; all were *B. burgdorferi sensu stricto* (RFLP group 1) type organisms. The sole

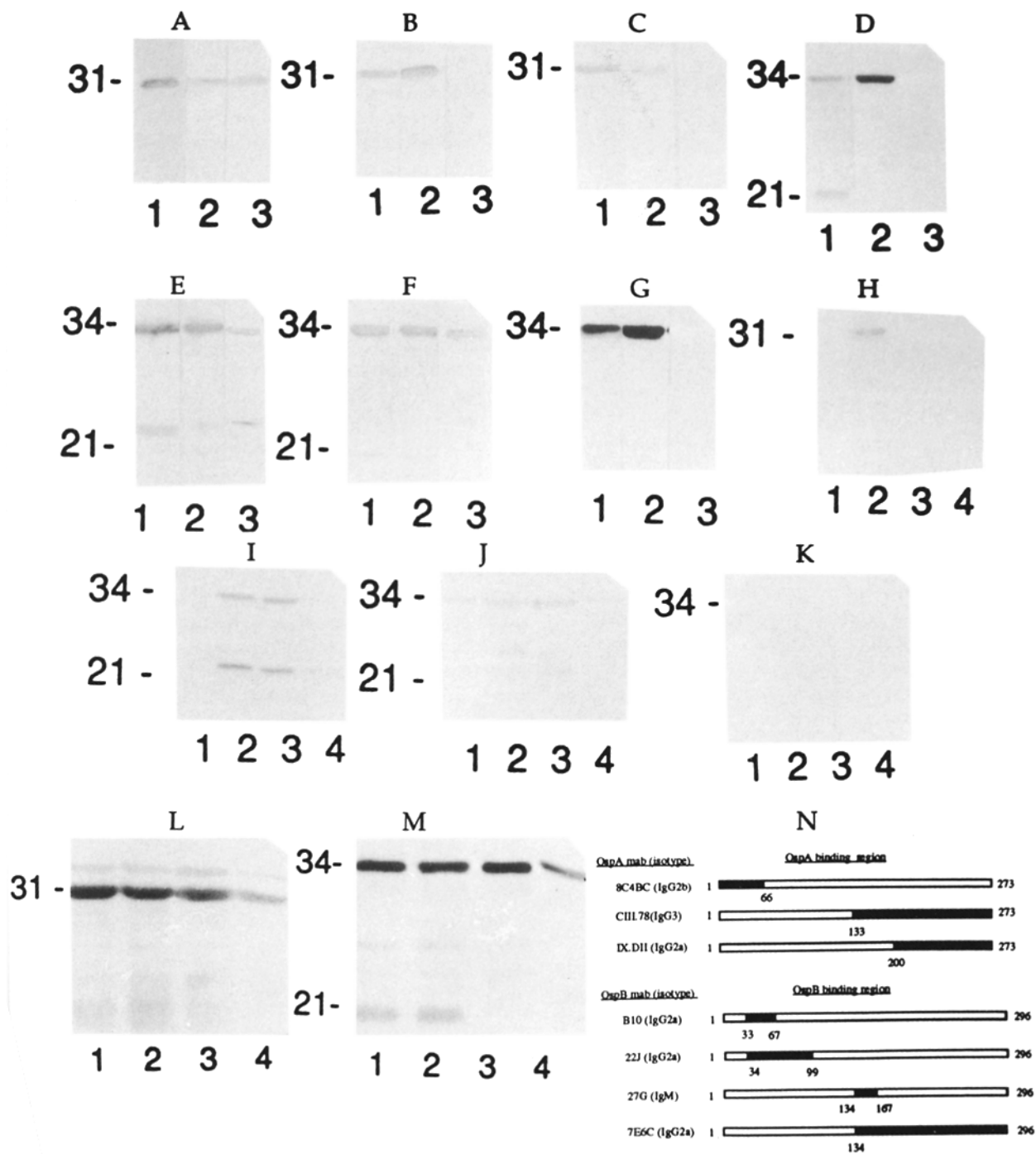
exceptions were one California isolate and the JD-1 strain (21) which were considered to be *B. burgdorferi sensu stricto* variants because of the presence of additional faint bands on Southern blot. These bands may be due to osp recombination, as has been observed with strain JD-1 (Lobet, Y., unpublished data) or OspA variability within *B. burgdorferi sensu stricto* such as occurs with *B. burgdorferi* 25015 (5, 15). All 11 German or Swedish isolates tested were *B. afzelii* (RFLP group 4) type spirochetes. *B. garinii* were not recovered from our studies and this may reflect the geographic regions used or the poor infectivity of *B. garinii* organisms in the hamsters or gerbils that initially served as hosts for the collected ticks. Vaccination with a single OspA or OspB antigen therefore protected mice against naturally occurring *B. burgdorferi* within different taxonomic groups, suggesting that immunity against vector-borne Lyme borreliosis is likely to be more comprehensive than previously believed.

