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Lyme Disease Assay Which Detects Killed *Borrelia burgdorferi*

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We developed an in vitro assay showing that *Borrelia burgdorferi* organisms were killed by serum from patients with Lyme disease. Twenty of 20 Lyme disease serum samples caused *B. burgdorferi* killing in a range of 36 to 99% compared with the mean number of viable spirochetes when sera from 10 healthy individuals were used. The percentage of killing of *B. burgdorferi* increased with convalescent serum from patients with early Lyme disease. The borreliacidal activity was detectable in some sera diluted 640-fold and was abrogated after treatment with anti-human immunoglobulin G. In contrast, pooled or individual normal human serum did not cause a decrease in the number of viable *B. burgdorferi*. Borreliacidal activity was also not detected in sera from patients with relapsing fever, rocky mountain spotted fever, syphilis, mononucleosis, rheumatoid factor, or DNA antibodies. Our results show that borreliacidal activity can be used as a specific serodiagnostic test for detecting Lyme disease.

Lyme disease in humans is acquired from ticks of the *Ixodes ricinus* (2) complex which are infected with the spirochete *Borrelia burgdorferi* (4, 20). This disease is presently the most common tick-associated illness in the United States (7). A classical Lyme disease scenario begins with the development of an expanding skin lesion, erythema migrans, sometimes followed weeks or months later by cardiac or neurologic complications and occasionally progressing into arthritis after weeks to years of infection (19). However, this progression of clinical manifestations is often not documentable since the skin, cardiac and neurologic systems, and joints may be affected at any time during the course of infection. In addition, Lyme disease symptoms frequently change and become intermittent, which contributes to a clinical picture that varies greatly and is often indistinct.

The determination of serum antibody levels to *B. burgdorferi* is the most frequently used laboratory method for Lyme disease diagnosis. Serodiagnosis is generally performed by using the indirect fluorescent-antibody (IFA) assay or various formats of the enzyme-linked immunosorbent assay (ELISA) (6). These tests can yield false-positive results due to cross-reactions with other bacterial or viral illnesses (9) or false-negative results when detectable antibody levels have not yet been produced (8). Therefore, serologic testing has not been recommended as the sole criterion for diagnosing Lyme disease.

Many laboratories have developed other methods to improve Lyme disease testing. These have ranged from capture assays (3) or Western blotting (immunoblotting) for detecting antibody (10) to DNA probes for detecting the *B. burgdorferi* organism (16). Recently, we showed that borreliacidal antibody developed in hamsters after infection with the Lyme disease spirochete (17). Borreliacidal activity was detected 7 days after infection, peaked at weeks 3 to 5, and gradually declined. The borreliacidal activity accurately reflected the levels of protective antibody determined by passive transfer of immunity studies. In addition, the borreliacidal activity correlated with the number of spirochetes observed in the tissues. Pavia et al. (15) also demonstrated that rats develop borreliacidal activity after infection with *B. burgdorferi*.

In this report, we present evidence that borreliacidal activity can be detected in serum from patients with Lyme disease. Our results suggest that borreliacidal activity can be used as a specific serodiagnostic test for detecting Lyme disease in humans.

MATERIALS AND METHODS

Organism. *B. burgdorferi* 297, originally isolated from human spinal fluid (20), was obtained from Russell C. Johnson (University of Minnesota). The strain was grown once in modified Barbour-Stoenner-Kelly (BSK) medium (1, 5) at 32°C for 5 days, and 500-μl samples were dispensed into 1.5-ml screw-cap tubes (Sarstedt Inc., Princeton, N.J.), sealed, and stored at −70°C until used.

Sera. Lyme disease sera were obtained from patients with erythema migrans (partial clearing and expanded to >5 cm wide) or involvement of the musculoskeletal, nervous, or cardiovascular system with serologic confirmation of infection. All patients were evaluated by rheumatologists or infectious-disease physicians. Pooled (n = 10) or individual (n = 10) normal human sera were obtained from 20 previously healthy individuals with no history of Lyme disease symptoms or evidence of *B. burgdorferi* antibody production (IFA titer, <1:64).

