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Antibody to a 39-Kilodalton *Borrelia burgdorferi* Antigen (P39) as a Marker for Infection in Experimentally and Naturally Inoculated Animals

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Borrelia burgdorferi **expresses a conserved, species-specific 39-kDa protein (P39) that can stimulate antibodies during human infection. To confirm that anti-P39 antibodies are produced consistently in animals exposed to infectious spirochetes, white-footed mice, *Peromyscus leucopus*, and laboratory white mice, *Mus musculus* (strain BALB/c), were experimentally inoculated with either infectious or noninfectious *B. burgdorferi* and the antibody response to P39 was determined by immunoblot at 21 days postinoculation. All mice inoculated with approximately 10^7 infectious *B. burgdorferi* produced anti-P39 antibodies and were cultured positive for this spirochete. Mice inoculated with similar numbers of inactivated or viable noninfectious *B. burgdorferi* still producing P39 did not induce anti-P39 antibodies. By contrast, putative antiflagellin antibodies were detected in <18% of the infected animals, which supports the notion that antibody reactive with flagellin may not be reliable as a marker for *B. burgdorferi* exposure as was originally thought. Mice infected with *B. burgdorferi* following exposure to ticks (*Ixodes dammini*) produced anti-P39 antibodies no later than 7 days postinfection, indicating that P39 is an effective immunogen in natural infections. Notably, anti-P39 antibodies were the predominant *B. burgdorferi* reactive antibodies detected early in the infection. Our results indicate that anti-P39 antibodies are produced in response to an active infection and are therefore reliable markers for infection in experimentally and naturally inoculated animals.**

The clinical and pathological manifestations resulting from a *Borrelia burgdorferi* infection continue to increase (5, 15, 20, 29, 31, 33–35, 40). As a result, the criteria for the diagnosis of Lyme borreliosis are not clearly defined and may depend on subjective clinical evaluations and unreliable laboratory tests. This problem, which is compounded by the tendency for Lyme borreliosis to mimic numerous immune disorders and inflammatory diseases, coupled with the increasing incidence of Lyme disease cases throughout North America (10, 11) and Europe and Asia (1, 14, 37), has created a demand for new and/or improved serological tests. Indirect immunofluorescent-antibody staining (30,36), enzyme-linked immunosorbent assays (24, 25), and immunoblot methods (2, 18) have been used as serological tests for Lyme borreliosis. These tests, however, have used either whole-cell extracts or cellular fractions as the diagnostic antigen, and this has led to problems of cross-reactivity with antibodies induced by other bacteria.

A 39-kDa antigen, designated P39, that is specific to *B. burgdorferi* and conserved among North American and European isolates was recently identified and its gene was cloned (42). Immune electron microscopy and immunoblot analysis revealed that P39 was distinct from previously described *B. burgdorferi* antigens, yet it reacted with serum specimens collected from Lyme borreliosis patients. Anti-P39 antibodies were also detected in a significant proportion of serum specimens determined to be seronegative by indirect fluorescent-antibody staining (42). These observations and the lack of antibodies to P39 in sera from patients with clinically similar diseases suggested that anti-P39 antibodies may be useful diagnostic markers for *B. burgdorferi* infections. A 39-kDa band in gel profiles that reacted with serum specimens from Lyme borreliosis patients has been described in other studies (18, 21, 25, 28), but the immunological specificity, if any, of these antigens was not documented. It is possible, however, that these reports also describe the P39 antigen. Unfortunately, the true prevalence of antibodies to P39 in humans with Lyme borreliosis is difficult to determine because not all serum specimens can be unequivocally linked to patients exposed to *B. burgdorferi*. Further studies assessing the immunological response to P39 in infected animals are therefore necessary.

Previous studies have reported that the flagellin of *B. burgdorferi* stimulates the earliest antibody response in the course of an infection (2, 13, 18). Antiflagellin antibodies, however, were not detected in many of the anti-P39-positive serum specimens from Lyme borreliosis patients (42), although immunoglobulin M (IgM) antibodies were probably not detected in the assay. It may be that anti-P39 antibodies have previously been mistaken for antiflagellin antibodies when human sera were tested by Western immunoblot. Brandt et al. (7) have suggested a similar possibility but described an antigen with a molecular mass of 37 kDa being more reactive than the flagellin. It remains to be determined whether the 37-kDa antigen is distinct from P39.

The white-footed mouse, *Peromyscus leucopus*, is a major reservoir of *B. burgdorferi* in the wild and is believed to be an integral part of the cycle that ensures that tick populations remain infected (6, 8, 26). In addition, many other vertebrates are naturally infected and appear to be useful sentinels for emerging Lyme disease endemic areas (9, 16, 22, 23). The isolation of cultivable spirochetes is the most definitive method for confirming an infection in these ani-

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mals, but this process is time-consuming and often unreliable because failure to isolate the organism may not exclude the existence of an infection. Serological confirmation is the most practical means, but as with current human serological tests, reactive antibodies are not necessarily specific indicators of a *B. burgdorferi* infection.

In the present study, several species of animals were examined for anti-P39 antibodies after experimental inoculation with different preparations of *B. burgdorferi* or following exposure to infected ticks. Our objective was to determine, under controlled experimental conditions, the reliability of the humoral response to P39 in animals as a diagnostic test for infection and compare this response with that of the flagellin.

