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Analysis of *Leptospira* spp., *Leptonema illini*, and *Rickettsia rickettsii* for the 39-Kilodalton Antigen (P39) of *Borrelia burgdorferi*

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Five serovars of *Leptospira interrogans*, *Leptospira biflexa*, *Leptonema illini*, and *Rickettsia rickettsii* were examined and found not to contain the 39-kDa antigen (P39) of *Borrelia burgdorferi*, the Lyme disease spirochete. The specificity of this antigen and its reactivity with human Lyme disease sera should exclude the possibility of false-positive serum samples from patients having had either leptospirosis or Rocky Mountain spotted fever, as well as tick-borne relapsing fever and syphilis, as reported previously (W. J. Simpson, M. E. Schrumpf, and T. G. Schwan, J. Clin. Microbiol. 28:1329–1337, 1990).

Diagnosing Lyme borreliosis in humans is a clinical decision that may or may not be supported by a positive serological test. Ruling out the disease is also complicated by serological tests being falsely positive because of cross-reactive antibodies produced during other bacterial infections. Recently, a 39-kDa antigen (P39) of *Borrelia burgdorferi*, the causative agent of Lyme borreliosis, was identified, cloned, and expressed in *Escherichia coli* (16). This antigen was present in all of the North American isolates and the one European isolate of *B. burgdorferi* examined but was not observed in five other *Borrelia* species. Additionally, this antigen was reactive with sera from patients having had Lyme borreliosis but was not reactive with sera from normal individuals or patients having had either tick-borne relapsing fever or syphilis (16). White-footed mice, *Peromyscus leucopus*, infected with *B. burgdorferi* by tick bite also produced antibodies to P39 as early as 2 to 7 days after the ticks had completed feeding (15).

Studies by other investigators demonstrated that a low percentage of sera from patients having had either leptospirosis or Rocky Mountain spotted fever reacted in serological tests for identifying antibodies to *B. burgdorferi* (5, 7, 10). In previous studies (15, 16), we did not examine *Leptospira* or *Rickettsia* spp. for P39 or antigens similar to it that might cause serological cross-reactivity. The presence of this antigen in only *B. burgdorferi* and in none of the other *Borrelia* species examined and the lack of reactivity of the antigen with sera from syphilis patients led us to believe that this antigen was unlikely to be present in more distantly related bacteria. However, given the continued need to improve upon the currently available serological tests for confirming or ruling out Lyme borreliosis in humans and other domestic and wild mammals and the potential for specific recombinant antigens like P39 to increase the specificity of such tests, we extended the earlier investigation of Simpson et al. (16) by examining *Leptospira* spp., *Leptonema illini*, and *Rickettsia rickettsii* for the P39 antigen of *B. burgdorferi*. Although little is known about the natural history of *L. illini*, it and *Leptospira* spp. infect domestic mammals and could stimulate the production of cross-reactive antibodies when animal sera are tested against whole-cell lysates of the Lyme disease spirochete.

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B. burgdorferi B-31 (ATCC 35210) and Sh-2-82 originated from *Ixodes dammini* ticks collected on Shelter Island, N.Y. (4, 12), and were maintained in BSK-II medium in our laboratory (1). *Leptospira interrogans* serovars ballum, canicola, copenhageni, grippotyphosa, and pomona, *Leptospira biflexa* serovar patoc, and *L. illini* were obtained as pellets of frozen cells from D. Denee Thomas, The Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, N.C. *R. rickettsii* R originated from a wood tick, *Dermacentor andersoni*, from the Bitterroot Valley in western Montana (3) and was most recently maintained in Vero cell tissue cultures. *Escherichia coli* containing pSPR33, which expresses the P39 antigen, and *E. coli* containing only the cloning vector were described previously (16). Polyclonal antiserum to the *E. coli* recombinant expressing P39 was produced in a rabbit (16) and was used at a dilution of 1:500 to identify the P39 antigen in immunoblot analyses. A monoclonal antibody to P39 was produced (13a) and used in immunoblots at a dilution of 1:1,000. Convalescent-phase sera from human patients with Rocky Mountain spotted fever were provided by Burt Anderson, Centers for Disease Control, Atlanta, Ga., and used in immunoblots at a dilution of 1:100. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western immunoblotting, and ^{125}I-labeled protein A autoradiography were done as described previously (13, 16), as was an indirect immunofluorescence assay (IFA) with *B. burgdorferi* B-31 (4).

