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Sensitivity and Specificity of the Borreliacidal-Antibody Test during Early Lyme Disease: a "Gold Standard"?

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The serodiagnosis of early Lyme disease has been plagued with problems of sensitivity and specificity. We found that the flow-cytometric borreliacidal-antibody test had a sensitivity of 72% for the detection of patients with early Lyme disease. By contrast, the sensitivity of the enzyme immunofluorescence assay was 28%. The enhanced sensitivity of the borreliacidal-antibody test was due to the use of *Borrelia burgdorferi* 50772, which lacks OspA and OspB. When *B. burgdorferi* 297, which expresses both OspA and OspB, was used, the sensitivity of the borreliacidal-antibody test was 15%. Our results also showed that the borreliacidal-antibody test was specific. No borreliacidal activity was detected in normal sera or in sera from patients with mononucleosis, rheumatoid factor, or syphilis. These results demonstrate that the flow-cytometric borreliacidal-antibody test may be the laboratory "gold standard" for the serodiagnosis of Lyme disease.

Lyme disease is a tick-associated zoonosis caused by *Borrelia burgdorferi* sensu lato. This multisystem disorder has emerged as the most common tick-transmitted disease in the world (4). The illness usually begins with a localized infection of the skin characterized by an erythema migrans (EM) lesion. If the illness is left untreated, the spirochetes spread systemically to various body tissues, such as those of the heart, nervous system, or joints (38). Weeks to years after infection, more severe clinical manifestations may develop, such as meningitis, facial palsy, atrioventricular heart block, encephalopathy, polyneuropathy, or chronic arthritis (23, 26, 28, 39). An additional late manifestation, acrodermatitis chronica atrophicans, is seen primarily in European Lyme disease patients (2).

The variety of symptoms that can develop after infection with *B. burgdorferi* contributes to the difficulty of making an accurate diagnosis of Lyme disease. Thus, serodiagnostic tests are often necessary to aid in the clinical diagnosis. Unfortunately, the serodiagnosis of Lyme disease has been hampered by problems of sensitivity and specificity which have caused and continue to cause gross inaccuracies in Lyme disease testing. The economic and health impacts of these problems have been significant. Steere et al. (40) reported that Lyme disease could be diagnosed with certainty in only 25% of patients referred to a Lyme disease specialty clinic. Lightfoot et al. (20) estimated that the incidence of false-positive serologic results in patients with nonspecific myalgia or fatigue exceeded fourfold the incidence of true-positive results in patients with nonclassical *B. burgdorferi* infections. In addition, they estimated that for each Lyme disease patient treated $86,221 is spent for the treatment of patients with conditions other than Lyme disease.

We (7–9) and others (17, 33, 34) have demonstrated that humans infected with *B. burgdorferi* develop specific lethal antibodies against the Lyme disease spirochete. Detection of these antibodies can be used as a highly specific serodiagnostic test for Lyme disease (7–9, 11). We report here the sensitivity and specificity of the flow-cytometric borreliacidal-antibody test for detecting Lyme disease in early Lyme disease patients. The results confirm the utility of borreliacidal-antibody detection as a "gold standard" serodiagnostic test for Lyme disease.

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MATERIALS AND METHODS

Normal and potentially cross-reactive sera. Normal serum was collected from 80 healthy adult volunteers 18 to 60 years of age residing in an area where Lyme disease is endemic (6). Sera were not tested for *B. burgdorferi* antibodies by conventional assays. Volunteers, however, filled out a questionnaire of medical history, and individuals with previous Lyme disease-related symptoms were not included. An additional 15 serum samples from individuals who were without current Lyme disease-related symptoms but who had previously been diagnosed with Lyme disease or who had evidence of past exposure to *B. burgdorferi* (enzyme immunoassay [EIA] range, 0.39 to 1.8; 10 serum samples had values of >1.0) were also included. Potentially cross-reactive sera were obtained from 50 patients with rheumatoid factor ($n = 10$), antinuclear antibodies ($n = 17$), or antibodies against *Treponema pallidum* ($n = 18$) or Epstein-Barr virus ($n = 5$).