MATERIALS AND METHODS

Bacterial strains. *B. burgdorferi* Sh-2-82, B31, BR4-3028, CT21305, CT26816, CA-3-87, ECM-NY-86, and G2, described previously (41), were cultured at 32°C in BSK-II medium unless otherwise indicated. Passages 2 (P2), 6 (P6), and 246 (P246) of strain Sh-2-82 were obtained by serially passaging the organism twice a week in BSK-II medium. *Escherichia coli* XL1-blue (Stratagene) carrying either pSPR33 or the cloning vector pBluescript SK has been described before (42). Plasmid pSPR33 carries the gene encoding the *B. burgdorferi* protein P39. Clones of P6 and P246 (strain Sh-2-82) were obtained by limiting dilution as described previously (3).

Animal inoculations. BALB/c laboratory white mice and white-footed mice were females of 6 to 8 and 8 to 12 weeks of age, respectively, at the time of inoculation. Mice were from colonies maintained at Rocky Mountain Laboratories, Hamilton, Mont., and were free of *B. burgdorferi*. Animals were bled 1 day prior to inoculation from the retro-orbital sinus.

Mice were inoculated with viable or heat-inactivated spirochetes as described previously (38). Briefly, after recovery from a stationary-phase BSK-II culture, spirochetes were resuspended in phosphate-buffered saline plus 5 mM $MgCl_2$ (pH 7.4). Cell numbers were adjusted to an optical density of 0.2 at 600 nm (2×10^7 to 5×10^7/ml), and 0.5 ml of this suspension was administered to each animal intraperitoneally. Mice were inoculated with the same number of heat-inactivated (56°C for 30 min) spirochetes after cell disruption on ice by sonication (15-s bursts for a total of 4 min at an output of 4; Sonifier-cell disrupter model 185 [Branson Sonic Power Co., Danbury, Conn.]).

Mice were killed at either 21 days or 6 months postinoculation, and the spleens and urinary bladders were cultured in BSK-II medium as described previously (38).

Exposure of mice to ticks. Infected *Ixodes dammini* nymphs were from two cohorts with infectivity rates of close to 100% (group A) and <50% (group B). Tick cohorts were initially infected by feeding uninfected adults on white-footed mice inoculated with a P1 of strain Sh-2-82. Each of 10 white-footed mice was exposed to four nymphs. Five mice received nymphs from group A and five mice received nymphs from group B. Ticks were allowed to feed to repletion, and the day on which the last tick dropped off was designated day 0 post-tick exposure. Mice were bled from the retro-orbital sinus 1 week before tick exposure, 2 days post-tick exposure, and on various days thereafter until day 63 post-tick exposure. At this time, mice were killed and their spleens, urinary bladders, and skin scrapings were assessed for cultivable spirochetes in BSK-II medium.

Immunoblot procedures. Rabbit anti-P39 antiserum (anti-pSPR33) was prepared as described previously (42) and was used at a dilution of 1:500. Antibodies to *Peromyscus* serum immunoglobulins of all classes were prepared in a rabbit and used at a dilution of 1:1,000 (39). This antiserum detects IgM and IgG antibodies in sera from infected mice that react with *B. burgdorferi* antigens (39). Immunoblot analysis of animal sera was performed as described previously (42). Briefly, *B. burgdorferi* and *E. coli* cells were recovered from liquid cultures by centrifugation and resuspended in phosphate-buffered saline to give an optical density of 0.2 at 600 nm. Cells from 2 ml of this suspension were resuspended in 100 μl of distilled water and 50 μl of sample buffer (0.2 M Tris [pH 6.8], 30% [vol/vol] glycerol, 3% [wt/vol] sodium dodecyl sulfate [SDS], 0.002% [wt/vol] bromophenol blue). Mercaptoethanol (14.3 M) was added to a final concentration of 10% (vol/vol), and the samples were boiled for 4 min. A 20-μl portion was loaded onto a 12.5% SDS–polyacrylamide gel, and electrophoresis, immunoblotting, and the detection of bound antibody with ^{125}I-labeled protein A were done as described previously (39). All animal sera were tested at a dilution of 1:100 for antibody that bound to the recombinant P39 and to antigens in *B. burgdorferi* whole-cell lysates, as described in Results.

RESULTS

Mice inoculated with infectious organisms. To determine the association of anti-P39 antibodies and Lyme borreliosis in experimentally inoculated animals, 12 white-footed mice and 10 BALB/c mice were inoculated with a low passage (P6) of *B. burgdorferi* Sh-2-82. On 0, 7, 14, and 21 days postinoculation, mice were bled and the sera were tested for antibody to P39 by Western blot. P39-specific antibodies were detected in all sera collected on 7, 14 (data not shown), and 21 days (Fig. 1) by comparing immunoblot profiles of *E. coli* cells that do and do not express P39. The presence of anti-P39 antibodies was indicated by the presence of an immunoreactive 39-kDa band in lysates of cells that express P39. Spleens or urinary bladders evaluated 21 days postinoculation from all mice were cultured positive for *B. burgdorferi*. A 39-kDa band was also observed in whole-cell lysates of *B. burgdorferi* immunoblotted with serum collected from infected animals (data not shown). Anti-P39 antibodies continued to be detected in six additional mice of both species for at least 6 months after seroconversion. These mice were also cultured positive for *B. burgdorferi*. All preimmune sera were negative for P39 antibodies. Thus, sera from mice inoculated with infectious spirochetes contained anti-P39 antibodies as early as 7 days and until at least 6 months postinoculation.

