The diagnosis of Lyme borreliosis has been compromised by the lack of definitive serology. Use of cultured preparations of the causative agent, *Borrelia burgdorferi*, to generate a whole cell sonicate for ELISA and Western blot analysis has yielded only marginal results (1–6). Recent reports have focused on a number of bacterial antigens that may be more useful in serodiagnosis, including outer surface proteins A and B (OspA and OspB), flagellin, and other proteins, designated P21, P39, P66, and P83 for their estimated molecular masses (7–12). The vast majority of the published data analyzing the utility of these bacterial antigens in serology testing have used a system of needle inoculation of cultured spirochetes or purified proteins to assess immune responsiveness (4–16).

In nature, this infection is transmitted by the bite of spirochete-infected *Ixodes* ticks. The reservoir of the infection is commonly the white-footed mouse, *Peromyscus leucopus*, and the disease can be transmitted to many mammalian species, including dogs and humans (17, 18). It is important to establish whether there is a difference in the immune response to the infection when it is transmitted by tick bite as compared with experimental infection by needle inoculation.

Two important aspects of *Borrelia* infection come under consideration here. First, is there a difference between *B. burgdorferi* as it exists in the tick gut and salivary glands versus the spirochete grown in culture? And second, what effect does the transmission of the bacteria via a tick bite have on the establishment and progress of the infection and the immune response to bacterial antigens?

This report presents data from Western blot analysis of sequential sera taken from a panel of inbred strains of mice infected with *B. burgdorferi* by exposure to *I. dammini* ticks infected with the B31 isolate of the spirochete. Results show the complexity of the antibody response to a number of the protein antigens derived from *B. burgdorferi* and identify proteins that may be useful in serodiagnosis of Lyme disease.

Abbreviation used in this paper: Osp, outer surface protein.
Materials and Methods

Mice. 4-6-wk-old mice were obtained from The Jackson Laboratory (Bar Harbor, ME) (BALB/c, B10, B10.BR, and B10.D2) or from the specific pathogen-free (SPF) colony of Institute for Cancer Research (Philadelphia, PA) outbred mice maintained at the Division of Vector Borne Infectious Diseases, National Center for Infectious Disease, Centers for Disease Control (Fort Collins, CO). The MHC and Ig haplotypes and allotypes of these mice are given in Table 1.

Maintenance of Infected Tick Colonies. I. dammini larvae, 1-2 mo posthatching, were fed on ICR outbred mice infected with the B31 strain of B. burgdorferi and subsequently maintained at 21°C at 97% relative humidity. Nymphs, 1-3 mo postmolting, were used to infect mice by the natural route of tick exposure. All infected ticks were maintained by a cycle of infected ticks transmitting the spirochete to mice, and these mice were used to infect the next batch of tick larvae. The rate of infection was 95% within the tick colony, determined by dissection of ticks and identifying spirochetes by dark field microscopy. Infection of the mice was determined by ear punch biopsy as described previously (19).

Infection of Mice by Tick Bite. Groups of four mice from each strain as well as ICR outbred mice were numbered by ear punch, and preimmune serum samples were drawn. The mice used in this study were BALB/c (H-2b), C57BL/10J (B10) (H-2a), and the recombinant inbred strains on the B10 background B10.BR (H-2b) and B10.D2 (H-2b). Each mouse was exposed to 10-12 nymphal ticks infected with B. burgdorferi strain B31. Each mouse had from 4 to 12 nymphs feed to repletion. All mice were shown to be infected by culturing B. burgdorferi from ear punch biopsies at 30 d postfeeding.

Preparation of Borrelia Antigens. Strains B31 and JD-1, both originally isolated from I. dammini (20, 21), were inoculated into culture from either low-passage (P6 for B31, and P1 for JD-1) or high passage (>P30) frozen stocks. These were expanded in BSK II medium supplemented with 6% rabbit serum (Pel-Freez Biologicals, Rogers, AR). 1-liter expansion cultures were harvested in late log phase (day 5), pelleted by centrifugation at 10,240 × g for 20 min at 20°C. Spirochetes were washed with PBS, 5 mM MgCl₂, and centrifuged again. Pellets were resuspended in 10 mM Tris, 1 mM EDTA (TE) at 30 ml/g wet weight of cells. The spirochetes were lysed in a Dounce homogenizer, and protein concentration was adjusted to 2 mg/ml. These preparations were then mixed with equal volumes of 2x SDS-PAGE sample buffer, aliquoted, and stored at -70°C.