Lyme disease sera. Thirteen Lyme disease serum samples were collected from individuals from whom *B. burgdorferi* organisms had been recovered from primary or secondary EM lesions after culture in Barbour-Stoenner-Kelly (BSK) medium. Ten case-defined Lyme disease serum samples were obtained from Gundersen Clinic patients. Five of these patients had clinically documented EM, and five had recurrent Lyme arthritis. An additional 47 early Lyme disease serum samples were culture or case defined. These represent a subset of a serum bank from Westchester County, N.Y., sponsored by the National Institutes of Health and the Centers for Disease Control and Prevention. Westchester County has the highest reported number of Lyme disease cases in the United States. Some of these sera had previously been used to validate the Western blot (immunoblot) as a serodiagnostic test for the detection of early Lyme disease (1). Donors of Lyme disease, normal, or potentially cross-reactive sera had not received antimicrobial therapy during the previous 30 days.

Organisms. Low-passage *B. burgdorferi* sensu stricto 297, isolated from human spinal fluid, and *B. burgdorferi* sensu stricto 50772, isolated from an *Ixodes scapularis* tick, were grown once in BSK medium at 32°C to a concentration of approximately 5×10^7 organisms per milliliter. After examination by dark-field microscopy, 500-μl aliquots were dispensed into 1.5-ml screw-cap tubes (Sarstedt, Newton, N.C.), which were sealed and stored at −70°C until used. *B. burgdorferi* 297 expresses multiple outer surface proteins, including outer surface protein A (OspA) and OspB. Isolate 50772 does not naturally express OspA or OspB since it lacks both the *ospA* and *ospB* genes.

Detection of borreliacidal antibody by flow cytometry. The borreliacidal-antibody test was performed as previously described (8). Briefly, a suspension of *B. burgdorferi* organisms was thawed and a 200-μl amount was inoculated into 6 ml

TABLE 1. Borreliacidal antibody titers of serum from patients with localized (EM) or disseminated (Lyme arthritis) Lyme disease against *B. burgdorferi* 297 and 50772

Serum type and sample	Titer[a] of borreliacidal antibody against:	
	297	50772
EM		
1	10	2,560
2	80	320
3	10	160
4	NRD[b]	2,560
5	NRD	2,560
Lyme arthritis		
1	≥40,960	80
2	2,560	160
3	1,280	10
4	2,560	20
5	320	20

[a] Reciprocal of last dilution of serum with significant borreliacidal activity.
[b] NRD, no response detected.

of fresh BSK medium. The culture was then incubated for 72 h at 32°C. After incubation, the number of spirochetes was determined and the spirochetes were diluted with fresh BSK medium to a concentration of 10^5 organisms per milliliter. Each human serum sample was diluted 1:5 with fresh BSK medium, sterilized by passage through a 0.22-μm-pore-size microcentrifuge filter (Costar, Cambridge, Mass.), and heat inactivated for 10 min at 56°C. One hundred microliters of the *B. burgdorferi* suspension (10^4 organisms) and 10 μl of sterile guinea pig serum complement (Sigma Chemical, St. Louis, Mo.) were then added to each sample. The assay suspensions were gently mixed and incubated at 32°C for 40 to 48 h. Pooled normal serum and complement were used as a growth control. Serum samples with immunoglobulin M (IgM) and IgG borreliacidal antibodies (7) were also included as controls.

After incubation, 100 μl of the assay suspensions was diluted 1:5 with phosphate-buffered saline (0.01 mol/liter at a pH of 7.2) containing acridine orange (final concentration, 5.4×10^{-9} mol/liter) and analyzed by using a FACScan single-laser flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). For each sample, events were acquired in the list mode for 3 to 6 min. The sample fluid flow rate was set at "low" (121 μl/min) to reduce signal variability. Analyses were performed with Lysys II research software (Becton Dickinson). Spirochetes were differentiated from BSK and complement particles by using side scatter and fluorescence intensity parameters. Nonrectangular gates on the side scatter-versus-FL1 fluorescence dot plots were used to identify spirochetes for analysis. Fluorescence signals were logarithmically amplified and converted to a linear scale for comparison after analysis. Serum samples were considered positive for borreliacidal activity when they showed a ≥13% increase in fluorescence intensity compared with normal serum.

EIA. The IgM-IgG fluorescence EIA was performed according to the manufacturer's instructions (BioWhittaker, Inc., Walkersville, Md.). Test values were calculated on the basis of reactivity with controls supplied with the assay system. An index value of ≥1.00 was considered positive.