White-footed mice were infected with *B. burgdorferi* CA-3-87, CT26816, CT21305, and ECM-NY-86, all of which had previously been shown to express P39 (42) (data not shown). Anti-P39 antibodies were detected in all mouse sera tested 21 days postinoculation (data not shown). Although only one mouse per strain was tested, the results indicate that different strains of *B. burgdorferi* stimulate antibodies reactive to the recombinant P39. Therefore, it is unlikely that the antibody response to P39 is restricted to strain Sh-2-82 and its derivatives.

Mice inoculated with inactivated organisms. To determine whether the immunological response to P39 was merely due to the immunogenicity of the inoculum rather than an actual infection, additional experiments were done with inactivated P6 organisms. After cells were heated and disrupted by

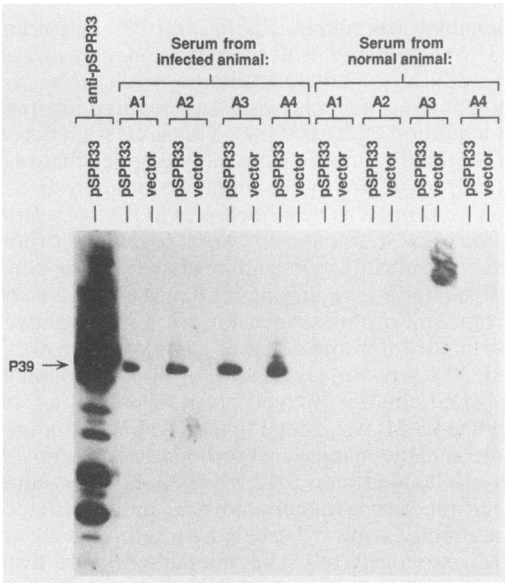

FIG. 1. Immunoblot showing the detection of anti-P39 antibodies in serum specimens collected from *B. burgdorferi*-infected mice. Western-blotted whole-cell lysates of *E. coli* plus pSPR33 (pSPR33) and *E. coli* plus vector (vector) were incubated with serum specimens collected from white-footed mice (A1 to A4) before inoculation (normal animal) and 21 days postinoculation (infected animal). Bound antibody was detected with ^{125}I-labeled protein A and autoradiography with an exposure of 6 h. The left lane was incubated with anti-pSPR33 serum to locate P39. Not all serum specimens from the 22 mice tested are shown.

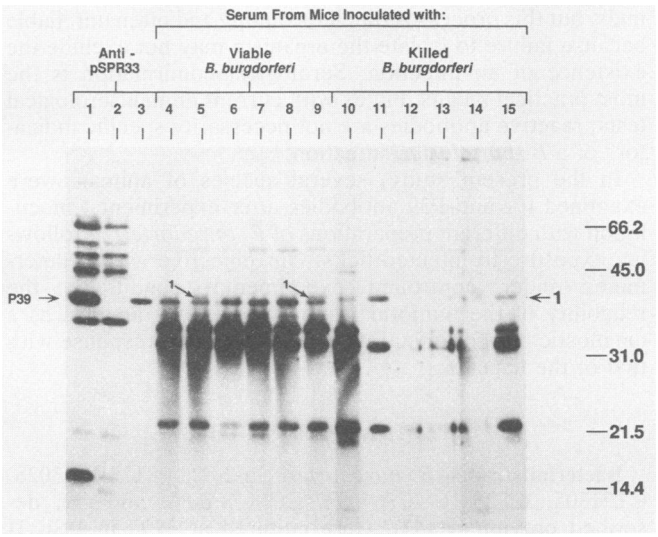

FIG. 2. Immunoblot analysis of serum specimens collected from mice inoculated with low-passaged (P6) *B. burgdorferi* that were either viable (Viable) or sonicated after heat inactivation (Killed). Western-blotted whole-cell lysates of *E. coli* plus pSPR33 (lane 1), *E. coli* plus vector (lane 2), and *B. burgdorferi* Sh-2-82 (P6) (lanes 3 to 15) were incubated with either anti-pSPR33 serum or serum collected from inoculated BALB/c mice. Bound antibody was detected with ^{125}I-labeled protein A and autoradiography with an exposure of 2 h. Arrow 1 indicates the position of the 41-kDa band, and the position of the molecular weight markers (10^3) are indicated on the right.

sonication, six BALB/c mice were inoculated with this preparation and the animals' antibody responses at 21 days postinoculation were compared with those for six BALB/c mice inoculated with untreated (viable) P6 organisms. By Western blot, serum specimens from each animal were tested for reactivity with whole-cell lysates of *B. burgdorferi* (Fig. 2, lanes 4 to 15), *E. coli* expressing P39, and *E. coli* carrying only the cloning vector (data not shown). Lanes 1 to 3 were incubated with anti-pSPR33 serum to identify the position of P39 (Fig. 2). Despite a strong humoral response to numerous *B. burgdorferi* antigens, mice that received inactivated and lysed organisms did not produce anti-P39 antibodies. These antibodies, however, were detected in all serum specimens from mice inoculated with the viable spirochetes.