We determined the antigenic and immunologic heterogeneity of OspA and OspB from 32 selected cultures from our tick transmission studies by probing the *B. burgdorferi* with selected mAbs (against *B. burgdorferi* N40) that bind to different epitopes on *B. burgdorferi* N40 OspA or OspB (Fig. 1). OspA mAb CIII.78 and OspB mAb 7E6C protected mice from infection in passive immunization studies and bound to COOH-terminal conformational epitopes, whereas OspB mAb B10 was not protective and recognized an epitope at the NH<sub>2</sub> terminus (1, 20, 24). We generated other Osp mAbs (against *B. burgdorferi* N40) and now show that OspA mAb IX.D11 protects mice from infection in passive immunization studies

**Table 3.** Immunoblot and RFLP Analyses of Selected *B. burgdorferi* Recovered from Selected Ticks or Mice Used in Immunization Studies

| <i>B. burgdorferi</i><br>isolate | RFLP type   | Immunoblot with Osp antibodies |         |        |          |     |     |      |
|----------------------------------|---|--------------------------------|---------|--------|----------|-----|-----|------|
|                                  |   | OspA mAb                       |         |        | OspB mAb |     |     |      |
|                                  |   | 8C4BC                          | CIII.78 | IX.D11 | B10      | 22J | 27G | 7E6C |
| California 1-8                   | <i>B. burgdorferi sensu stricto</i><br>(RFLP group 1)                 | +                              | +       | +      | +        | +   | +   | +    |
| California 9                     | <i>B. burgdorferi sensu stricto</i> variant<br>(RFLP group 1 variant) | +                              | +       | +      | +        | +   | +   | +    |
| Northeast US 1-12                | <i>B. burgdorferi sensu stricto</i><br>(RFLP group 1)                 | +                              | +       | +      | +        | +   | +   | +    |
| Germany 1-6                      | <i>B. afzelii</i><br>(RFLP group 4)                                   | +                              | -       | -      | +        | +   | +   | -    |
| Sweden 1-5                       | <i>B. afzelii</i><br>(RFLP group 4)                                   | +                              | -       | -      | +        | +   | +   | -    |
| JD-1                             | <i>B. burgdorferi sensu stricto</i> variant<br>(RFLP group 1 variant) | +                              | +       | +      | +        | +   | +   | +    |

RFLP analysis of *B. burgdorferi* DNA, digested with HindIII and probed with radiolabeled *ospA* and *flagellin* was performed as described and correlated with the three newly described *B. burgdorferi* species: *B. burgdorferi sensu stricto*, *B. afzelii*, and *B. garinii* (16). Immunoblots were performed using OspA and OspB mAbs as described.