SDS-PAGE and immunoblotting were used to examine whole-cell lysates of the various bacteria for the P39 antigen. A Coomassie brilliant blue-stained 12.5% acrylamide gel of the whole-cell lysates of the *Leptospira* spp. and *L. illini* showed numerous polypeptides ranging in apparent size from over 92 kDa to under 14 kDa (Fig. 1). Identical amounts of lysates were separated in two additional gels, electroblotted onto nitrocellulose, and incubated with either the rabbit anti-recombinant P39 antiserum (Fig. 2A) or the mouse anti-P39 monoclonal antibody (Fig. 2B). Whole-cell lysates of *B. burgdorferi* Sh-2-82 (passage 5), the *E. coli* recombinant expressing P39, and *E. coli* containing only the cloning vector were included as controls for identifying the P39 antigen in its native and recombinant forms. While both the polyclonal and monoclonal antibodies clearly identified P39 in *B. burgdorferi* and the *E. coli* recombinant, neither of the antibodies reacted significantly with any polypeptides in any of the serovars of *L. interrogans*, *L. biflexa*, or *L. illini* (Fig.

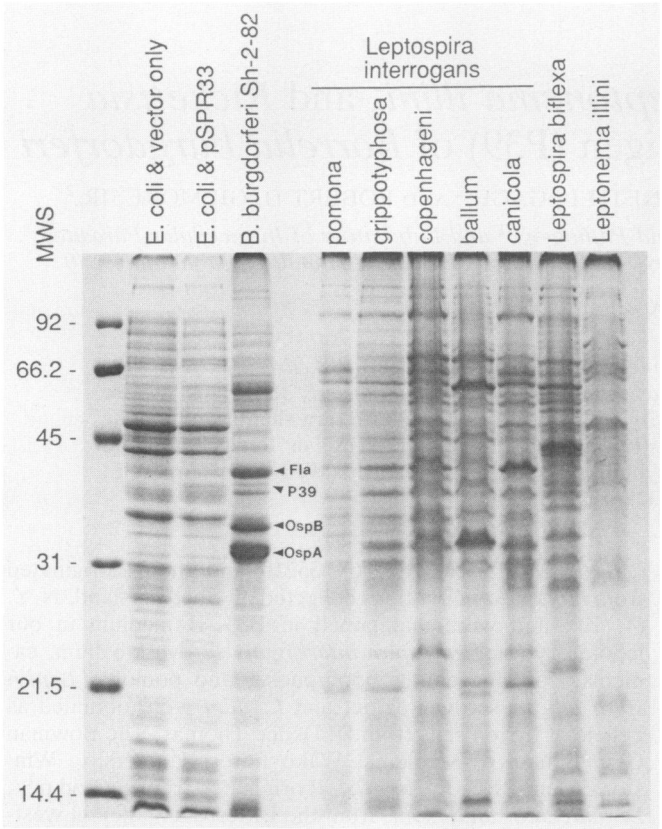

FIG. 1. SDS-PAGE of whole-cell lysates of the *E. coli* recombinant and spirochetes used in Western immunoblot analysis for P39. The gel was stained with Coomassie brilliant blue, and molecular weight standards (MWS) from Bio-Rad are shown on the left (in thousands). P39, flagellin (Fla), outer surface protein A (OspA), and outer surface protein B (OspB) are indicated by arrowheads.

2). When the film was exposed for several days to the blot incubated with the rabbit antiserum and then with ^{125}I-labeled protein A, weak reactivity with a few other antigens in the *Leptospira* spp. and *L. illini* was detected (data not shown). However, reactivity was strongest with large comigrating antigens (>92 kDa) present in all the lysates, including both *E. coli* preparations, and there was no reactivity with anything comigrating with P39 in the control lysates. Prolonged exposure of the film to the blot incubated with the monoclonal antibody did not reveal any reactivity other than that with P39 in *B. burgdorferi* and the *E. coli* recombinant (data not shown).