Potential cross-reactive sera were obtained from 16 patients with a previous diagnosis of relapsing fever, rocky mountain spotted fever, syphilis, mononucleosis, rheumatoid factor, or antinuclear antibodies and no evidence of *B. burgdorferi* exposure. Donors of Lyme disease, normal, or potential cross-reactive sera had not received antimicrobial therapy during the previous 60 days. The sera were heat inactivated at 56°C for 45 min, diluted with fresh BSK medium, filter sterilized by using a 0.22-μm Acrodisc filter (Gelman Sciences, Ann Arbor, Mich.), and stored at −70°C until used.

Determination of borreliacidal activity. A frozen vial containing a suspension of *B. burgdorferi* was thawed, inoculated into 6 ml of fresh BSK medium, and incubated for 72 h. After incubation, the number of organisms was quantitated

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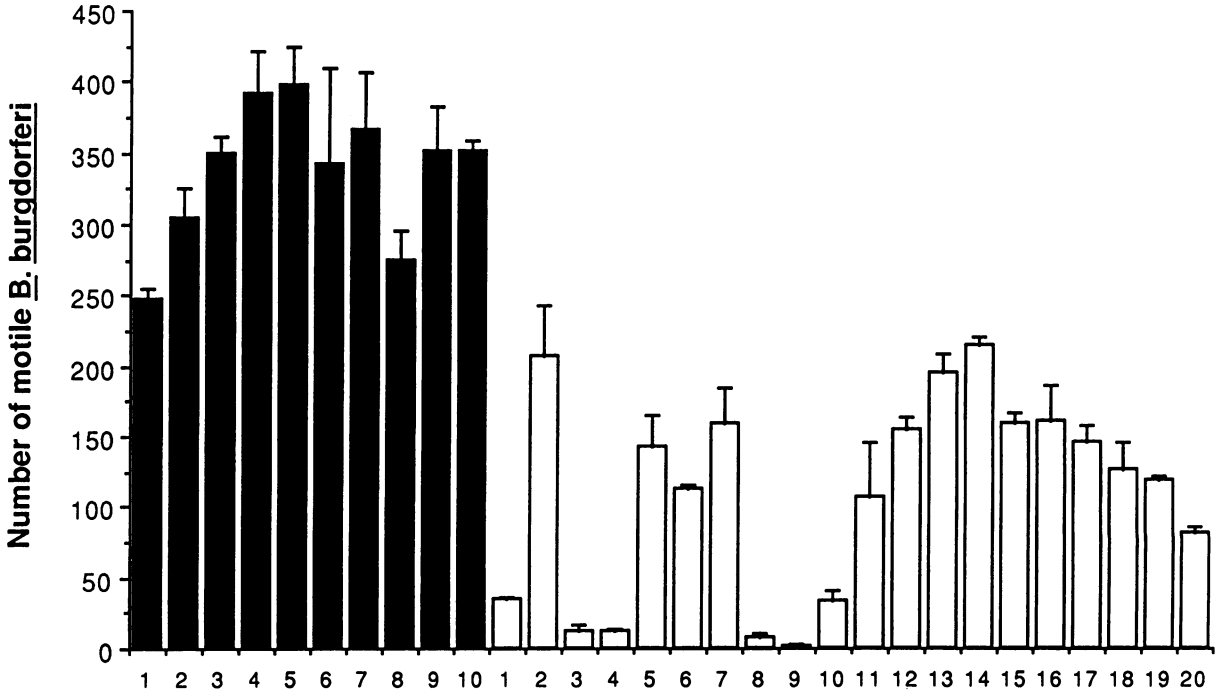

FIG. 1. Number of motile *B. burgdorferi* detected in 10 normal (■) and 20 Lyme disease (□) serum samples. Error bars represent the standard error of triplicate counts.

by using a Petroff-Hauser counting chamber, and 100-μl suspensions of *B. burgdorferi* from cultures containing approximately 10^7 organisms per ml were added to screw-cap polystyrene tubes (13 by 100 mm; Becton Dickinson, Lincoln Park, N.J.). A 100-μl amount of each human serum sample, diluted 20-fold, and 10 μl of sterile guinea pig serum complement (GIBCO Laboratories, Grand Island, N.Y.) were then added to the 100-μl suspensions of *B. burgdorferi*. Modified BSK medium, alone or with pooled or individual normal serum, and complement were used as *B. burgdorferi* growth controls. All assays were performed in duplicate or triplicate.