SDS-PAGE and Electrophoretic Transfer to Nitrocellulose Filters. Samples were run in a discontinuous SDS-PAGE system using a vertical slab gel electrophoresis system (SE600; Hoefer Scientific Instruments, San Francisco, CA). The stacking and resolving gels were 4 and 10% acrylamide, respectively. Bacterial lysates were run at 80 µg/gel in a single preparative well. Molecular mass standards (14-106 kD) were run in flanking lanes. Proteins were transferred to 15 × 15 cm nitrocellulose filters, 0.2-µm pore size (Bio Rad Laboratories, Richmond, CA), using a transblot system (Semiphor TE 70; Hoefer Scientific Instruments) at 100 mA for 50 min.

Abs. Antibodies specific for B. burgdorferi antigens OspB and flagellin (H6831 and H9724) were obtained from Symbicon (Umeå, Sweden) as concentrated culture supernatants. These antibodies were originally described by Barbour et al. (22, 23).

Western Blot Analysis. Nitrocellulose filters were placed in a blocking solution containing 3% BSA fraction V, 0.9% NaCl, 10 mM Tris-HCl, pH 7.4, and 0.1% sodium azide for 16 h at 4°C. Filters were washed in 0.05% Tween 20, 0.9% NaCl, and 10 mM Tris-HCl, pH 7.4 (wash buffer), for 20 min. Filters were mounted in a Miniblotter 25 (Immunetics, Cambridge, MA) and washed again. Wells were suctioned dry and loaded with a 1:100 dilution of antisera or appropriate dilution of mAb in wash buffer. The indicated dilution of primary antibody was incubated for 3 h at room temperature, filters were washed three times each for 5 min, and alkaline phosphatase--conjugated goat anti-mouse Ig G + M (H and L chains) (Jackson Immunoresearch, Vineland, PA) at 1:1,000 dilution was added to all wells. After 1 h, filters were washed twice, removed from the apparatus, and washed once more. Filters were quickly washed twice with deionized water, and bromochloroindolyl phosphate nitro blue tetrazolium (BCIP/NBT) substrate (Kirkgaard & Perry Laboratories, Inc., Gaithersburg, MD) was added for exactly 5 min to detect bound alkaline phosphatase.

Results

Antibody Response to B. burgdorferi Infection Resulting from Tick Bite. Serum samples were drawn on days 5, 12, 19, 26, and 60 postinfection, and these sera were used in Western blot analysis using whole lysates of B. burgdorferi, strain B31, low passage, as antigen. Preimmune sera as well as day 5 sera from all animals were negative in Western blot using B. burgdorferi strain B31. Fig. 1 shows that on day 12, all mice had serum antibodies to a variety of Borrelia proteins, however, inbred strains of mice reacted differentially to these antigens.

For example, the B10 animals all showed a strong response to a protein migrating at a molecular mass of just above 80 kD, most likely the P83 reported by LeFebvre et al. (12). The balance of the animals in this study did not respond to this protein at this early stage of infection. There was a variable response to P17 antigen described by Wilski et al. (11) in that only the B10 congenics responded to this protein at day 12. The antigen migrating at 24 kD also induced a weak response in all strains, and there appeared to be a variable response to a group of proteins in the 20-22-kD range, as well as the 55-60-kD range. The antibody response was still very weak to these antigens at this early stage of the infection.

Proteins in the 39-kD range induced an early response in all animals, and this group of proteins includes P39, described by Simpson et al. (8). There are at least two bands detected in this region, but we do not believe these include the 41-kD flagellin protein since the mAb to flagellin detected a band that migrates just above the P39 doublet. Two other proteins migrating at 14 and 18 kD also induced a variable response.

Table 1. MHC Haplotypes and Ig Allotypes of Mouse Strains Used

| Mouse strain | MHC haplotype | Ig allotype | Background genes |
|--------------|---------------|-------------|------------------|
| ICR outbred  | NA*           | NA          | ICR outbred      |
| BALB/c       | d             | a           | BALB/c           |
| B10          | b             | b           | B10              |
| B10.BR       | k             | b           | B10              |
| B10.D2       | d             | b           | B10              |

* Not applicable.
this early in infection. Finally, there was a significant response by all mice to a protein migrating close to the 45-kD marker. Previously, Roehrig et al. (24) described this protein in natural infection in hamsters and designated the antigen P43 in that report.