**Figure 1.** Immunoblots of different *B. burgdorferi* probed with various monoclonal and polyclonal antibodies to *B. burgdorferi* N40 OspA and OspB. (A and H) OspA mAb 8C4BC. (B) OspA mAb CIII.78. (C) OspA mAb IX.DII. (D) OspB mAb B10. (E and I) OspB mAb 22J. (F and J) OspB mAb 27G. (G and K) OspB mAb 7E6C. (L) Rabbit OspA antiserum. (M) Rabbit OspB antiserum. (A-G) Lane 1, California *B. burgdorferi*; lane 2, Northeastern *B. burgdorferi*; and lane 3, Swedish *B. burgdorferi*. (H-M) Lanes 1-4, German *B. burgdorferi*. The molecular masses are described in kilodaltons. (N) Diagram depicts the regions of OspA or OspB to which the various mAbs bind. The binding epitopes of OspA or OspB are shown in black and the corresponding amino acids are numbered.

(Chi square test,  $p \leq 0.001$ ), whereas OspA mAb 8C4BC and OspB mAbs 22J and 27G did not have protective capabilities against *B. burgdorferi* infection (2) (Table 4). mAb IX.D11 bound to a COOH-terminal epitope of OspA and mAbs 8C4BC, 22J, and 27G bound the NH<sub>2</sub> terminus or central

region of OspA or OspB (2, 24). All of the Osp mAbs bound to all 21 of the Northeastern and Californian spirochetes whereas protective mAbs (OspA mAbs CIII.78 and IX.DII and Osp B mAb 7E6C) did not bind with *B. burgdorferi* from Germany and Sweden (Fig. 1). Some mAbs showed

**Table 4.** Passive Immunization of Mice against Challenge with *B. burgdorferi* N40

| Passive immunization | <i>B. burgdorferi</i> infection |
|----------------------|---------------------------------|
| OspA mAb             |                                 |
| CIII.78              | 0/5                             |
| IX.D11               | 0/5                             |
| 8C4BC                | 5/5                             |
| OspB mAb             |                                 |
| 7E6C                 | 0/5                             |
| 22J                  | 5/5                             |
| 27G                  | 5/5                             |

The passive transfer of antibody and challenge of mice with a syringe inocula of *B. burgdorferi* was performed as described and *B. burgdorferi* infection was documented by recovery of spirochetes from cultures of the animals blood, spleen, bladder, or skin and/or histopathologic evidence of arthritis or carditis.

binding to more than one band of some *B. burgdorferi* on immunoblot, further showing antigenic differences among the spirochetes—these may represent truncated Osps or antigens that arose from *osp* recombination (2, 7, 12, 13). These data suggest that there are conserved regions of OspA and OspB that are recognized by some protective antibodies in all *B. burgdorferi* isolates tested. Indeed polyclonal rabbit anti OspA-N40 or anti OspB-N40 reacts with all the United States, Swedish, and German isolates tested; a representative immunoblot from

four German strains demonstrates the strong reactivity we observed with all of our challenge isolates (Fig. 1, L and M).

Immunization against tick-borne Lyme borreliosis using a single recombinant OspA or OspB appears to be more efficacious than would be expected on the basis of *B. burgdorferi* classification systems, in vitro killing assays, or in vivo studies using syringe-mediated challenge. Therefore, destruction of *B. burgdorferi* within the tick before spirochete transmission may be the main mode of protection and may depend on less specific interactions than those observed within the vertebrate host. In cases where partial eradication of spirochetes occurred in ticks, the pathogenicity of the remaining *B. burgdorferi* and the ability of partially debilitated organisms to transmit disease may be diminished. Indeed, *B. burgdorferi* with a truncated OspB have reduced infectivity in mice (25). Although such spirochetes may evade Osp-mediated protection in the tick, the clinical significance of infection by poorly infectious or avirulent spirochetes is unclear. Therefore it may not be necessary to elicit a protective immune response against *B. burgdorferi*, with atypical Osps that may occur in nature. In addition, the cross-protective effect may be due to antibodies that bound to conserved epitopes among the *B. burgdorferi* isolates—these antibodies may not be overtly protective in standard syringe inoculation and in vitro assays but are sufficiently effective within the tick. Studies of immunity to other major vector transmitted diseases such as malaria and leishmaniasis rely, for the most part, upon artificial laboratory studies that may greatly influence the experimental outcome. The natural mode of transmission should be used in studies of vaccination against vector-borne agents.

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