

Borrelia burgdorferi-Specific Immune Complexes in Acute Lyme Disease

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LYME DISEASE (LD), A TICK-borne infection caused by the spirochete *Borrelia burgdorferi* is increasing in incidence and geographic distribution.¹⁻⁴ Diagnostic laboratory tests are needed to detect active infection. They are particularly needed early when the hallmark clinical sign of infection, erythema migrans (EM), is not seen, as occurs in at least one third of cases.^{3,5} Existing antibody tests require a lag phase, often of several weeks, to become positive and can be inconclusive.⁶ Culture is not useful in the clinical setting since it is rarely positive except in EM lesions and takes many weeks for growth. The Lyme vaccine,^{7,8} since it produces seropositivity, will make the diagnosis of infection in vaccinated persons even more difficult.⁹

The objective of this study was to examine whether *B burgdorferi*-specific immune complexes can be detected during active infection using a simple specific immunologic assay.¹⁰ If this assay correlated with active infection it could be used in difficult-to-diagnose populations, such as seropositive people with nonspecific symptoms; early infected seronegative people without pathognomonic EM, including tick-bitten individuals; and seropositive vaccinated people who may not be protected. We felt that it was imperative first to evaluate the performance of the *B burgdorferi* immune complex assay in serum samples from controls and well-

Context Diagnosis of infection with *Borrelia burgdorferi*, the cause of Lyme disease (LD), has been impeded by the lack of effective assays to detect active infection.

Objective To determine whether *B burgdorferi*-specific immune complexes are detectable during active infection in LD.

Design, Setting, and Patients Cross-sectional analysis of serum samples from 168 patients fulfilling Centers for Disease Control and Prevention surveillance criteria for LD and 145 healthy and other disease controls conducted over 8 years. Tests were performed blinded.

Main Outcome Measure Detection of *B burgdorferi* immune complexes by enzyme-linked immunosorbent assay and Western blot.

Results The *B burgdorferi* immune complexes were found in 25 of 26 patients with early seronegative erythema migrans (EM) LD; 105 of 107 patients with seropositive EM LD; 6 of 10 patients who were seronegative with culture-positive EM; 0 of 12 patients who were treated and recovered from LD; and 13 of 13 patients with neurologic LD without EM. Among 147 controls, *B burgdorferi* immune complex was found in 0 of 50 healthy individuals; 0 of 40 patients with persistent fatigue; 0 of 7 individuals with frequent tick exposure; and 2 of 50 patients with other diseases.

Conclusion These data suggest that *B burgdorferi* immune complex formation is a common process in active LD. Analysis of the *B burgdorferi* immune complexes by a simple technique has the potential to support or exclude a diagnosis of early as well as active LD infection.

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characterized patients with LD who met the Centers for Disease Control and Prevention surveillance definition for LD.⁵ If the assay were positive in a high number of these patients, while negative in well-characterized other disease controls and healthy people from the same endemic areas, it would support the potential use of this assay in the individual patient who is suspected of or is concerned about having LD, but does not have a classic EM rash. Diagnosis is particularly important early in the course of infection when there is the best chance for cure. The approach stems from the normal humoral immune response to an infection in which the antibody is produced and binds to antigens of the infectious agent.¹¹ In active diseases, relevant antibody may be detected by techniques that liberate the

antibody from its bound immune complexed form.¹²⁻¹⁴ Unlike free antibody, which does not distinguish past from present infection, complexed antibody is more likely to reflect an active process. Therefore, we examined serum samples from 168 patients meeting criteria for LD to evaluate the *B burgdorferi* immune complex assay as a possible marker of active infection.