Lysates of *B. burgdorferi* prepared by heating and sonication still contained P39 antigen that bound anti-P39 antibodies when tested by immunoblot with either anti-pSPR33 or sera from infected animals (data not shown). Furthermore, *E. coli* cells expressing P39 that were heated and disrupted by sonication stimulated the production of anti-P39 antibodies in six white-footed mice tested. These antibodies bound to P39 produced by *B. burgdorferi* (Fig. 3, lanes 3 to 7) and to the recombinant P39 (data not shown), indicating that the recombinant P39 is still antigenically similar to P39 expressed by *B. burgdorferi*. As a control for nonspecific reactivity, it was shown that *E. coli* cell-plus-vector lysates prepared in the same manner did not induce antibodies reactive with P39 or any other significant *B. burgdorferi* antigens (Fig. 3, lanes 14 to 17). Therefore, the lack of P39 antibodies in mice immunized with heat- and sonically treated whole-cell lysates of *B. burgdorferi* is unlikely to be due to alterations induced in the antigen by heating or sonication. Therefore, anti-P39 antibodies are stimulated during an infection that follows inoculation with approximately 10^7 viable organisms but not when mice are exposed to a similar number of inactivated *B. burgdorferi*. This is despite the fact that killed spirochetes do have antigenically reactive P39 associated with their lysates.

Absence of antiflagellin antibodies in infected mice. Sera from the 40 mice infected with strain Sh-2-82 so far described reacted with P39, but only seven of these serum specimens had detectable antibodies that bound a 41-kDa band, which for the present study we presume to be flagellin; the 41-kDa band may be composed of several unrelated antigens, any of which could be reacting with serum from infected animals (Fig. 2; data not shown). Furthermore, for those sera that did react to the flagellin, the associated signal was always weaker than the corresponding P39 signal. For example, lanes 5 and 9 (Fig. 2) have detectable flagellin bands, but the signals for these two bands are weaker than the corresponding P39 signals. Attempts to enhance the flagellin signal by using rabbit anti-*Peromyscus* serum, which detects IgM and IgG antibodies, were unsuccessful (data not shown). Notably, stronger, more consistent reactivity to the flagellin was observed with the serum specimens from six mice inoculated with *B. burgdorferi* lysates (Fig. 2, lanes 10 to 15; Fig. 3, lanes 8 to 13). This indicates that these animals are capable of responding to the flagellin, but apparently this response is best when animals are inoculated with cell lysates. Sera from the four mice infected with strains CA-3-87, CT26816, CT21305, and ECM-NY-86 also lacked antibodies that bound to the flagellin (data not shown), indicating that the apparent absence of these antibodies is not peculiar to Sh-2-82-infected animals.

Mice inoculated with viable noninfectious organisms. Ex-

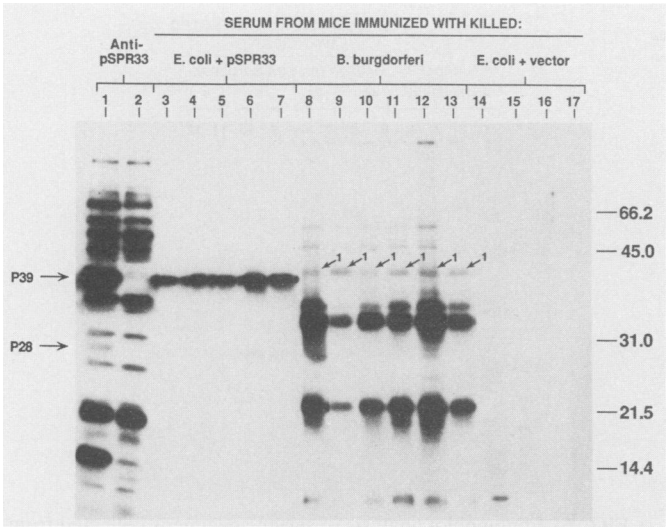

FIG. 3. Immunogenicity of P39 associated with heat-killed organisms that were lysed by sonication. Western-blotted whole-cell lysates of *B. burgdorferi* Sh-2-82 (P6) (lanes 3 to 17) were incubated with serum specimens collected from BALB/c mice immunized with cell lysates of *E. coli* plus pSPR33, *B. burgdorferi* Sh-2-82 (P6), and *E. coli* plus vector. Lysates represented in lanes 1 (*E. coli* plus pSPR33) and 2 (*E. coli* plus vector) were incubated with anti-pSPR33 serum to confirm the location of P39. Bound antibody was detected with ^{125}I-labeled protein A and autoradiography with an exposure of 4 h. Not all serum specimens tested are shown, and the arrows denote the locations of P39 and P28. P28 is a *B. burgdorferi* antigen expressed by pSPR33 (42) and is not discussed further in this study. Arrow 1 indicates the position of the 41-kDa band, and the position of the molecular weight markers (10^3) are noted on the right.