At this stage of the infection, we detected no response to OspA, OspB, or flagellin, all proteins that induce an early response from these as well as other strains of mice when challenged by needle inoculation of cultured *B. burgdorferi* (13). Though there was a marginal reactivity with bands at 30 kD (close to OspA) and 32 kD (OspB), this banding is significantly different from the Coomassie blue staining of the acrylamide gel (not shown) or the Ponceau S stain of the nitrocellulose filter (Fig. 1, right hand margin), where OspA and OspB are very prominent bands. This reactivity is likely to be an antibody response to different proteins migrating coincident with OspA and OspB. We confirmed this in the case of OspA by analyzing these sera in Western blot and ELISA using purified, whole lipidated OspA from a cloned gene product expressed in *Escherichia coli* and detected no anti-OspA antibody in the sera of any mice used in this study (Golde et al., manuscript in preparation).

By day 19 postinfection, the antibody response to a number of bacterial antigens increased as detected on Western blots of B31 low passage (Fig. 2A). The set of five bands migrating at 14, 17, 18, 22, and 24 kD was detected by sera from all five mouse strains with the exception that B10 mice did not respond to the 18-kD band. Sera from the B10.BR mice did not detect the 30-kD band migrating just below OspA, and there was a variable response to a group of proteins migrating coincident to OspB. All mice responded to the P39 band(s), and one B10.BR (no. 5) mouse had antibodies specific for flagellin (41 kD). All mice maintained the response to P43 and a band or bands migrating at ~52–55 kD. B10.BR and,

![Figure 1. Western blot analysis using sera from five mouse strains drawn on day 12 postinfection with *B. burgdorferi*. The antigen was 80 µg of *B. burgdorferi*, strain B31, low-passage run on a 10% SDS-PAGE and electrophoretically transferred to a 0.2-µm nitrocellulose filter. The relative migration of prestained molecular mass markers is illustrated in the left hand margin, and Ponceau S staining of the molecular mass standards is shown in the right hand margin. The last lane shows direct staining of total bacterial protein by Ponceau S on the nitrocellulose filter. mAbs H6831 (lane B) and H9724 (lane F), specific for OspB and flagellin, respectively, were used at a final dilution of 1:20.](image)
Figure 2. Western blot analysis using sera from five mouse strains drawn on day 19 postinfection with *B. burgdorferi*. (A) Binding of these sera to strain B31 low passage; (B) binding of the same sera to the same concentration of protein derived from strain B31 high passage. Molecular mass standards are given in the left hand margin of each panel, and H9724 (antiflagellin) was used at 1:100 (B, lane F).

to a lesser degree, B10.D2 detected a band slightly higher at 58 kD, and as on day 12, only the B10 animals responded to the 83-kD band.

When the same sera, drawn on day 19 postinfection, were used to blot B31 high passage, a very different pattern was seen (Fig. 2 B). In the low molecular mass range, only outbred mice responded to P24 and all mice except the BALB/c's responded to P17. These sera did not detect the P14, P18, and P22 antigens observed in the blots of B31 low passage. A similar reactivity to the band at 30 kD was seen with all mice responding except B10.BR. No reactivity was detected between 31 and 39 kD, and all had antibody that detected the P39 band(s). There was no reactivity with P41 (flagellin), P43, or any other bands in this molecular mass range seen in the Western blots of B31 low passage. The response to the band at 52–55 kD was detectable but the B10 response to P83 was not seen in this blot.

Immunoblots performed with sera drawn on day 26 postinfection continued to show an increase in the response to many *Borrelia* antigens (Fig. 3). The strong response to the five bands in the 14–24-kD range was observed in all mice except for the weak response to P18 by the B10 animals. There was a reactivity to a protein at ~28 kD that was only detected in serum from the B10.BR animals, and there was still a variable and minimal response to antigens in the same molecular mass region as OspA (31 kD). The variability in the response to proteins in the 32–34-kD range, possibly including OspB, was maintained at day 26. For example, outbred and B10.BR mice had an antibody response to multiple bands in this region, as did the B10 mice, with the exception of mouse no. 3. The B10.D2 and BALB/c mice (both H-2*^d*) did not respond to these proteins, with the exception of BALB/c mouse no. 3 responding to a single band. Apparently these proteins are not efficiently presented by H-2*^d* MHC proteins.

All animals made antibody specific for the P39 bands, and by day 26 all the B10.BR animals were responding to P41 flagellin. As seen at earlier time points, all animals responded to P43. A pair of bands at 50 and 52 kD gave a minimal signal using these sera, and all animals had antibodies to a series of proteins in the 55–65-kD region that includes the heat shock proteins. At this time in the course of the infection only the B10 animals responded to P83.