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METHODS**Patients**

Patients came from our clinics, including control patients, except for serum samples that came from EM studies by independent investigators. The studies were approved by the institutional review boards at the collaborating institutions, and patients gave informed consent. Samples were accumulated seasonally over 8 years and all serum samples were frozen at -70°C. The LD samples were used from patients who met the Centers for Disease Control and Prevention Surveillance Definition⁵ and all received negative VDRL test results. Serum samples were not included in the groups with LD if they did not meet the Centers for Disease Control and Prevention definition or if there was a significant codiagnosis of neurologic or immunologic disease. The technicians performing the assays were blinded as to whether the sample came from a patient with LD.

The Centers for Disease Control and Prevention Surveillance Case Definition is as follows⁵:

Case classification. Confirmed: (a) a case with erythema migrans or (b) a case with at least one late manifestation (as defined below) that is laboratory confirmed.

Late manifestations include any of the following when an alternate explanation is not found:

(1) Musculoskeletal system. Recurrent, brief attacks (weeks or months) of objective joint swelling in one or a few joints, sometimes followed by chronic arthritis in one or a few joints. Manifestations not considered as criteria for diagnosis include chronic progressive arthritis not preceded by brief attacks and chronic symmetrical polyarthritides. Additionally, arthralgia, myalgia, or fibromyalgia syndromes alone are not criteria for musculoskeletal involvement.

(2) Nervous system. Any of the following, alone or in combination: lymphocytic meningitis; cranial neuritis, particularly facial palsy (may be bilateral); radiculoneuropathy; or, rarely, encephalomyelitis. Encephalomyelitis must be confirmed by demonstration of antibody production against *Borrelia burgdorferi* in the CSF [cerebrospinal fluid], evidenced by a higher titer of antibody in CSF than in serum. Headache, fatigue, paresthesia, or mildly stiff neck alone are not criteria for neurologic involvement.

(3) Cardiovascular system. Acute onset of high-grade (2nd-degree or 3rd-

degree) atrioventricular conduction defects that resolve in days to weeks and are sometimes associated with myocarditis. Palpitations, bradycardia, bundle branch block, or myocarditis alone are not criteria for cardiovascular involvement.

This surveillance case definition was developed for national reporting of Lyme disease; it is not intended to be used in clinical diagnosis.

The following groups with LD were studied:

(1) Patients who had EM and LD but were seronegative before treatment (n = 26). All had EM and had been exposed less than 12 weeks before testing.

(2) Patients who had EM and LD and were seropositive before treatment (n = 107). Fifty-five had sufficient serum samples to run both enzyme-linked immunosorbent assay (ELISA) and immunoblots for complexed antibody. This group of 107 also represented, as close as possible, the clinical equivalent to a criterion standard.

(3) Patients who had EM and LD confirmed by culture, but were seronegative positive (n = 10). These were selected after a completed pilot study of 25 blinded samples collected from 13 patients. They were provided and documented by the Centers for Disease Control and Prevention to be seronegative in their conventional 2-tier test (requires a positive whole *B burgdorferi* ELISA and a Western blot with IgM: 2 of 3 bands of 23, 39, 41 kd or IgG: 5 of 10 bands of 18, 21, 28, 30, 39, 41, 45, 58-60, 66, 93 kd).¹⁵ For the focus of this study on early disease, we evaluated the IgM *B burgdorferi* immune complex ELISA results on these 10 serum samples, classified as false-negative results under the 2-tier approach.

(4) Patients without EM who had neurologic LD and were seropositive (n = 13). These patients had Lyme meningitis, radiculoneuritis, or facial nerve palsy.

(5) Asymptomatic patients who had recovered from LD (n = 12). These patients had EM previously and had received standard antibiotic therapy (amoxicillin, doxycycline, or ceftriaxone). They were tested for *B burgdorferi* immune complexes when they were

clinically asymptomatic (6 to 24 months later).

The following control groups were studied:

(6) Non-EM healthy outdoor controls with frequent tick exposure (not engorged) (n = 7). Serial serum samples were collected and analyzed from 7 individuals teaching at a nature and wilderness school who had frequent exposure to ticks.