RESULTS

The amounts of borreliacidal activity against *B. burgdorferi* 297 and 50772 differed significantly during localized (EM) and disseminated (Lyme arthritis) Lyme disease (Table 1). High titers of borreliacidal antibodies against isolate 50772 were detectable during early Lyme disease, while high titers of borreliacidal antibodies against isolate 297 were present after dissemination of the spirochetes. Similar results were obtained with sera from patients with EM lesions that were culture positive for *B. burgdorferi* (Table 2). When *B. burgdorferi* 297 was used, borreliacidal antibodies were detected in only 2 of 13 (sensitivity, 15%) serum samples from patients with culture-defined primary or secondary EM lesions. By contrast, the flow-cytometric borreliacidal-antibody test was positive for five of nine patients with primary EM (sensitivity, 56%) and three of four patients with secondary EM (sensitivity, 75%) when isolate 50772 was used. Thus, the sensitivity of borreliacidal-antibody detection during early localized or early disseminated Lyme disease was greatly increased by using *B. burgdorferi* 50772. When both isolates were used, the sensitivity of the borreliacidal antibody test increased to 77%.

TABLE 2. Number of culture-defined Lyme disease serum samples from patients with localized (EM) Lyme disease with significant borreliacidal-antibody activity against *B. burgdorferi* 297 and 50772

Serum sample type (*n*)	No. (%) of serum samples positive for:	
	297	50772
Primary lesion (9)	1 (11)	5 (56)
Secondary lesion (4)	1 (25)	3 (75)
Total (13)[a]	2 (15)	8 (62)

[a] Sensitivity was 77% when both isolates were used.

The detection of borreliacidal antibodies against *B. burgdorferi* 50772 was also highly specific. No borreliacidal activity was detected in normal sera from individuals with no previous Lyme disease-related symptoms (Table 3). One (7%) serum sample from an asymptomatic individual previously diagnosed with Lyme disease in April 1993 was positive for borreliacidal antibody. In addition, no positive reactivities against isolate 50772 were detected in sera from patients with syphilis, mononucleosis, or rheumatoid factor. Borreliacidal activity was detected in 1 (6%) of 17 serum samples with antinuclear antibodies.

The flow-cytometric detection of borreliacidal antibodies in culture- or case-defined (EM) early Lyme disease sera from patients in Westchester County was also highly sensitive. Of 47 such serum samples, 13 (28%) were positive for *B. burgdorferi* antibodies by EIA, whereas 34 (72%) were positive by the borreliacidal-antibody test (9 [19%] when isolate 297 was used [of these 9, 3 had significant borreliacidal-antibody activity against isolate 297 only] and 31 [66%] when isolate 50772 was used). Borreliacidal-antibody detection was thus almost threefold more sensitive than EIA (72 versus 28%) when *B. burgdorferi* 297 and 50772 were used as the test organisms.

DISCUSSION

It is well established that serum from Lyme disease patients contains borreliacidal antibodies (7–9, 17, 33, 34). We (8, 9) and others (33, 34) have demonstrated the high specificity of this antibody response. Thus, it is puzzling why the development of a diagnostic test to exploit this finding has not been widely pursued. The detection of borreliacidal antibodies has

TABLE 3. Borreliacidal antibody activity of various serum samples against isolate 50772

Serum sample type (*n*)	No. (%) with significant activity
Normal (80)	0 (0)
Previous exposure or infection (15)	1 (7)[a]
Syphilis (18)	0 (0)
Mononucleosis (5)	0 (0)
Rheumatoid factor (10)	0 (0)
Antinuclear antibody (17)	1 (6)
Total	2 (1.4)

[a] The positive sample was from a patient previously diagnosed with Lyme disease in 1993.

previously been limited primarily to serum from patients with Lyme arthritis (9, 17, 33). In addition, these antibodies have most often been observed to be against OspA or OspB (7, 10, 17, 21, 24, 30, 32, 34). Antibody responses against these proteins are most often detectable during late Lyme disease (5, 18). Thus, the diagnostic utility of the borreliacidal antibody test may have appeared to be limited to these patients.

In this study, titers of borreliacidal antibody against OspA and/or OspB were significantly higher after dissemination of the spirochetes, since serum from patients with Lyme arthritis had higher titers of borreliacidal antibodies against *B. burgdorferi* 297. However, OspA- and/or OspB-specific borreliacidal antibodies could also be detected during localized infection, since some sera from patients with early Lyme disease killed only *B. burgdorferi* 297. Schutzer et al. (35) and Kalish et al. (19) also demonstrated OspA-specific IgM antibodies in 10 (91%) of 11 and 10 (100%) of 10 early Lyme disease patients, respectively.