Sera from day 60 postinfection had high titers of antibodies reactive with all of the five proteins in the lower molecular mass range using low-passage B31 (Fig. 4 A). At this later stage of infection, all animals responded to the protein migrating at ~28 kD. The response in the region of OspA (31 kD) continued to be variable between the strains tested in this study. The response to proteins migrating in the region of OspB had become more pronounced and consistent between strains, with the exceptions of B10 mouse no. 6, as noted at earlier time points, and B10.D2 mice nos. 7 and 8. Again, all animals responded to the P39 bands, and now all sera had antibodies to P41 flagellin. As has been seen throughout the infection, all animals had a strong response to P43. At this later stage of infection all animals responded to the pair of bands at ~50 and ~52 kD, although the B10.BR and B10.D2 response was very weak. There was a
variability in the intensity of the signal in the response of these mice to the triplet of bands in the 55-65-kD region. By day 60 postinfection, all strains generated antibody to P83 with the exception of B10.D2 and BALB/c animals nos. 2 and 4, all H-2d haplotypes.

When these sera were used to blot strain B31 high passage as antigen, the blotting pattern was very different (Fig. 4 B). The only antigen detected in the low molecular mass region of the gel was P17. A minimal response to P22 and P24 was seen in the outbred BALB/c and B10.D2 animals, and the B10 and B10.BR were negative. Because the antibody binding to P17 in the B31 low-passage and B31 high-passage gel were comparable, the difference in detection of the other proteins using these sera indicates a qualitative difference in the bacterial proteins expressed by the two derivatives of B31. These sera also failed to detect the P28 antigen and show variable binding in the OspA and OspB region. All sera again detected the P39 bands but not P41 flagellin. There was no reactivity in the region of P43 or the 52-54-kD range, yet a similar binding pattern was seen in the region of 55–65 kD. The ability of these sera to bind the P83 antigen was exactly as in the low passage blot (Fig. 4 A).

In general, when comparing the immunoblots on B31 low passage and high passage using the same amount of protein antigen, sera from mice infected with B. burgdorferi by tick bite taken at both days 19 and 60 detected many more antigens in the low-passage material. In the case of the antigens detected in both lysate preparations, the signal in the low passage blots was more intense than the same reactivity detected against high-passage derivative, with the exception of P17 in the blots with day 60 sera as discussed above. Long-term culture of B. burgdorferi leads to significant changes in the expression of a majority of the bacterial antigens that can be detected by murine antibodies.

**Detection of Antigens from Heterologous Strain of B. burgdorferi**. Data in Fig. 5 show the ability of these sera to detect the same antigens from a different strain of B. burgdorferi. In Fig. 5 A, a low-passage isolate of the strain JD-1 was probed using sera drawn on day 12 postinfection. There was no reactivity with the five low molecular mass bands but a similar binding pattern with the band at 30 kD was observed with B31 low-passage immunoblots. All mice responded to the P39 bands and there was a very marginal detection of P43. There was also a strong response to the P58 band. Compared
with the blots using these sera on the low-passage isolate of strain B31, fewer bands were detected from JD-1 and these bands showed less intensity.

Sera from day 19 postinfection (data not shown) showed a stronger signal than the bands detected by day 12 sera, with the only addition being that some variable reactivity to P17 and P18 was now detectable. Using day 26 sera (Fig. 5 B), the reactivity to P17 by all animals was apparent, except for B10. The response to P18 was more consistent across these mouse strains but B10 animals had a marginal response and serum from B10.BR no. 6 did not detect this antigen. There was a variable detection of an antigen migrating just below OspA and all sera detected the P39 bands. All of these sera gave a good signal to P43 and the reactivity to the 58-62-kD antigens was more pronounced. As in the case of B31 strain used as antigen, only sera from B10 animals detected P83.

Sera from day 60 postinfection blotted against low-passage strain JD-1 showed a consistent crossreactivity with the P17 and P18 bands without binding to P14, P22, and P24. There was a tremendous variability to all of the bands migrating between 28 and 35 kD and, as with earlier bleeds, all sera detected the P39 doublet as well as the P43 antigen. The reactivity to the band at 58 kD was consistent with all sera and the bands above that were variably detected. Specifically, P83 was detected by all B10 and B10.BR sera, variably by BALB/c and outbred sera, and not at all by B10.D2. As in the case of using high-passage strain B31 as antigen, all of these blots showed less intensity of signal even though they were done with the same serum samples on the concentration of protein antigen.

Finally, Fig. 6 shows the results of using sera drawn on day 60 postinfection on a high-passage isolate of JD-1. Clearly, the only prominent reactivity detected on this high-passage B. burgdorferi was the anti-P39 response. There was marginal detection of the band at 58 kD by all sera and only sera from BALB/c and outbred mice showed marginal detection of P18. There was a barely detectable binding of serum antibodies from B10 animals for the P83 band. This striking result indicates how different a high-passage isolate of a different strain, JD-1, is from the infective agent used in this study, strain B31 in infected ticks.