(7) Seronegative endemic area controls with fatigue (n = 40). These patients had persistent fatigue without other specific or major compatible signs of LD.

(8) Other disease controls (n = 50). The group included subjects with other immunologic and infectious diseases (2 with allergic rhinitis, 1 with asthma, 1 with polymyositis, 10 with systemic lupus erythematosus, 10 with chronic fatigue, 1 with syphilis, 25 with multiple sclerosis), who were expected to have elevated levels of immune complexes.

(9) Normal healthy controls (n = 50); all subjects came from Lyme endemic areas in New Jersey and New York except for 2 city dwellers.

Immune Complex Isolation and Dissociation

The basis for the test is that if the starting serum sample contains free and bound antibody, polyethylene glycol (PEG) precipitates the bound antigen-antibody complex, leaving the free antibody in solution, which is then removed. The precipitated antigen-antibody complex is dissociated with alkalization, separating the complex into newly unbound antigen and antibody of each immunoglobulin isotype. These dissociated complexes are then applied to ELISA and Western blots.

Immune complexes were isolated by PEG precipitation and dissociated as described, using PEG concentrations shown not to precipitate free IgG.¹⁶ This PEG technique does not precipitate detectable quantities of free IgG to *B burgdorferi* as confirmed by performing immunoglobulin equivalences between serum and immune complexes.¹⁷ Briefly, 0.5 mL of the serum sample was added to an equal volume of 7% PEG in phos-

phate-buffered saline, and incubated overnight (or alternatively it can be incubated for 2 hours) at 4°C, then centrifuged at 8320g for 15 minutes. Pellets were washed twice using 3.5% PEG and resuspended in 0.5 mL of 0.1 mol-sodium borate with a pH of 10.2, yielding the dissociated components.

ELISA for Free Serum Antibodies to *B burgdorferi*. Serum samples were tested for anti-*B burgdorferi* B31 antibody in an ELISA as described.^{17,18} This determined the clinical laboratory designation of seropositive or seronegative. A positive result was indicated by optical density readings greater than 3 SDs above the mean of a panel of 10 healthy controls without a history of *Borrelia* infection. In the clinical laboratory these 10 controls represented the range of optical density readings from more than 100 healthy control samples such that they yielded the same mean \pm 3 SDs as the larger group.

ELISA for Complexed Serum Antibodies to *B burgdorferi*. Dissociated PEG precipitates were diluted 1:10 and run by ELISA¹⁷ to separately detect IgG and IgM reactive to *B burgdorferi* anti-

gens. Immune complexes on ELISA were considered to be *Borrelia*-specific when the optical density readings of the PEG precipitate was greater than 3 SDs above the mean of at least 10 normal controls run on each plate.¹⁷

Western Blots. *B burgdorferi* B31 sonicates (provided by Marc Goldsmith, PhD) or recombinant outer surface proteins of *B burgdorferi*, outer surface protein A (OspA), and outer surface protein B (OspB) were electrophoresed in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto immunoblots as previously described. The blots were used for both free and complexed antibody analysis.^{10,17} In a subset of experiments, serum was used at more concentrated levels or at immunoglobulin equivalence to eliminate the theoretical possibility of concentrating specific-free antibody in apparent seronegative samples. Our dilutions of 1:100 of the serum samples, similar to that used in commercial assays, still provide a greater concentration of free antibody, thereby minimizing the theoretical possibility noted

above. Analyses for the antigen within the complexes was performed by first blotting the dissociated complex material (as described^{10,17}), followed by probing the blot with monoclonal antibodies directed against flagella (41 kd), OspA (31 kd), OspB (34 kd) (provided by Alan Barbour, MD), or polyclonal antibody from high-titer animals or humans.

Recombinant Outer Surface Protein. The entire OspA lipoprotein recombinant gene product that had been expressed in *Escherichia coli* and purified by Triton X-114 extraction and ion exchange chromatography¹⁹ was used for the blots above (provided by J. Dunn, PhD). Recombinant OspB was similarly prepared and provided.