We also demonstrated significant borreliacidal activity against other proteins during early Lyme disease. In many cases, borreliacidal antibodies were detectable only when *B. burgdorferi* 50772, which does not express OspA and OspB, was used. OspA and OspB may hinder the interaction of borreliacidal antibodies with other outer surface or internal proteins. These results confirm our previous findings that adsorption of Lyme disease sera with OspA diminished but did not abolish borreliacidal activity (7).

Other investigators have also demonstrated borreliacidal activity against *B. burgdorferi* proteins besides OspA and OspB (3, 25, 37). Antibody responses against several of these proteins are often detectable during early Lyme disease by conventional diagnostic Lyme disease assays. For example, Scriba et al. (37) demonstrated in vitro borreliacidal activity against the 39-kDa *B. burgdorferi* periplasmic protein. Engstrom et al. (14) also showed that the predominant antibody response in patients with early Lyme disease was against this protein. OspC is another intriguing candidate. OspC expression is upregulated by *B. burgdorferi* after the attachment of infected ticks to the host (36, 41). Anti-OspC antibodies are also among the first antibodies detected by conventional tests (13, 27). In addition, this protein has been shown to be highly protective when used as an immunogen in experimental animals (29). Probert and LeFebvre (30), however, did not detect borreliacidal antibodies in vitro after immunization of mice with OspC. Anti-OspC borreliacidal antibodies may be detectable by a more sensitive assay than the growth inhibition assay (33), or they may be detectable if spirochetes which do not express OspA and OspB are used. Other outer surface proteins are also likely candidates. The vaccination of mice with OspE and OspF causes partial destruction of *B. burgdorferi* spirochetes within feeding ticks (25). Thus, it is not surprising that borreliacidal antibodies are detectable during early Lyme disease.

Borreliacidal antibodies can be either IgM or IgG. We previously showed that IgM and IgG borreliacidal antibodies were detectable during early and late Lyme disease, respectively (7). Also, Aydintug et al. (3) detected IgM and IgG borreliacidal antibodies after infection of rhesus monkeys. In a preliminary study, borreliacidal activity against *B. burgdorferi* 50772 could be abrogated by the removal of IgM antibodies (data not shown). Additional studies to determine the kinetics of the IgM and IgG borreliacidal-antibody response are ongoing.

The borreliacidal-antibody test had a sensitivity of 72%, compared with a sensitivity of 28% obtained with EIA. Killing by borreliacidal antibodies causes a significant increase in acridine orange uptake by dead organisms (8). By monitoring fluorescence intensity, borreliacidal activity can be objectively and accurately detected. Flow cytometry also enabled testing with 10^4 spirochetes. The increased sensitivity is crucial since treatment is easier before dissemination of the spirochetes. After dissemination, treatment failures of 50% or more have been reported (12).

Our results confirmed the exquisite specificity of borreliacidal antibodies. We (8) and Sambri et al. (34) previously demonstrated highly specific borreliacidal antibodies using *B. burgdorferi* isolates which primarily reacted with borreliacidal antibodies in serum from patients with late Lyme disease sequelae. In the present study, we showed the high specificity of borreliacidal antibodies produced during early infection. When non-Lyme disease sera were tested, borreliacidal antibodies were detected only in one individual with antinuclear antibodies and in one asymptomatic individual with previously treated Lyme disease. The patient with antinuclear antibodies may have had Lyme disease, although the medical records could not be reviewed. The individual previously treated for Lyme disease could still have been infected. Preac-Mursic et al. (28) showed that the Lyme disease spirochete could survive in patients despite antibiotic therapy.

Many methodologies to increase the sensitivity and/or specificity of serologic Lyme disease testing have been developed. Unfortunately, laboratory tests have continued to be hampered by technical difficulties and subjectivity problems. In addition, improvements in sensitivity or specificity have most often been at the expense of other variables. In an effort to improve Lyme disease testing, the Centers for Disease Control and Prevention and the Association of State and Territorial Public Health Laboratory Directors have recommended that sera submitted for serology be tested by EIA or indirect fluorescent-antibody assay and that borderline or positive samples be confirmed by Western blotting (31). However, recent reports continue to demonstrate the lack of sensitivity and specificity of this approach (15, 16, 22). Thus, there remains a serious need for a sensitive and specific serodiagnostic test. Our results demonstrate that the flow-cytometric borreliacidal-antibody test may be the gold standard for the serodiagnosis of Lyme disease.

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