A serum sample from a human having had Lyme borreliosis was used to examine a whole-cell lysate of *R. rickettsii*. Although this antiserum recognized P39 in both *B. burgdorferi* and the *E. coli* recombinant, it did not react with *R. rickettsii* antigens (Fig. 3A). Additionally, seven serum samples from human patients with Rocky Mountain spotted fever did not react with P39 in either *B. burgdorferi* or the *E. coli* recombinant, although they did react with numerous *R. rickettsii* antigens (one example is shown in Fig. 3B). With a 3-h exposure of film, autoradiographs showed that three of these seven samples contained antibodies to one to three antigens of *B. burgdorferi*, while with a prolonged 26-h exposure, autoradiographs showed that all seven samples had some reactivity with various spirochetal antigens other than P39 (data not shown). When these same samples were tested by an IFA with whole cells of *B. burgdorferi* at eight serial twofold dilutions from 1:16 to 1:2,048, two were not reactive, one reacted at 1:16, three reacted at 1:32, and one reacted at 1:64. As shown by immunoblot analysis, these low reactivities were not likely due to antibodies cross-reactive with the 39-kDa antigen.

The enzyme-linked immunosorbent assay (ELISA) and the IFA were adapted soon after the discovery of the

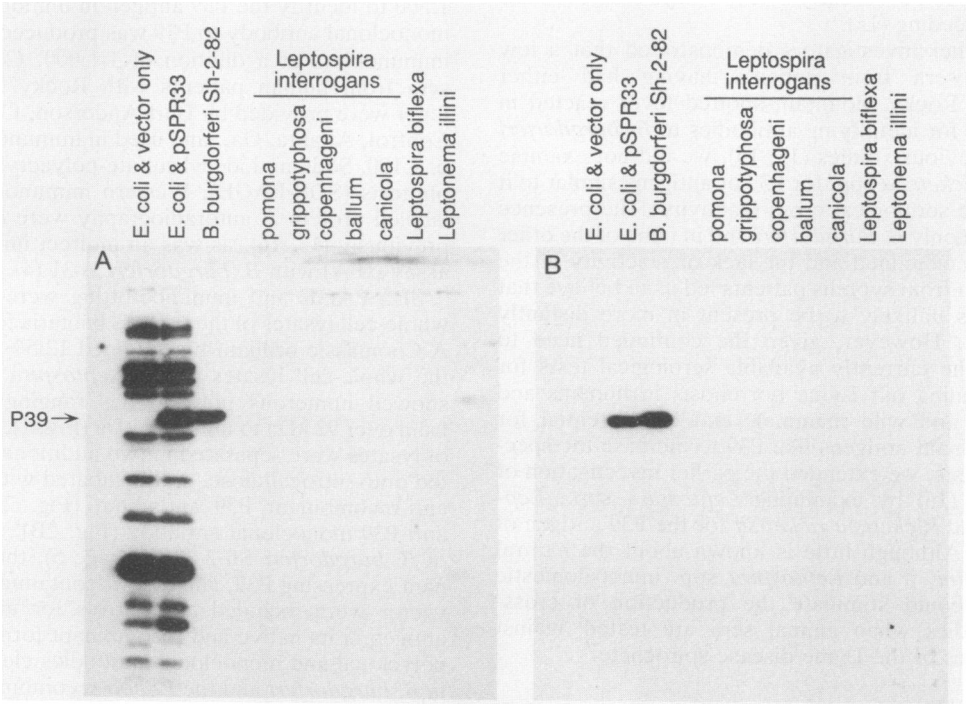

FIG. 2. Western immunoblot analysis for the presence of the P39 antigen, as detected by the rabbit anti-recombinant P39 antiserum (A) and the monoclonal antibody (B) with ^{125}I-labeled protein A autoradiography.