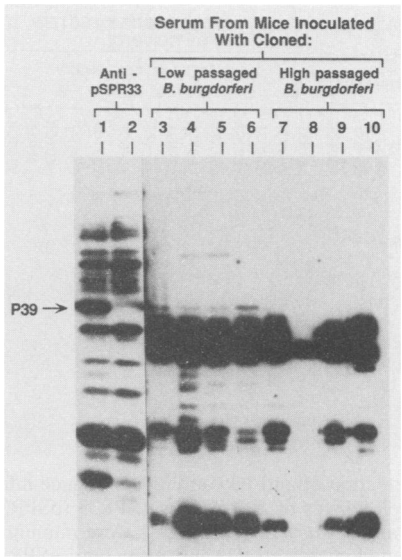

FIG. 4. Immunoblot analysis of serum specimens collected from mice inoculated with either infectious or noninfectious cloned *B. burgdorferi*. Western-blotted whole-cell lysates of *B. burgdorferi* Sh-2-82 (P6) (lanes 3 to 10) were incubated with serum specimens collected from BALB/c mice inoculated with either infectious (Low passaged) or noninfectious (High passaged) *B. burgdorferi*. Cell lysates in lanes 1 (*E. coli* plus pSPR33) and 2 (*E. coli* plus vector) were incubated with anti-pSPR33 serum to identify the position of P39 (arrow). Bound antibody was detected with ^{125}I-labeled protein A and autoradiography with exposures of 4 (lanes 1 and 2) and 8 (lanes 3 to 10) h. Not all serum specimens tested are shown.

cept for the presence of either anti-P39 or antiflagellin antibodies, the two groups of sera, one from infected mice and the other from mice immunized with heat-treated *B. burgdorferi* lysates, had relatively similar reactivities (Fig. 2 and 3). In this study, antibodies that bound antigens in Western blots were detected with ^{125}I-labeled protein A, so it is likely that only the IgG antibodies were detected. It appears, then, that the single most significant difference in IgG antibodies between infected and uninfected mice inoculated with approximately 10^7 spirochetes is the animal's response to P39. This conclusion is further supported by the observation that viable noninfectious spirochetes did not induce an antibody response to P39 in mice in spite of a strong humoral response to other *B. burgdorferi* antigens. In this experiment, six BALB/c mice were inoculated with a high-passaged variant (P246) of strain Sh-2-82 that was cloned by limiting dilution. This strain expressed a 39-kDa antigen that reacted with anti-pSPR33 serum and to sera from infected mice. It is concluded, therefore, that this strain expresses P39. At 21 days postinoculation, mice were bled and their sera were tested for reactivity with whole-cell lysates of *B. burgdorferi* Sh-2-82 (Fig. 4, lanes 7 to 10), *E. coli* expressing P39, and *E. coli* carrying only the cloning vector (data not shown). These sera were compared with six serum specimens from similar mice inoculated with viable P6 organisms that had also been cloned and shown to express P39 (Fig. 4, lanes 3 to 6). Anti-P39 antibodies were detected only in the sera from the mice inoculated with P6 organisms. Sera from animals inoculated with P246 organisms did not react with P39 produced by P246 organisms (data not shown), which eliminates the possibility that anti-P39 antibodies are produced in these animals but are not detected using P39 expressed by either P6 organisms or the *E. coli* recombinant. Testing sera for reactivity with *E. coli* cell lysates confirmed that the antibodies binding the 39-kDa band in cell lysates of *B. burgdorferi* (lanes 3 to 6) were anti-P39 in their specificity (data not shown). Notably, P39 signals observed with sera from animals infected with the cloned organisms (Fig. 4) were relatively weaker than signals associated with sera from animals infected with uncloned cells (Fig. 2). The reason for this is not clear, but it could simply be due to different concentrations of P39 in different batches of cells (either *B. burgdorferi* or *E. coli*) used in the various immunoblots. Alternatively, cloning of the spirochete may reduce its ability to elicit an anti-P39 response.

Spirochetes were isolated from urinary bladder triturates of the anti-P39-positive animals but not from any of those that received viable P246 organisms. The uncloned, high-passaged (>50 in vitro passages) *B. burgdorferi* strains P246, B31, BR4-3028, and G2 were also unable to stimulate an anti-P39 antibody response (data not shown), in spite of their ability to produce P39 when cultured in vitro. Thus, our data indicate that, like inactivated organisms, viable but noninfectious *B. burgdorferi* do not elicit an anti-P39 antibody response in mice, even though they induce a considerable antibody response to other *B. burgdorferi* antigens (Fig. 4, lane 7 to 10).