Reproducibility Assessment. Intra-laboratory and interlaboratory testing reproducibility were evaluated. Positive and negative serum samples were subjected to repetitive runs on different days. A set of 5 positive serum samples was run blinded in 2 independent research laboratories for comparison. Samples from 10 non-LD cases, seronegative for free antibody in a clinical laboratory, were analyzed for *B burgdorferi* immune complexes in each of the 2 research laboratories and immune complex dissociation were reapplied to the clinical laboratories' ELISA plate as well. Thirty serum samples that were positive for LD were periodically rerun as positive controls. Those results were assessed retrospectively.

Table. Detection of Complexed Antibody (IgM and/or IgG) to *Borrelia burgdorferi* Antigens by Western Blot and Enzyme-Linked Immunosorbent Assay*

Patient Group and Status at Sampling Time	No. of Patients	No. of Patients
Patient Group	With Complexed Antibody†	Without Complexed Antibody
Seronegative‡ pretreatment EM Lyme disease (n = 26)	1	25
Seropositive‡ pretreatment EM Lyme disease (n = 107)§	2	105
Seronegative‡ EM Lyme disease but culture positive (n = 10)¶	3	6
Non-EM neurologic Lyme disease (n = 13)	4	13
Asymptomatic, recovered Lyme disease (n = 12)	5	0
Non-EM healthy outdoor controls with frequent tick exposure (not engorged) (n = 7)	6	0
Seronegative‡ endemic area controls with fatigue (n = 40)	7	0
Other disease controls in endemic area (n = 50)	8	2
Healthy controls in endemic area (n = 50)	9	0

*EM indicates erythema migrans.

†Statistical significance of $P < .01$ for the positive *B burgdorferi* immune complex results compared with the non-Lyme disease controls was confirmed by Fisher exact test.

‡Refers to status by conventional assays.

§A total of 55 patients had sufficient samples for both enzyme-linked immunosorbent assay and Western blot.

¶Represents postperformance analysis of a selected group of 10 samples that were culture positive. These samples were classified as seronegative by the Centers for Disease Control and Prevention using the 2-tier testing approach, which requires both a positive enzyme-linked immunosorbent assay for whole *B burgdorferi* and a positive Western blot. They were tested by an IgM *B burgdorferi* immune complex enzyme-linked immunosorbent assay that had a low content of outer surface protein C in antigen preparation, which may have limited the positive results.

||Subjects were asymptomatic 6 to 24 months after therapy. At sample time, 5 were seronegative and 7 were seropositive.

RESULTS

Detection of *B burgdorferi* Immune Complexed Antibody and Antigen

The majority of patients were white, aged 18 to 70 years, with approximately equal sex distribution. The neurologic patients with LD had an average age of 44 years and a distribution of 2 men to 1 woman. The patients with chronic fatigue syndrome had an average age of 34 years and an approximate 1:1 male to female distribution.

Of those with active LD (groups 1-4), 149 (96%) of 156 patients had detectable *B burgdorferi* immune complexes

(TABLE). In contrast, none of the patients who had recovered from LD (group 5) were positive, and only 2 (1%) of 147 controls (groups 6-9) were positive. These 2 were being evaluated for probable multiple sclerosis and possible concomitant LD. Selected samples positive for *B burgdorferi* immune complexes were examined for antibody reactivity to recombinant *B burgdorferi* proteins¹⁰ as well as for complexed *B burgdorferi* antigens. In group 1, of the 23 patients with EM and early infection who were seronegative by conventional antibody assays, 22 (96%) had *B burgdorferi* immune complex-containing antibody that reacted to OspA, an organism-specific protein. Fifteen (68%) of the 22 had complexed antibody that reacted to OspB. Seven of those who had complexed antibody to OspA also had IgM, which reacted to outer surface protein C (OspC) (another *B burgdorferi*-specific protein). Ten of 11 samples with *B burgdorferi* immune complexes examined contained OspA antigen analyzed by immunoblot. The top of the FIGURE shows a representative immunoblot from a patient with EM positive for *B burgdorferi* immune complexes and compares free and complexed antibody reactivity to *B burgdorferi* sonicate proteins. The bottom of the Figure shows a representative positive sample for *B burgdorferi* immune complexes probed for complexed *B burgdorferi* antigen. Thirty of 31 randomly chosen samples demonstrated immune complex antigens that comigrated with known B31 antigens when run using SDS-PAGE and blotted with polyclonal antibody. In contrast, this was not found in any of 10 healthy controls.