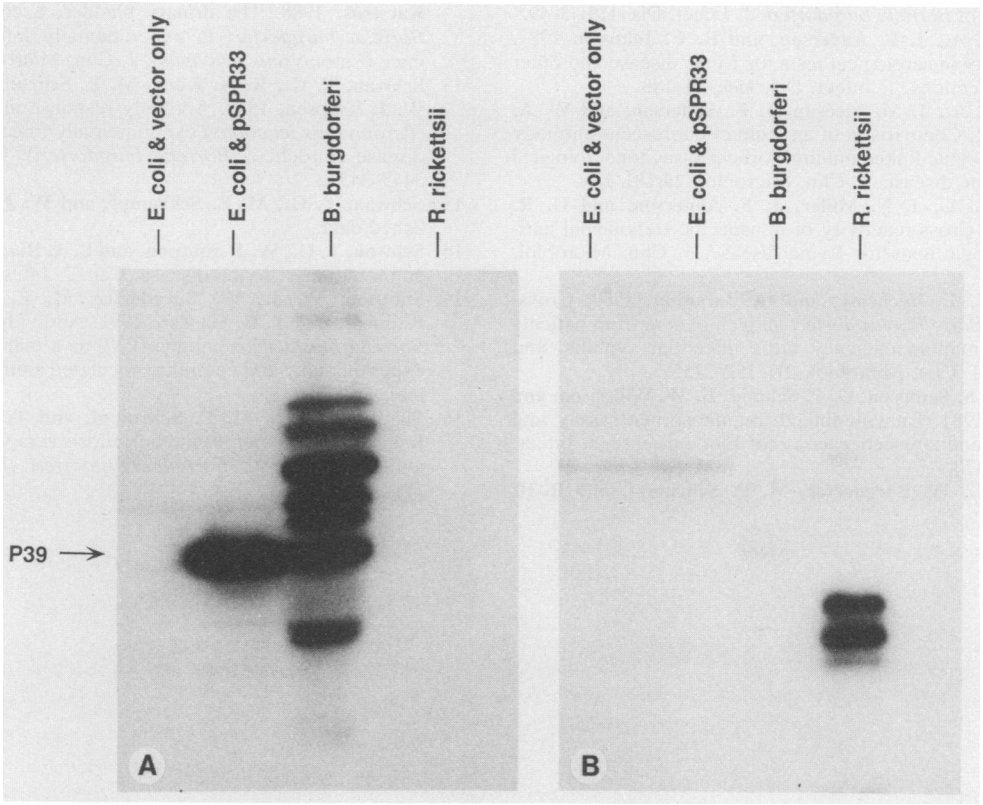

FIG. 3. Western immunoblot analysis to detect the P39 antigen with human anti-*B. burgdorferi* serum (A) and human anti-*R. rickettsii* serum (B).

causative agent of Lyme borreliosis to aid in the laboratory confirmation of this disease (4, 8, 11). Although these two tests are the most practical and widely used serological tests for detecting antibodies to *B. burgdorferi* (2, 14), problems still exist with specificity because of the large number of borrelial antigens included, some of which have shared epitopes with antigens of other species of bacteria (7, 9). Previous studies with *B. burgdorferi* in either an ELISA or an IFA demonstrated that some serum samples from human patients with leptospirosis and Rocky Mountain spotted fever reacted positively (5, 7, 10). Although it was possible that some of the samples came from patients having been exposed to *B. burgdorferi* as well, it is clear that some people exposed to other spirochetal infections will have false-positive reactions in serological tests with whole cells or whole-cell lysates of Lyme disease spirochetes (7–10). Our results demonstrate that *Leptospira* spp., *L. illini*, and the one strain of *R. rickettsii* tested do not have antigens that are cross-reactive with the P39 antigen of *B. burgdorferi* and that serum samples from patients having had Rocky Mountain spotted fever are not reactive with either the native or the recombinant form of this antigen. Therefore, antibodies to P39 should allow one to confirm as positive Lyme disease sera that first reacted positively in an ELISA or an IFA with total antigen preparations from *B. burgdorferi*. When Magnarelli et al. (6) fractionated total antigens of *B. burgdorferi* and used the different preparations in an ELISA, the fraction containing antigens of 34, 39, 59, and 68 kDa was the most specific. If the 39-kDa antigen in their fraction B is the same as the P39 antigen that has been cloned and shown to be specific and highly immunoreactive with sera from Lyme disease patients (16), then the increased specificity of that fraction is understandable. However, recombinant DNA techniques are more likely to provide methods for obtaining adequate amounts of specific antigens of *B. burgdorferi* to improve on the currently available tests for confirming cases of Lyme borreliosis (14). Serological tests for Lyme disease antibodies with P39 should exclude false-positive serum samples from cases of leptospirosis and Rocky Mountain spotted fever, as well as tick-borne relapsing fever and syphilis, as described previously (16).

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