Anti-P39 antibodies in mice infected by tick bite. To determine whether antibodies to P39 correlate with infections derived from tick bites, two groups (A and B) of *I. dammini* nymphs representing two cohorts infected with *B. burgdorferi* Sh-2-82 were fed to repletion on white-footed mice. By

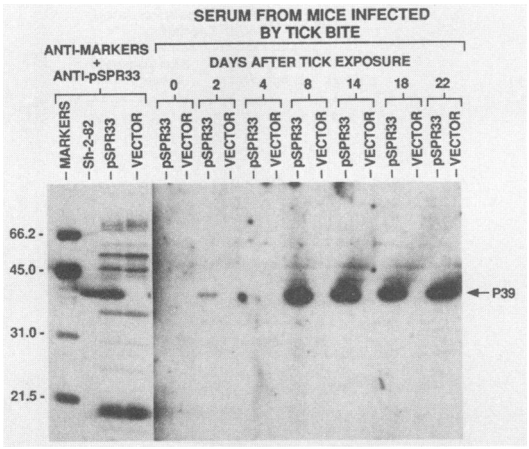

FIG. 5. Detection of anti-P39 antibodies in mice infected by tick bite. Whole-cell lysates of *E. coli* plus pSPR33 (pSPR33) or vector only (vector) and *B. burgdorferi* Sh-2-82 were immunoblotted with sera from infected mice, anti-P39 antiserum (anti-pSPR33), or rabbit serum raised to the molecular weight markers (anti-markers). Bound antibody was detected with ^{125}I-labeled protein A and autoradiography with an exposure of 2 (anti-pSPR33/anti-markers) and 16 (serum from mice) h. Molecular weights (10^3) of the marker proteins (markers) are indicated on the left. Serum from only one animal is shown, and 0 days corresponds to serum collected before ticks were placed on the animal.

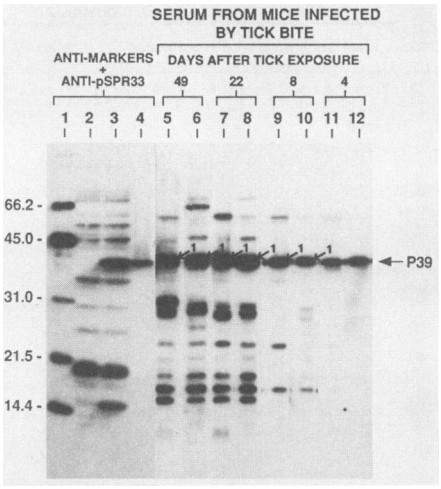

FIG. 6. Immunoblot analysis of serum specimens collected from mice infected by tick bite. Western-blotted whole-cell lysates of *E. coli* plus vector (lane 2), *E. coli* plus pSPR33 (lane 3), and *B. burgdorferi* Sh-2-82 (P6) (lanes 4 to 12) were incubated with sera from infected mice, anti-P39 antiserum (anti-pSPR33), or antibodies reactive with the molecular weight markers (anti-markers). Bound antibody was detected with ^{125}I-labeled protein A and autoradiography with an exposure of 2 (anti-pSPR33/anti-markers) and 8 (serum from mice) h. Serum specimens from only two mice are shown: lanes 5, 7, 9, and 11, animal 1; lanes 6, 8, 10, and 12, animal 2. Molecular weights (10^3) of the immunoreactive marker proteins (lane 1) are indicated on the left.

immunofluorescence staining of gut tissue from ticks, it was shown that group A had a higher infection rate than group B (data not shown). Only four of the five animals exposed to nymphs from group A and two of the five animals that received group B nymphs produced anti-P39 antibodies, and in all cases these could be detected as early as 2 days post-tick exposure (Fig. 5). Antibodies to P39 could still be detected 63 days post-tick exposure. Notably, it was not until 8 to 16 days post-tick exposure that antibodies to other *B. burgdorferi* antigens could be detected (Fig. 6), including a 41-kDa band, presumably flagellin (Fig. 6, arrow 1). It should be noted that band 1 and the corresponding P39 band were observed as two distinct bands in all lanes with a 2-h exposure. This exposure, however, reduced the signal for many of the other *B. burgdorferi* antigens below a detectable level. Because sera from these animals collected after 2 weeks reacted similarly and bound to at least eight *B. burgdorferi* antigens in Western blots (Fig. 6) and five of the six mice were cultured positive for *B. burgdorferi* (spirochetes were recovered from spleen, bladder, or skin scrapings), it is concluded that these animals were infected. Mice that did not produce anti-P39 antibodies lacked other *B. burgdorferi*-reactive antibodies and were cultured negative for the infectious agent, indicating that they were not infected. The reactivity of a 41-kDa band with all sera from animals infected by tick bite is in contrast to the relative absence of these reactive antibodies when animals were infected experimentally.

Influence of growth temperature on P39 production. Because P39 expression by *B. burgdorferi* was demonstrated by immunoblot with spirochetes cultured at 32°C although rodents can have average body temperatures exceeding 37°C, the effect of elevated temperatures on P39 expression was examined. P2 (infectious) and P246 (noninfectious) organisms produced the same amounts of P39 at 32°C, but for P246 organisms levels were considerably reduced at temperatures above 37°C (Fig. 7). All lanes contained the same amount of total cellular protein, which was confirmed by staining gels with silver and Coomassie brilliant blue (data not shown). In contrast, P2 organisms, the low-passaged strains CT26816, CA-3-87, CT21305, and ECM-NY-86, and the high-passaged strains B31 (Fig. 7) and BR4-3028 (data not shown) produced similar levels of P39 at all temperatures. P246 organisms appeared to grow as well as B31 and BR4-3028 at the elevated temperatures, indicating that the reduced P39 production in P246 organisms at these temperatures is unlikely to be due to differences in cell growth.