The assay suspensions were gently mixed and incubated at 32°C for 18 h. After incubation, triplicate 10-μl aliquots from each assay were mounted onto clean glass slides, and the numbers of remaining motile and nonmotile spirochetes in 25 random fields (×400) were determined by dark-field microscopy. Spirochetes which exhibited motility were considered viable (11).

We also determined whether the nonmotile *B. burgdorferi* organisms were viable. After exposure to Lyme disease or normal serum, 6 ml of fresh modified BSK medium was added to each assay. Subsequently, the assays were incubated at 32°C for 3 days, and the numbers of motile *B. burgdorferi* were determined every 24 h by examination of three 10-μl aliquots.

Neutralization of borreliacidal activity. Borreliacidal assays were performed with the following modifications. Briefly, 75 μl of anti-human immunoglobulin G (IgG; The Binding Site Inc., University of Birmingham Research Institute, Birmingham, United Kingdom) was added to 5 μl of normal or Lyme disease serum, and the mixture was incubated for 2 h at 37°C. After centrifugation at 2,200 × *g* for 10 min (Surespin; Helena Laboratories, Beaumont, Tex.), the supernatant was diluted 1:20 with modified BSK medium and 100-μl aliquots were transferred to tubes containing 100 μl of approximately 6×10^5 *B. burgdorferi*. Complement (10 μl) was added, and the number of viable spirochetes was determined after incubation for 6 h at 32°C. The borreliacidal activity of the normal and Lyme disease sera without treatment with anti-IgG was determined by substituting 75 μl of phosphate-buffered saline (pH 7.2) for anti-human IgG and assaying as described above.

Statistical analysis. The numbers of motile spirochetes obtained in the experimental and control groups were tested by analysis of variance. The Fisher least-significant-difference test (18) was used to examine pairs of means when a significant F ratio indicated reliable mean differences. The alpha level was set at 0.05 before the experiments were started.

RESULTS

The ability of case-defined Lyme disease sera to immobilize and kill *B. burgdorferi* was determined. Sera from 20 of 20 antibiotic-free Lyme disease patients caused reductions of 36 to 99% compared with the mean number of viable spirochetes when sera from 10 healthy individuals were used (Fig. 1). When pooled normal serum (10 = donors) was used as the control, similar results were obtained.

Lyme disease serum samples 2, 13, and 14, which demonstrated the least amount of borreliacidal activity (36 to 42% compared with the mean of 10 normal serum samples), were obtained from patients with erythema migrans. However, these patients had no constitutional symptoms and were unavailable for testing of borreliacidal activity with 3-week convalescent serum. Convalescent serum was obtained from seven additional patients with early Lyme disease whose sera also demonstrated low levels of borreliacidal activity. In each case, borreliacidal activity was enhanced significantly ($P \leq 0.01$) when the convalescent serum was used. Serum samples 1, 3, 4, 8, 9, and 10, which were from

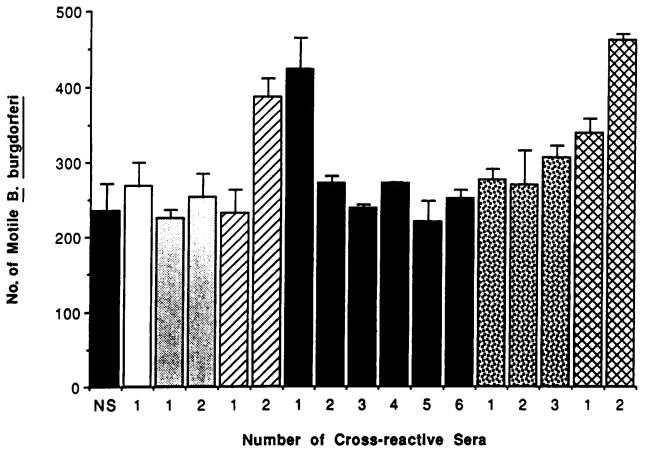

FIG. 2. Number of motile *B. burgdorferi* detected in pooled normal serum (■) and serum from patients with rocky mountain spotted fever (□), relapsing fever (□), mononucleosis (▨), syphilis (■), rheumatoid factor (▩), and antinuclear antibodies (▩). Error bars represent the standard error of triplicate counts.