Reproducibility

Blinded analysis of replicates of 5 samples from patients with EM in 2 different research laboratories of the investigators revealed almost identical positivity. Four samples were categorized as positive in both laboratories. One sample that was positive above the cutoff of the mean and 3 SDs in 1 laboratory was borderline positive in the other. However, that sample was only 0.1 SD below the cutoff in 1

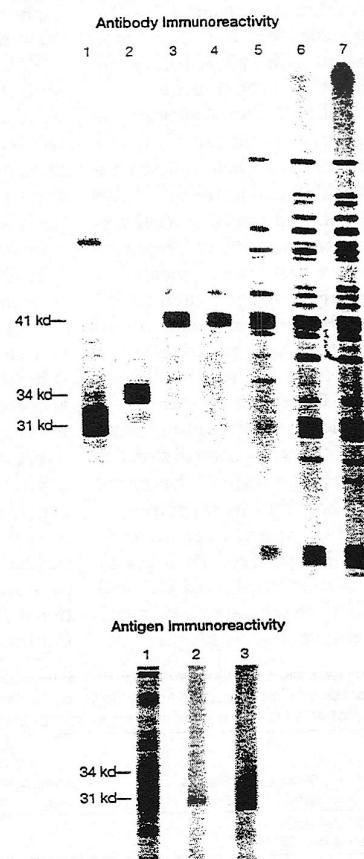
laboratory and 0.2 SD above the cutoff in the other laboratory. Thirty positive samples run on at least 2 occasions on different days with different controls that were reviewed retrospectively were positive on repeat runs. A set of 10 seronegative non-LD samples from a clinical laboratory was negative in both research laboratories. ELISA results were concordant with the Western blots.

COMMENT

The results demonstrate that formation of specific *B burgdorferi* immune complexes is common in LD. *B burgdorferi* immune complexes were found in patients with EM before they have had time to seroconvert as well as those who had seroconverted. Not all the patients with EM were seropositive. This may be because a finite period of at least 4 days is required before B cells differentiate into antibody-secreting cells. This, coupled with the variable time of appearance of EM (average of 4-14 days after exposure), can account for some negative test results even by a sensitive *B burgdorferi* immune complex assay. The EM patients had *B burgdorferi* immune complexes that contained, among a variety of *B burgdorferi* antigens, at least some of those that seem to be unique to *B burgdorferi* such as OspA, OspB, and OspC, and proteins 39, 66, and 83/93 kd. To prove the principle in this preliminary study, we chose cases with incontrovertible evidence of LD. However, the people most likely to benefit from this specific immune complex analysis are those in whom the diagnosis of LD may be difficult to establish. Examples would be suspected early cases, such as individuals bitten by ticks or those with an unidentifiable rash, who would otherwise have to wait several weeks before a conventional test could detect a specific antibody; seropositive reinfected people; vaccinated individuals; and people within endemic areas of exposure without EM who have compatible but nonspecific symptoms. Further studies in these populations will determine the clinical utility of this test.