DISCUSSION

When evaluating an immunological response to a pathogen, particularly when the resulting disease has such a varied clinical picture as does Lyme borreliosis, human patients are not always the best study group. The difficulty in both confirming exposure to the pathogen and correlating specific antibody responses to an actual infection makes a controlled study nearly impossible. To overcome these difficulties, we infected laboratory-maintained mice to evaluate the immunological response to P39. In the present study, anti-P39 antibodies were consistently detected in infected animals inoculated experimentally with approximately 10^7 organisms. By contrast, these antibodies were not elicited when animals were exposed to a similar number of live noninfectious organisms. We also showed that these antibodies could be detected in naturally infected animals as early as 2 days post-tick exposure, but because these antibodies were probably of the IgG class, animals exposed to ticks were most likely infected several days before the last tick had dropped off; ticks on each animal fed for 4 to 5 days, indicating that animals may have been infected on the first day of feeding,

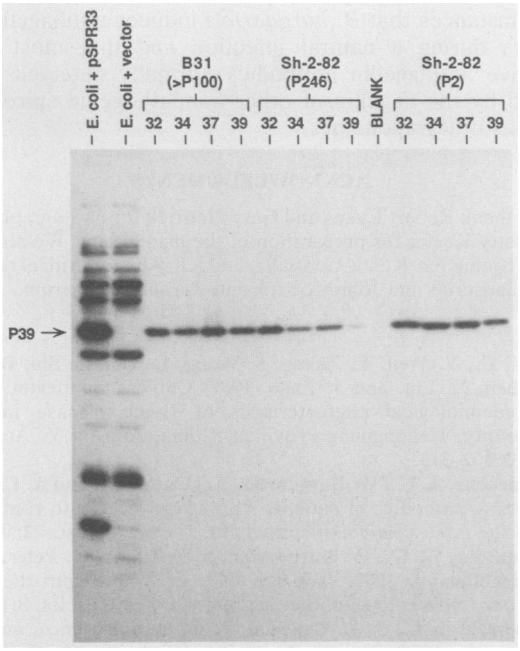

FIG. 7. Effect of growth temperature on production of P39 by infectious (P2) and noninfectious (P246 and >P100) *B. burgdorferi*. Western-blotted whole-cell lysates of *E. coli* plus pSPR33, *E. coli* plus vector, and *B. burgdorferi* B31 (>P100), Sh-2-82 (P246), and Sh-2-82 (P2) were incubated with anti-pSPR33 serum. Numbers refer to the temperatures (°C) at which the cells were cultured prior to lysate preparation. The position of P39 is indicated by the arrow.

which would mean that 2 days post-tick exposure translates to 7 days postinfection. Several rabbits inoculated with infectious *B. burgdorferi* also produced anti-P39 antibodies but did not when inoculated with inactivated spirochetes (unpublished observation). Although only a small number of rabbits have so far been examined, this indicates that mice are not the only laboratory-maintained animals capable of responding to P39 antigen expressed by infectious organisms. Thus, within the limits of the inoculum sizes used in this study, it is concluded that anti-P39 antibodies are relatively specific for the infected state and are reliable markers for confirming an infection in experimentally inoculated or tick-exposed animals. Furthermore, because P39 antibodies are both detected in human patients with Lyme borreliosis and apparently specific for this disease, it has been suggested that anti-P39 antibodies may be reliable markers of human Lyme borreliosis (42). The data presented in this study strongly support that notion.

Studies of the pathogenic mechanisms used by spirochetes to infect animals and perhaps cause disease may now be possible without the need to kill the animals or culture skin biopsies to confirm an established infection. This is an important point, as exposure to live spirochetes should, at least following experimental inoculation, induce an antibody response but only anti-P39 antibodies can confirm whether an infection has actually been established, assuming that the infectious dose is no greater than that used in our study. Thus, the serological surveillance of animal populations in the wild, with respect to actual infections versus the potential for passive exposure to *B. burgdorferi*, may now be possible. Although further studies are necessary, it is hoped that the use of P39 as a diagnostic marker will aid in a better epidemiological assessment of Lyme borreliosis.

Although viable noninfectious spirochetes produced P39, they did not elicit anti-P39 antibodies after their inoculation into animals. The reason for this is unknown, but it suggests that, for P39 to stimulate an antibody response, given the limited number of spirochetes in the inoculum ($\leq 10^7$), an infection must first be established. This is the first report of a *B. burgdorferi* protein with this characteristic and distinguishes it from other antigens reported to elicit an antibody response during an infection by this spirochete. Conceivably, after infectious *B. burgdorferi* cells are injected into the animal, P39 may be modified, rendering it immunogenic perhaps in a way similar to hapten-conjugate formation. Alternatively, once an infection has been established, P39 may increase in concentration beyond what can be obtained in vitro. When animals are inoculated with noninfectious organisms, the rapid removal of the organisms would presumably inhibit the accumulation of P39 or its putative modification or both. If the antibody response to P39 is merely dependent on the amount of P39 (i.e., there is a minimum inducing level), then presumably animals exposed to noninfectious organisms could elicit anti-P39 antibodies if the dose administered is greater than what was used in this study. A dose of such magnitude, however, is unlikely to be encountered naturally, and experimental infections can be readily established with considerably fewer organisms (32). Even if noninfectious organisms can induce anti-P39 antibodies when given to animals in exceptionally high doses, this would not affect the usefulness of these antibodies as markers for *B. burgdorferi* infections in nature or laboratory experiments. The above possibilities assume that the P39 antigens produced by infectious and noninfectious organisms or by different infectious strains are identical: all of these organisms produced similar-sized antigens sharing immunoreactive epitopes that induced cross-reactive antibodies in animals infected with the different *B. burgdorferi* strains. All things considered, the relatively specific association of P39 antibodies with the infected state is highly significant and could mean that *B. burgdorferi* virulence is dependent, at least in part, on the expression of P39.