Discussion

Infection of laboratory mice with B. burgdorferi transmitted by the feeding of infected Ixodes ticks gave a very different antibody response pattern than the equivalent infection using cultured spirochetes transmitted by needle inoculation. One of the primary goals of this study was to more clearly understand the serology of natural Borrelia infection and we chose to do this in the context of recombinant inbred strains of mice. A number of bacterial proteins appear to induce a response restricted to certain MHC haplotypes in mice, even in this limited study of three MHC haplotypes in four mouse strains. For example, until late in the infection, only B10 animals respond to the P83 protein. Since B10.BR and B10.D2 animals are perfect genetic matches except for the MHC locus, apparently H-2^d and H-2^a are relatively inefficient at presenting this protein when compared with H-2^b.

Other proteins appear to induce an unrestricted response.
in these mice, but when crossreactivity between different bacterial strains is analyzed these antigens are not detected. Similarly, there are antigens that induce an MHC-nonrestricted response when assayed on homologous strain B31, but respond to the heterologous strain JD-1 antigen in a restricted fashion. The antigen we refer to as P17 is detected in both B31 strains, the strain used to infect, and JD-1, a different strain of *B. burgdorferi* with the exception that B10 animals do not detect the JD-1 version of this protein until late in infection.

A third class of bacterial antigens, including P18, P39, and P43, induced a good response in all strains of mice, and this reactivity is detected in both B31 and JD-1 low-passage preparations of bacteria. This crossreactivity is significant considering these strains of *Borrelia* fall into two different ribotyping classifications as determined by dos Santos and Mayer (25). Though this study surveys only two ribotypes of *B. burgdorferi*, this may indicate a significant conservation of this group of bacterial antigens.

A comparison of these results with the use of long-term culture preparations of bacteria for antigen shows that a number of these protein antigens are lost or modified by multiple passages. In the case of either strain of bacteria used in this study, the reactivity of the mouse sera with lysates of high-passage B31 and JD-1 is remarkably less than with the low-passage derivative of the same strain. In fact, a significant number of protein bands are no longer detected at all, including P14, P18, P22, P24, P28, a number of bands in the 31–38-kD range, P43, P52, and some of the 55–65-kD bands. The detection of the P39 bands is consistent throughout.

### Table 2. Summary of Antibody Binding Data

| JD-1          | B31 MHC restricted | B31 MHC nonrestricted |
|---------------|--------------------|-----------------------|
| nonreactive   | P29                | P24                   |
| crossreactive | P83                | P14                   |
| MHC restricted| P52-P65            | P17                   |
| MHC nonrestricted | P41 (fla) | P34 |
|               | P28                |                       |
|               | P30                | P43                   |
|               | P39                | P39                   |

### Figure 5. Western blot analysis of sera from five mouse strains binding *B. burgdorferi*, strain JD-1 low passage. (A) Sera drawn on day 12 postinfection; (B) sera drawn on day 26 postinfection; (C) sera drawn on day 60 postinfection. Molecular mass standards are given in the left margins. mAbs H9724 (antiflagellin, lane F) and H6831 (anti-OspB, lane B) were used at 1:100 and 1:50, respectively.
the screen of all these different sources of antigen with all of the mouse sera. A summary of these data is given in Table 2.

This study presents the primary data from an analysis of the response of recombinant inbred strains of mice differing at the MHC locus to the natural, tick-transmitted infection of *B. burgdorferi*. The object of this initial report was to analyze the overall polyclonal antibody response of the mouse strains to whole cell lysates of various strains and cultured isolates of the infectious spirochete. These results give us a preliminary indication of the potential antigenicity of various bacterial products in the murine MHC. Using these data we are generating mAbs from these tick-infected animals to begin to dissect the different bacterial molecules that induce an immune response. Once we can isolate the individual antigens, we can then analyze which mouse strains respond to a given antigen and what is the nature of that response, specifically, MHC restriction, kinetics, crossreactivity between bacterial strains, and antigenic variation between early isolates and high-passage culture derivatives of the same bacterial strain.

Finally, these results indicate that there are a subset of proteins that may have the potential to be more accurate diagnostic reagents than those presently in use. These are the P18, P39, and P43 proteins that induce an MHC-nonrestricted response and are crossreactive between at least two strains of *B. burgdorferi*. The P39 protein has already been cloned (8) and a mAb specific for P39 has been generated (T. Schwan, personal communication). We are using the mice from this study to generate mAb to the P18 and P43 proteins. Our results predict that all three of these bacterial antigens have diagnostic potential for Lyme disease and may improve Lyme serology in the very near future.
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