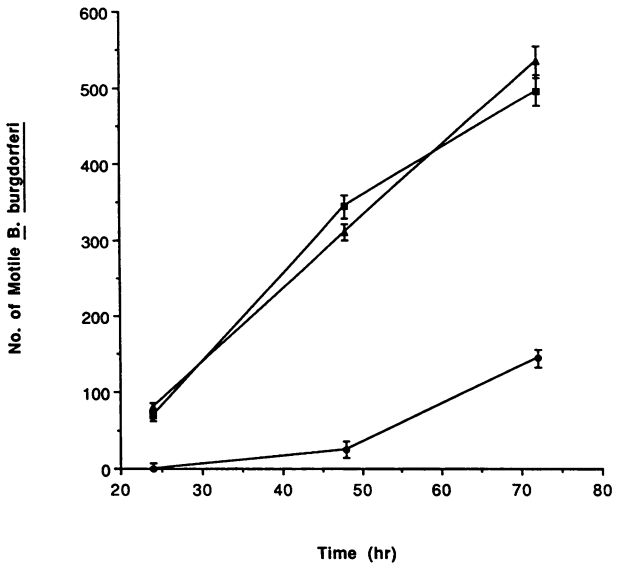

FIG. 3. Number of motile *B. burgdorferi* detected in BSK medium (■) and BSK medium containing pooled normal (▲) or Lyme disease (●) serum after the addition of 6 ml of BSK medium and incubation for 24, 48, and 72 h. Error bars represent the standard error of duplicate counts.

patients with Lyme disease arthritis, had considerable borreliacidal activity. The remaining sera were from patients with various durations of *B. burgdorferi* infection. No significant ($P \geq 0.05$) borreliacidal activity, compared with the mean number of spirochetes obtained using pooled serum from 10 healthy individuals, was detected in sera from one patient with rocky mountain spotted fever, two patients with relapsing fever, two patients with mononucleosis, six patients with syphilis, three patients with rheumatoid factor, and two patients with antinuclear antibodies (Fig. 2).

The immobilization and killing of *B. burgdorferi* organisms occurred rapidly after the addition of Lyme disease serum and complement. Lyme disease serum from an arthritic patient caused a 97% decrease in motile *B. burgdorferi* organisms after 6 h of incubation compared with pooled normal serum. When 6 ml of fresh BSK medium was added to the separate assay suspensions, significantly higher numbers of motile spirochetes were detected in BSK medium and BSK medium-containing pooled normal serum compared with BSK medium-containing Lyme disease serum after 24, 48, and 72 h of incubation (Fig. 3).

Borreliacidal activity was abrogated (Fig. 4) when Lyme disease serum (diluted 640-fold) was treated with anti-human IgG. Similar results were obtained when these experiments were repeated with other Lyme disease sera. In addition, treatment of normal serum with anti-human IgG did not cause significant changes in the numbers of viable *B. burgdorferi* organisms compared with untreated normal serum.

DISCUSSION

We demonstrated that humans with Lyme disease develop borreliacidal antibody. When Lyme disease serum was incubated with *B. burgdorferi* organisms, killing of spirochetes occurred within 6 h. In contrast, serum from humans not infected with *B. burgdorferi* could not kill the spirochete, and borreliacidal antibody was not detected in potential cross-reactive sera.

The Lyme disease patients demonstrated various amounts of borreliacidal activity. These results are not surprising since patients evaluated in this study presented at various intervals after infection with *B. burgdorferi*. We showed previously that borreliacidal activity varied during the course of *B. burgdorferi* infection in the hamster (17). Borreliacidal activity was detected 1 week after infection, peaked at weeks 3 and 5, and then gradually declined. The waning of borreliacidal antibody correlated with clearance of spirochetes from the tissues and resolution of disease. In this study, the highest levels of borreliacidal antibody were detected in patients with arthritis. Lower levels of borreliacidal antibody were detected in patients with early disease (erythema migrans) and in patients with a long duration of infection. Additional studies are needed to determine whether human borreliacidal activity correlates with the onset, severity, and resolution of Lyme disease. This information may be a useful prognostic indicator.