The *B. burgdorferi* inoculum used in our experiments, irrespective of whether the organisms were low or high passaged, presumably contained P39 in a concentration too low and/or in association with spirochete components so that it could not elicit anti-P39 antibodies until after an infection was established. This supports the above possibilities but is in direct contrast to what was shown for P39 associated with killed *E. coli* cells, which did elicit a good response. The explanation for this remains puzzling, although the answer may well lie in the ability of *E. coli* extracts to serve as an effective nonspecific immunostimulant or in some way modify the P39 antigen. This implies that *B. burgdorferi* extracts, including the putative endotoxin (19), do not have these characteristics. Other possibilities to consider are that P39 produced by *B. burgdorferi* may be presented to the immune system differently from the recombinant P39 or that P39 is merely in higher concentration in *E. coli* than in *B. burgdorferi* cells. With regard to the latter notion, it remains possible that animals produce P39 antibodies if immunized with lysed *B. burgdorferi* in adjuvants or with higher concentrations of P39 by way of increasing the number of organisms above the dose used in this study.

The observation that P39 expression can be influenced by temperature, albeit in one noninfectious variant, requires further investigation. Thermal regulation of several *B. burgdorferi* proteins has been described recently (12). In that study, the synthesis of a 39-kDa antigen was described, but

unlike P39 it increased in amount in response to elevated growth temperatures. Therefore, this antigen is probably not P39, although in the same study a 29.5-kDa protein that was produced in lower concentrations at higher growth temperatures was noted. This effect, however, was not dependent on the passage of the strain, as was the case for P39. The effect of in vitro passages on the stability of P39 expression by spirochetes grown at high temperatures was not seen for other isolates of *B. burgdorferi*. Therefore, although P39 production may be affected by temperatures at or above 37°C, this relative sensitivity appears to be restricted to a high-passaged variant. Thus, this phenomenon is probably not significant with respect to natural spirochete-host interactions, but such mutants may be useful for the genetic analysis of P39 regulation.

The OspA and OspB antigens also elicit antibodies in experimentally infected animals, but these antibodies appear to be considerably less prevalent in Lyme disease patients and naturally infected animals (4, 17). In this context, it could be argued that the use of experimentally inoculated animals to show a consistent anti-P39 antibody response to infection may similarly be peculiar to the type of inoculum and not necessarily reflect the true response to a natural exposure. This is unlikely, however, as anti-P39 antibodies were detected in white-footed mice exposed to *B. burgdorferi*-infected ticks.

The lack of detectable antiflagellin IgG antibodies in most experimentally infected animals, which was shown by the absence of detectable immunoreactive 41-kDa bands in Western blots, is different from what might be expected based on the previously reported immunogenic properties of this antigen (2, 13, 18). If flagellin elicits the earliest immunological response in humans, including IgM and IgG antibodies, we would have expected flagellin in our experimental system to have stimulated the production of detectable antiflagellin antibodies in most, if not all, of the infected animals. Although we did not evaluate in depth classes of immunoglobulins other than IgG, an attempt was made to enhance for antiflagellin IgM antibodies in Western blots by using rabbit anti-*Peromyscus* serum. This did not result in additional or stronger signals in the region of the flagellin, suggesting that an IgM response may also be absent. These results may be a further indication that antiflagellin antibodies are not as frequently induced during an infection, as was originally thought. However, antibodies reactive with a 41-kDa antigen, presumably flagellin, were detected in all serum specimens from six mice infected by tick bite. Although only a small number of animals were examined in this part of the study, it appears that experimental infections induce a response different from that established by tick bite. Greene et al. (17) have also noted a difference between experimentally and naturally infected animals in their immunological response to *B. burgdorferi*. These observations may require a more critical evaluation of animal models of Lyme borreliosis that depend on experimental inoculations if immunological mechanisms involved in protection are to be assessed.

The response to P39 as opposed to the flagellin is clearly stronger and more reliable in the animals examined in this study, whether experimentally or naturally infected. This supports our earlier observation (42) that human Lyme disease sera frequently react with P39 but not with the flagellin when tested by immunoblot. Magnarelli et al. (27) have recently reported the cross-reactivity of nonspecific treponemal antibodies in serological tests for Lyme borreliosis. Thus, the possibility exists that it is in only some circumstances that *B. burgdorferi* induces antiflagellin antibodies during a natural infection and that most of the reactive antiflagellin antibodies normally detected are induced by the flagellin of other nonpathogenic spirochetes, such as oral treponemes.

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