The *B burgdorferi* immune complex assay appears to allow early diagnosis

Figure. Western Blot Immunoreactivity From a Patient With Early Seropositive Erythema Migrans (Group 2)



Top, Immunoreactivity of serum and serum immune complex antibodies to antigens of whole *Borrelia burgdorferi* B31 sonicates. Lanes 1, 2, and 3 show reactivity using monoclonal antibodies. Lane 1 is the reactivity of monoclonal antibody H3TS to 31-kd outer surface protein A (OspA). Lane 2 is the reactivity of monoclonal antibody H68 to 34-kd outer surface protein B (OspB). Lane 3 is the reactivity of monoclonal antibody H9724 to 41-kd flagellin. Reactivity of the patient's free and immune complexed antibody is represented as follows: lane 4 is free IgG, lane 5 is immune complexed IgG, lane 6 is free IgM, and lane 7 is immune complexed IgM. Bottom, OspA antigen detection in the dissociated immune complexes. Isolated and dissociated serum immune complexes were electrophoresed, blotted, and probed with rabbit polyclonal antibody and rabbit anti-OspA. Lane 1 is whole *B burgdorferi* B31 sonicate, probed with the polyclonal antibody, shown for comparison. Rabbit anti-OspA reactivity is shown in lanes 2 and 3. Lane 2 is the patient's immune complexed antigen. Lane 3 is the recombinant OspA. Molecular weights determined by monoclonal antibodies are indicated on the left.

of infection before conventional antibody tests become positive. This is important because patients who are treated early have the best chance for a cure. The *B burgdorferi* immune complex test appears to have good specificity in healthy controls, those with theoretical exposure but without known tick bite or symptoms, and most of the other disease control subjects. Based on a case with a known tick bite and appearance of EM, the results demonstrate that complexed antibody can be found in the first week of infection.

Our approach to immune complex analysis was directed at identification of the relevant antibody and antigen. This does not imply that the symptoms of LD are due to immune complexes such as occurs in systemic lupus erythematosus or other immune complex diseases. On the contrary, our earlier data¹⁷ indicated that levels of immune complexes were essentially normal in patients with later-stage LD in whom *B burgdorferi*-specific components were identifiable in the immune complexes.

As has been noted in other infectious diseases, the level of free antibody is not a measure of active infection or recovery.¹¹ In contrast, the detection of complexed rather than free, specific anti-

body is more likely reflective of active infection than a past exposure or a nonspecific polyclonal response to another inciting stimulus. The occurrence of this in LD is supported by our published^{10,17,20} and current results. That these immune complexes are specific for *B burgdorferi* infection is supported by our previous study, which confirmed the early detection of unique proteins such as OspA.¹⁰ These findings suggest that one can adapt the analyses of immune complexes as new antigens or important sequences are discovered, particularly those preferentially expressed during *in vivo* infections. The assay can also be adapted to new recombinant proteins as illustrated by our findings of immune complexed antibody to protein 37 (data not shown). This protein is up-regulated early during mammalian infection.^{21,22} This sensitive and specific immune-complex assay may have practical advantages over polymerase chain reaction, which has been encumbered by a number of factors that may lead to diagnostic false-positive and false-negative results.²³

Our data show that the immune complex approach is superior to free antibody assays during the first weeks. In later stages, sensitivities may become similar.

However, direct comparison between free and immune-complexed antibody assays may be misleading. Theoretically, the existence of immune complex is a marker of active disease, while free antibody is a marker of prior exposure.

The results suggest that while we and others continue to investigate expression of unique and *in vivo* expressed *B burgdorferi* antigens and the immune response to them,²⁴ clinical utility is highly probable. The true broadscale clinical utility of the *B burgdorferi* immune-complex assay will become clearer once additional samples are analyzed from the population within an endemic region.

The diagnosis of LD is still based on clinical history and examination, and we support the immediate treatment of EM, regardless of a test result. However, our data suggest that analysis of *B burgdorferi* immune complexes has potential as an adjunctive test to support or exclude *B burgdorferi* infection. This potential can be further substantiated in future studies that would include non-EM cases.

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