In a preliminary study, we addressed whether borreliacidal activity was present in all IFA-positive sera. We tested 10 seropositive serum samples from patients from an

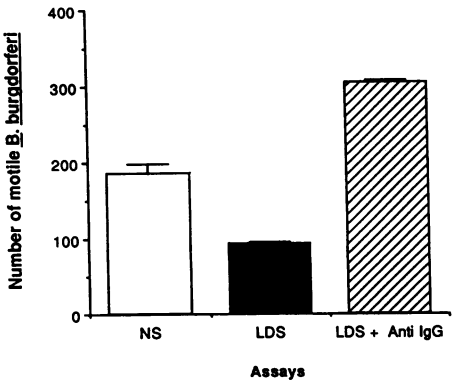

FIG. 4. Number of motile *B. burgdorferi* detected in pooled normal serum (NS; □) and Lyme disease serum (LDS) with (▨) and without (■) treatment with anti-human IgG. Error bars represent the standard error of duplicate counts.

endemic area who did not exhibit classic Lyme disease symptoms. Only one (10%) exhibited borreliacidal activity. In this individual, a convalescent serum sample contained increased levels of borreliacidal antibody and the patient was treated for Lyme disease. In addition, we detected high levels of borreliacidal activity in serum from IFA-seronegative patients with classic Lyme disease symptoms. These results suggest that assays which detect borreliacidal antibody may be more specific than currently available serodiagnostic tests. Additional studies are needed to confirm these findings.

The killing of *B. burgdorferi* organisms was mediated by antibody and complement. Killing and immobilization with extensive blebbing on the surface of *B. burgdorferi* occurred only when spirochetes were incubated with heat-inactivated Lyme disease serum and complement. Killing of *B. burgdorferi* was abrogated by treating Lyme disease serum with anti-human IgG. Kochi and Johnson (11) also showed that IgG from a Lyme disease patient could kill *B. burgdorferi* when complement was present.

When complement was not present in our assay suspensions, *B. burgdorferi* organisms formed clumps (5 to 100 organisms) which adhered tightly to the sides of the assay tubes. Growth assays confirmed that many of these organisms were not viable. Because this "strangulation effect" was consistently detected with serum having high levels of borreliacidal antibody, it could be used to screen serum for borreliacidal activity. Pavia et al. (15) also detected complement-independent killing of *B. burgdorferi* in rats. The mechanism of killing needs to be elucidated.

Immobilization has been used for decades to define the viability of another spirochete, *Treponema pallidum* (13). The treponemal immobilization test was regarded as the standard against which other techniques for the serodiagnosis of syphilis were judged (14). While this test remains the most specific syphilis test available, several technical factors have contributed to its demise. Perhaps most significant of these is the requirement for a continuous supply of syphilitic rabbits with orchitis. In addition, the stage of orchitis was critical since it influenced the quality of the spirochetes used for detecting immobilization.

Our results demonstrate that immobilization is effective for assessing the borreliacidal activity of serum from Lyme disease patients. Since *B. burgdorferi* can be cultivated in BSK medium, organisms are readily available for routine testing. When these organisms are incubated with Lyme disease serum and complement, borreliacidal activity can be detected within several hours. In addition, killing of *B. burgdorferi* organisms was specific since normal serum or serum from patients with relapsing fever, rocky mountain spotted fever, mononucleosis, syphilis, rheumatoid factor, or antinuclear antibodies failed to demonstrate borreliacidal activity. This is important because sera from these patients often produce false-positive results with the currently available IFA test or ELISA for diagnosing Lyme disease (9, 10, 12).

In conclusion, serum from Lyme disease patients can immobilize and kill *B. burgdorferi* organisms. Our assay detected no borreliacidal activity in potentially cross-reactive serum. Although the numbers of normal, potential cross-reactive, and Lyme disease sera evaluated were small, our results confirm previous findings in hamsters (17) and rats (15) that borreliacidal antibody plays a significant role during infection with *B. burgdorferi*. Further studies characterizing this interesting phenomenon should lead to a borreliacidal assay for diagnosing Lyme disease.

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